HEPATOTOXICITY OF PARACETAMOL AND STRUCTURAL ANALOGUES

Molecular mechanisms of bioactivation and of chemoprotection

Jos Bessems

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VRIJE UNIVERSITEIT

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SECTION I GENERAL INTRODUCTION AND REVIEW

Chapter 1 Introduction, aim and scope

INTRODUCTION

AIM AND SCOPE OF THE THESIS

Fate and behaviour of xenobiotics

Toxicology is the science involved with investigations on adverse effects that chemical compounds may have on living organisms. Whenever a chemical compound is not a normal constituent of a living organism, it is not endogenous and thus called a xenobiotic ('xenos' (G) = stranger; 'bios' (G) = life). Although even endogenous compounds may be toxic or harmful (Paracelsus: 'Sola dosis facit venonum'), toxicology is mostly limited to the study of xenobiotics. This ever increasing group of compounds includes medicines, food additives, (natural) food toxins, constituents leaching from food packages, residues of pesticides, biocides and veterinary medicines in food (or drinking water), industrial chemicals at the workplace, engine exhausts and environmental pollutants. Toxicology is an interdisciplinary science, mainly in between biology, (bio)chemistry, medicine, pharmacy and ecology, but even mathematics and statistics are important. The achievements of toxicology are used daily in e.g. clinical toxicology, occupational toxicology, food toxicology and ecotoxicology. Irrespective of this, toxicology is divided into explanatory toxicology and descriptive toxicology.

In descriptive toxicology, two questions are at the basis of what is referred to as toxicokinetics and what is referred to as toxicodynamics, i.e. what does the organism do with a xenobiotic and what does the xenobiotic do with the organism, respectively. Absorption, distribution, metabolism and excretion (ADME; see Figure 1) are phenomena for specialists in toxicokinetics and biotransformation. The effect of the xenobiotic for the organism is studied in toxicodynamics by e.g. molecular toxicologists, pathologists, immunotoxicologists, reproduction toxicologists. One tries to find the target organ and the critical effect(s). By testing several doses or concentrations in animal toxicity tests, one tries to find the No-Observed-Adverse-Effect-Level (NOAEL). By exploration from observations in the exposed human population, also a No-Observed-Effect-Level (NOEL) can be established. From basic to complex, toxicodynamics applies to the (sub)cellular level (e.g. induction and inhibition of enzymes, covalent protein binding, oxidative stress, redox cycling, membrane leakage, apoptosis), tissue level (e.g. cell proliferation, tissue damage, cell infiltration, vasoconstriction or dilatation), organ level (organ failure, tumour formation) and level of whole organism (increase plasma levels of endogenous waste products, coma, death). This is depicted in Figure 1.

In explanatory toxicology, it is studied how the ultimate adverse effect of a xenobiotic due to a specified exposure are qualitatively as well as quantitatively dependent on qualitative and quantitative reactions at the ultimate molecular target. The inherent chemical properties of the substance determine the kind of effect (hazard identification) whereas the concentration at the site of action determines the intensity

of the effect (dose-response relationship; hazard characterisation). Both join in hazard assessment. Molecular and biochemical toxicology are closely linked scientific subdisciplines of toxicology that deal with the following aspects: toxicokinetics, toxifying and detoxifying biotransformation reactions, reversible and irreversible interactions with cell or tissue components, (bio)chemical protection and repair mechanisms and consequences of the toxic effect for the organism (Vermeulen, 1996). Biochemical toxicology focusses mainly on elucidation of the biochemical disturbances that lead to dysfunctioning of the cell (cytotoxicity) or system, e.g. inhibition of a critical enzyme or disturbance of membranes. Molecular toxicology investigates the molecular aspects of the chemical substance itself in relation to the fate of the molecule and its inherent toxic properties. What physicochemical properties determine the trip of a substance through an organism down to the subcellular and molecular level? How do the functionalities in a molecule predispose for adverse molecular effects such as oxidation of and covalent binding to (macro)molecules such as NADPH, glutathione, lipids, proteins and DNA? Molecular toxicology also studies the biotransformation into metabolites which is normally aimed at making a compound more hydrophylic in order to enhance biliary or urinary excretion. However, sometimes, inadvertent bioactivation of the parent compound or an already formed metabolite takes place. As the concentration of the ultimate toxin (metabolite in case the parent compound is a protoxin) at the molecular target is partly dependent on the ratio of bioactivation and detoxification, this ratio is of utmost importance. Subsequently, cell toxicology is the subdiscipline where the knowledge of molecular and biochemical toxicology is integrated to find a mechanistic explanation of cytotoxicity (Fig. 1).

Toxicological risk assessment, lastly, is the subdisclipine in toxicology in which all toxicological findings regarding a chemical substance are integrated and compared to the actual human exposure level. Findings on the toxicity mechanism and the animal toxicity testing or epidemiologic research, together with dose-response relationships, combine to a most critical effect (hazard characterization) in the hazard assessment (Dutch 'gevaarsidentificatie'). For this effect, a NOAEL or NOEL is established or estimated, that is subsequently taken as starting point for risk assessment. For local effects such as erythema or itching, but in principal also tumour formation of epithelia of the skin, airways and gastro-intestinal tract, eyes, often only external exposure is relevant. For systemic effects, internal exposure of the body (systemic exposure) is determining an eventual outcome in terms of an adverse effect. Xenobiotics may be taken up (unintentionally) or delivered (intentionally) by mouth, via skin, via the nose or via other entries. A risk is to be expected only if real exposure exceeds a predetermined exposure level (external or internal), referred to as 'limit value' or reference dose, that is expected not to result in any adverse effect (regarded to be safe). A limit value may be equal to the NOEL but is mostly derived by extrapolating from a NOAEL in animal toxicity testing (involving e.g. interspecies extrapolation, extrapolation for duration of toxicity test). This limit value or NOEL is further expressed as an Acceptable Daily Intake (ADI) in case of food additives or residues of plant protection products or veterinary medicines, a Tolerable Daily Intake (TDI) in case of therapeutics and contaminations or an Occupational Exposure Limit

(OEL) in case of chemicals that humans are exposed to in occupational settings. Thus, a risk is only to be expected whenever the (expected) exposure superseeds a safe reference level (i.e. the risk ratio expressed as the ratio of exposure and reference level >1).

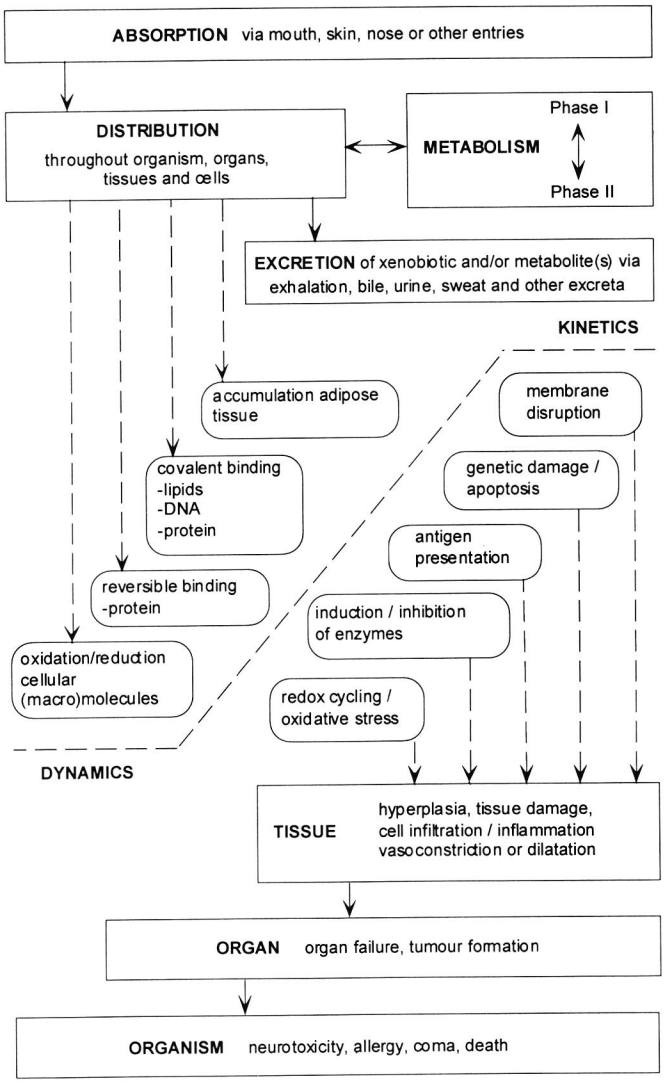

Figure 1: Concept of toxicokinetics and toxicodynamics of drugs and other xenobiotic chemicals. It should be noted that only examples of various processes are presented.

Toxicokinetics and biotransformation of paracetamol

This thesis is mainly dealing with the molecular and cell toxicology of paracetamol (acetaminophen, 4'-hydroxyacetanilide; see Fig. 2) and its structure analogues, and not so much with the toxicodynamic properties. Therefore, a brief introduction to toxicokinetic phenomena such as biotransformation, and the processes preceeding the cellular uptake, i.e. toxicokinetics, is presented here. The toxicodynamic aspects of paracetamol are mentioned only very briefly.

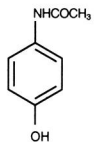

Figure 2: Paracetamol

Paracetamol (PAR) is mostly taken orally although i.v. or rectal delivery is also possible. After passage of the stomach, intestinal uptake of PAR via membrane passage is fast and almost complete, due to its physicochemical properties such as molecular weight, lipophilicity and pKa (because of their lipophilicity, many foreign compounds readily pass cell membranes leading to increased intracellular concentrations). Following intestinal uptake, delivery of the PAR via the portal vein to the liver is still presystemic. The systemic circulation is not reached before PAR has been transported via the hepatic vein. Maximum plasma levels in humans are reached within 0.5-2 h, the distribution volume is about 1 l/kg body weight and the elimination half-life is 1-4 h. At normal dosage (500-1000 mg, at maximum 3000 mg/day), reversible plasma protein binding is negligible.

At therapeutic levels of intake, the uptake of PAR by hepatocytes from the portal vein and the hepatic artery results in a high level of conjugation with glucuronic acid (approx. 60%) and sulphate (approx. 35%), thereby lowering the lipophilicity and enhancing aqueous solubility (percentages are presented for adults). The latter is necessary for excretion via plasma to the urine, although a small part of the administered dose (<5%) is excreted unchanged via urine. Glucuronidation and sulphation are so called phase II reactions as they may be subsequent to phase I reactions (see Fig. 3). Phase I reactions mostly introduce a functional group in xenobiotics as a substrate for phase II reactions where this functional group may be conjugated. Phase I reactions decrease the lipophilicity only slightly whereas conjugation with hydrophylic moieties greatly facilitates aqueous solubility. A small amount of PAR in the hepatocytes (approx. 3%) undergoes phase I oxidation by one or more enzymes of the microsomal cytochrome P450 family to a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI can be trapped readily by the intracellular soluble thiol containing tripeptide glutathione (GSH; γ-glutamyl-

cysteinylglycine), also in a phase II reaction, thereby preventing unwanted reactions with non-soluble protein thiols (Vermeulen *et al.*, 1992).

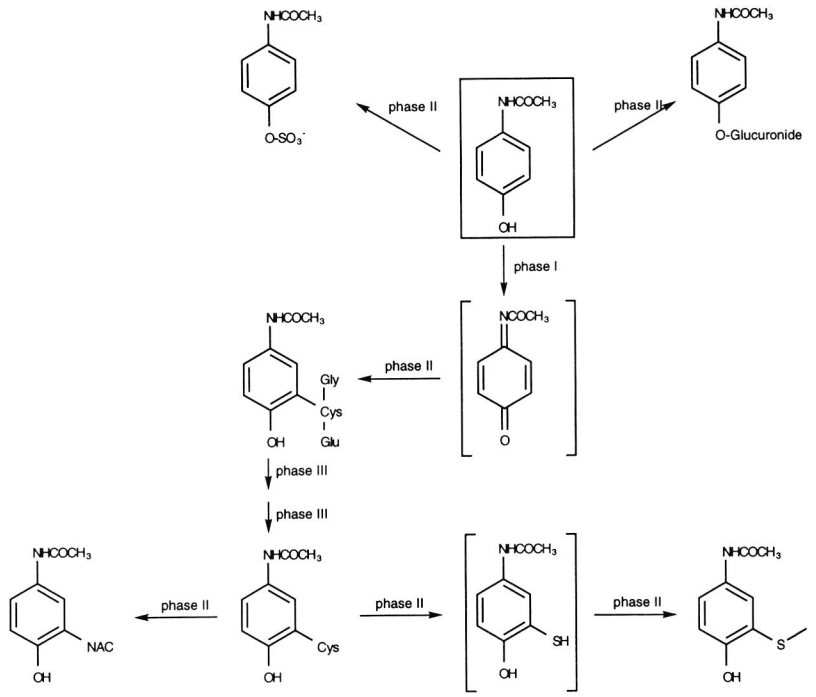

Figure 3: Biotransformation of paracetamol in humans

It should be mentioned that in humans as well as laboratory animals, several enzymes of the cytochrome P450 family are involved in oxidation of PAR. In humans, mostly probably CYP3A4 is involved at therapeutic concentrations. At high dose intake and intoxication, also CYP2E1 and CYP1A2 may become involved, respectively (Raucy *et al.*, 1989; Thummel *et al.*, 1993). Genetic polymorphism has been reported for CYP2E1 (Kadlubar, 1994; Park *et al.*, 1995). Furthermore, it has been established that heavy use of alcohol or specific medicinal drugs may predispose humans to PAR toxicity at intake levels only slightly above the recommended maximum daily intake (references in (Thomas, 1993)). The importance of these phenomena in observed interindividual differences in vulnerability to PAR toxicity remains to be established.

As indicated above, part of the internal dose of PAR in the liver, escapes hepatic phase I and II reactions during first passage of the liver (first pass metabolism) and is distributed as such to other organs such as kidneys and brains. In the brains, PAR can exhibit its analgesic and antipyretic activity. The latter activities are based on inhibition of microsomal prostaglandin endoperoxide synthase (PGES). The exact

mechanism of inhibition of PGES has not been elucidated yet but oxidation of PAR by PGES may be important in this. As the proximal tubules in the kidneys are also rich in PGES, oxidation of PAR may also pose a hazard to critical renal sites. Upon PGES-dependent oxidation of PAR, NAPQI or an even more reactive intermediate, the radical *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) may result. In addition, it has been shown in certain species that the phase II enzyme *N*-acetyltransferase may deacetylate PAR (synonym *N*-acetylaminophenol). This results in the formation of *p*-aminophenol, a well known nephrotoxicant. Thus, the ultimate outcome of exposure of a specific cell in a specific organ will be dependent on the ratio of bioactivation (to NAPQI, NAPSQI or *p*-aminophenol) and bioinactivation (glucuronidation, sulphation, GSH-conjugation of the metabolite NAPQI).

Upon ingestion of PAR at levels exceeding the recommended intake, high plasma and intracellular levels in hepatocytes will result. The phase II biotransformation cofactors uridine-5'-diphosphoglucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) may become exhausted in hepatocytes. As a result, larger quantities of PAR will be liable to P450- and PGES-dependent bioactivation to NAPQI and maybe NAPSQI. However, NAPQI can be reduced or conjugated by glutathione (GSH). Conjugation to GSH may be a pure chemical trapping reaction or a reaction catalysed by the enzyme glutathione *S*-transferase (GST). Both result in the formation of a GSH-conjugate of PAR which may be excreted via bile and urine. The latter is also a phase II biotransformation enzyme. Reduction of NAPQI by GSH gives rise to PAR and GSSG (oxidised glutathione; see Fig. 4), consequently the redox state of the cell will become impaired.

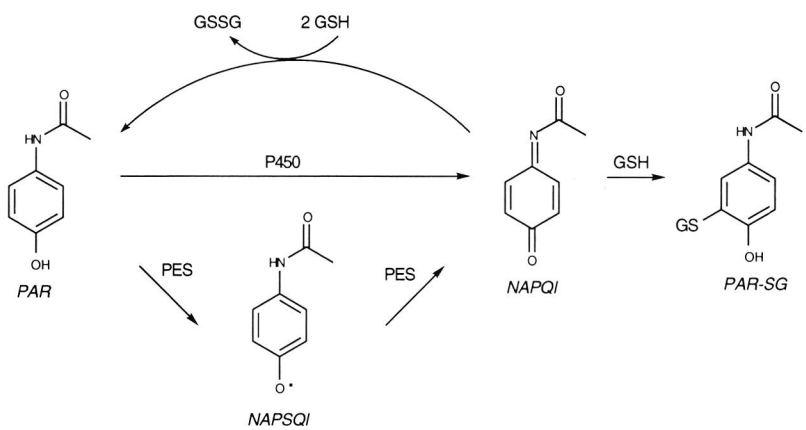

Figure 4: Oxidation of PAR by P450 and PGES to NAPQI followed by conjugation to GSH

The GSH-conjugate of PAR formed (PAR-SG), is also liable to enzymatic hydrolysis (phase II biotransformation reactions) of the tripeptide moiety, resulting in the γ-glutamylcysteinyl conjugate (PAR-CG) and the cysteine conjugate (PAR-Cys). All

three conjugates (PAR-SG, PAR-CG and PAR-Cys) have been detected in biological samples of one or more species including man. Subsequently, PAR-Cys may become *N*-acetylated, resulting in formation of the mercapturic acid of PAR, PAR-NAC. PAR-NAC is readily excretable via urine.

It has been speculated that in some species, the PAR-Cys conjugate is also a substrate for one of the phase II biotransformation enzymes, i.e. cysteine conjugate ß-lyase, or of enzymes also in gut flora (Möller-Hartmann and Siegers, 1991). A reactive thiol may result and react with another reactive thiol to a dimer. This thiol may also become *S*-methylated by *S*-methyltransferase (also a phase II enzyme), resulting in an *S*-methyl derivative. Both compounds (the dimer and the *S*-methyl derivative of PAR) have been found in some species. Lastly, in some laboratory animals but not in man, the *S*-methyl derivative of PAR may also be liable to *S*-oxidation, possibly by gut microflora, as the corresponding sulfoxide and sulfone were found.

Whenever upon unusually high intake of PAR the internal dose (intracellular concentration during a certain time period or area under the concentration vs. time curve) of NAPQI formed supersedes the amount of glutathione (GSH) available for trapping or reduction (see Fig. 4), critical proteins may become arylated or oxidised resulting in oxidative stress, lipid peroxidation and changes in the intracellular calcium homeostasis and energy status, ultimately leading to loss of cellular integrity and functioning of the hepatocyte (Vermeulen *et al.*, 1992) (Fig. 5).

Toxicodynamic and clinical effects of paracetamol

Upon therapeutic intake of PAR, only few side-effects occur in humans, e.g. allergic reactions. Also, although rarely, agranulocytosis (upon long-term use), thrombocytopenia and haemolytic anaemia have been observed. Occasionally, interstitial nephritis upon very long term use of high doses has been reported. Quantities of 6 g and higher may already result in liver damage, higher quantities cause irreversible liver necrosis. Lastly, hepatic damage has been reported upon chronic use of PAR of 3-4 grams each day.

Upon intake of an overdose, when the capacity of the organism to cope with PAR has been overloaded, liver damage will occur (jaundice and hepatic necrosis) as can be monitored by measuring serum ALT and AST. This may happen already at quantities of 8 g or more. One dose of 25 g is regarded to be mortal. The first symptoms of poisoning are anorexia, nausea and vomiting. Unconsciousness mostly doesn't occur. Nevertheless, emergency treatment is necessary in order to avoid irreversible liver damage. Indicated are rinsing of the stomach followed by repeated oral administration of active charcoal (adsorbent) and sodium sulphate (laxans). Indicated is also i.v. administration of *N*-acetylcysteine in sodium sulphate (150 mg/kg; 500 ml) and repetition is dependent on the measured plasma levels of PAR (Thomas, 1993; Sdu, 1998).

General outline of this thesis

In 1990, at the start of the project of which the results are described in this thesis, it was hypothesised that structural modification of PAR could be useful for

investigating the toxicological properties of PAR including potential built-in protection properties as well as the molecular pharmacological properties at a molecular level. Several investigators had used phenyl-substituted analogues of PAR before (Dearden and O'Hara, 1976; Fernando *et al.*, 1980; Rosen *et al.*, 1984; Fischer *et al.*, 1985; Harvison *et al.*, 1986; Van de Straat *et al.*, 1986; Porubek *et al.*, 1987; Van de Straat *et al.*, 1987a; Van de Straat *et al.*, 1987b; Harvison *et al.*, 1988; Rossi *et al.*, 1988; Rundgren *et al.*, 1988; Birge *et al.*, 1989). Extending the series of analogues that were investigated already would enlarge the potential of such investigations. It was argued that the hypothesised single hydrogen atom abstraction mechanism of PAR by P450 could be investigated properly as potentially more stable semiquinone imine radicals, compared to the very reactive and unstable NAPSQI, could be formed. Also, not only the effects of ring-substution of PAR on the ease of enzymatic oxidative bioactivation in phase I biotransformation reactions by peroxidases and liver microsomal cytochrome P450 (P450) and the reactions of once-formed reactive metabolites with GSH and proteins could be studied but also the effects on the cyclooxygenase-inhibiting properties of PAR.

In **Chapter 2** of **Section I - General introduction and review**, a review of molecular and biochemical mechanisms and protective agents with respect to PAR-induced (cyto)toxicity is presented. Mainly, the developments of the past decade are presented although for a complete understanding also earlier findings are described. Shortly, with intake of prescribed doses, PAR is mainly glucuronidated and sulphated to readily excretable conjugates (see Figure 1). Part of the dose (only a few percent) will be oxidised by liver microsomal P450 and PGES in liver and e.g. kidneys, respectively.

However, with intake of higher doses, an increasing portion of PAR will be bioactivated by hepatic P450 to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which can react with soluble and non-soluble thiol groups in glutathione (GSH) and proteins, respectively. With intake of lethal doses of PAR, cellular thiol pools will become exhausted, essential proteins (enzymes) will become oxidised and/or covalently modified by NAPQI. The irreversible (sub)cellular and subsequent tissue damage will culminate in fulminant liver necrosis, organ failure and ultimately death. At chronic intake, the oxidation of PAR by renal PGES may result in chronic irritation of proximal tubule tissue in the kidneys. In the past decades, numerous attempts have been described to improve the risk/benefit ratio of PAR by adding protective agents to therapeutic PAR formulations or by modification of the chemical structure of PAR with prevention of its inherent cytotoxic and retainment of its analgesic properties.

In **Section II - Synthesis of analogues, screening of biological activities and purification of P450 enzymes**, the work is described that was necessary to start the investigations described in the Sections III and IV, i.e. the synthesis of new paracetamol analogues, screening of biological activities and purification of P450 enzymes. Before the start of the experiments that are described

in this thesis, substantial work had already been performed on 3,5-alkylated analogues of PAR. It was known that blocking of one or both of the aromatic positions *ortho* to the phenolic group, was important in inhibition or even prevention of the *in vitro* and *in vivo* hepatotoxicity of PAR. Substitution of one and both positions with an alkyl group diminished and prevented, respectively, the cytotoxicity in freshly isolated rat hepatocytes and the *in vivo* hepatotoxicity in rats (Van de Straat *et al.*, 1986; Van de Straat *et al.*, 1987b). Several of these 3,5-dialkylated substrates that were used again in this thesis were already present in our laboratory (Dearden and O'Hara, 1978; Van de Straat *et al.*, 1986).

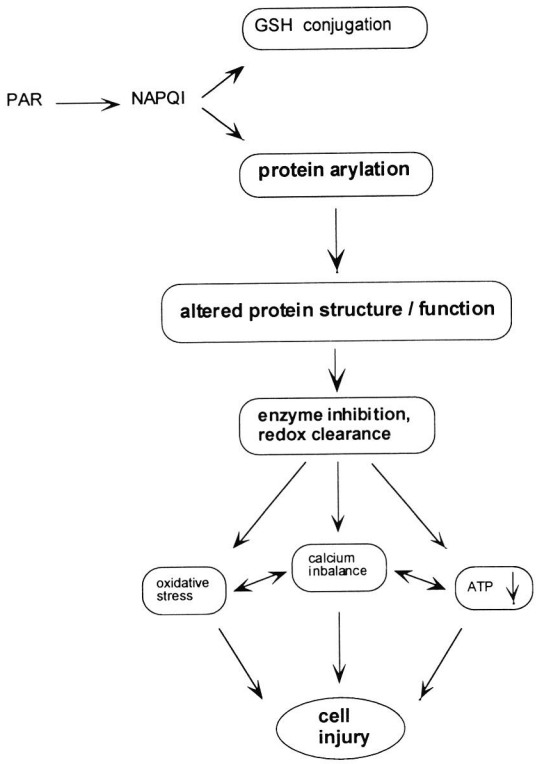

Figure 5: Covalent modification of proteins by NAPQI followed by loss of cellular integrity

In **Chapter 3**, the synthesis is described of some novel 3,5-disubstituted analogues of PAR that were used in molecular, biochemical and toxicological investigations due to exposure to PAR. As it was expected that (cyto)toxicity of PAR could be modified at various levels of bioactivation and detoxication, it was decided to investigate the effects of substitution on one- and two-electron oxidation by P450 (hydrogen abstraction

mechanism). It was chosen to expand the series of 3,5-dialkylated PAR analogues (of which the substituents *ortho* to the phenolic hydroxyl group exhibit electron-donating properties towards this hydroxyl group) with substituents R = $-OCH_3$ and $-SCH_3$. Furthermore, as the effects of electron-withdrawing groups were to be investigated, 3,5-dihalogenated analogues of PAR (R = -F, -Cl, -Br and -I) were synthesised. Investigation of their cytotoxic properties as well as their analgesic properties *in vitro* using freshly isolated hepatocytes and mouse brain microsomal fractions respectively, is described.

As it was expected at the beginning of the investigations described that one-electron oxidation of PAR by P450 might be important in the toxicity mechanism of PAR, experiments for the detection of radicals using electron spin resonance (ESR) were planned. As radicals mostly are very reactive chemical entities, being capable of oxidation of ribonucleic acids, proteins and lipids with subsequent quenching of their radical properties, the possibility of detection of these radicals would be increased by working in enzymatic systems as clean as possible. That is to say, the presence of other proteins, next to the NADPH-dependent monooxygenase system, should be diminished. At that time, rat liver microsomal cytochromes P450 2E1 and 1A1 (CYP1A1 and CYP2E1) were known to be involved in oxidative bioactivation of PAR. Therefore, a preparative scale purification of rat hepatic CYP1A1 and CYP2E1 together with NADPH-dependent cytochrome P450 reductase by high-performance ion-exchange chromatography was initiated as described in **Chapter 4**.

In **Section III - Mechanistic investigations - Cytochrome P450-dependent oxidative biotransformation**, mainly the oxidation of PAR and 3,5-disubstituted analogues is investigated at the subcellular level, using microsomal fractions, purified P450 enzymes and horseradish peroxidase (HRP). In **Chapter 5**, ESR-analysis is described as used to investigate the mechanism of hydrogen atom abstraction of 3,5-disubstituted analogues of PAR by P450. As many similarities exist between P450s and peroxidases with respect to substrate oxidation, horseradish peroxidase (HRP) was used as a cheap and clean (no protein or lipid 'impurities') model for P450. Furthermore, *ab initio* calculations were performed in order to rationalise the ESR-observations with respect to the energetical aspects of potential oxidation reactions and the potential hydrogen abstraction from the phenolic hydroxyl group compared to the acetyl amino group. Subsequently, in **Chapter 6**, rat liver P450-dependent oxidation of 3,5-disubstituted analogues of PAR is investigated by UV-spectroscopy as well as by monitoring GSH oxidation and formation of GSH-conjugates. Additionally, structural analysis of GSH-conjugates is used to test our hypothesis that halogen atoms in the NAPQI derivatives and in the parent PAR derivatives, could be liable to nucleophylic addition as well as addition-elimination reactions by GSH.

In **Section IV - Cellular effects and protective aspects in paracetamol cytotoxicity**, experiments are described to investigate various aspects of toxicity of PAR and 3,5-dihalogenated analogues and protection against their toxicity in freshly

isolated hepatocytes. In **Chapter 7**, investigations are described into the cytotoxicity of PAR and 3,5-dihalogenated analogues including the role of (inhibition of) cytochrome P450 (CYP)1A1 and the role of depletion of intracellular GSH. Also, possible phase II biotransformation of the new 3,5-dihalogenated analogues, possible site-specific GSH conjugation and protein thiol adduct formations is described. Lastly, attempts are presented to explore possible structure-activity relationships of PAR and 3,5-dihalogenated derivatives. In **Chapter 8**, the protection of ebselen, an anti-inflammatory agent, against PAR-induced toxicity in freshly isolated rat hepatocytes is described. In order to elucidate the mechanism of protection, biochemical assays of P450 activity in microsomes isolated from these hepatocytes are used. In addition, the reaction between sythetical ebselen selenol, the thiol derivative of ebselen, and NAPQI is described in order to investigate the mechanism of protection.

Lastly, in **Chapter 9** of **Section V - Overall summary**, an overall summary is presented including some general conclusions and future perspectives.

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Chapter 2 Paracetamol-induced toxicity. Molecular and biochemical mechanisms, analogues and protective approaches

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Paracetamol-induced toxicity

Molecular and biochemical mechanisms, analogues and protective approaches

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List of abbreviations: 3'-HAA, 3'-hydroxyacetanilide; 3',4'-HAA, 3',4'-hydroxy-acetanilide = 3-hydroxyparacetamol; 3-OH-PAR, 3-hydroxyparacetamol; ALT, alanine aminotransferase (ALAT); an indicator of hepatotoxicity; AST, aspartate aminotransferase (ASAT); an indicator of hepatotoxicity; ßNF, ß-naphthoflavone; CYP, cytochrome P450; Cys, cysteine*; γGT, γ-glutamyltranspeptidase = γ-glutamyltransferase; GSH, glutathione (γ-glutamylcysteinylglycine); GSSG, oxidised glutathione; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NAPSQI, *N*-acetyl-*p*-benzosemiquinone imine; NAT, *N*-acetyltransferase; ßNF, ß-naphthoflavone; P450, cytochrome P450; PAP = *p*-aminophenol; PAR, paracetamol = acetaminophen = 4'-hydroxyacetanilide; PAR-CG, cysteinylglycine conjugate of PAR; PAR-Cys, cysteine conjugate of PAR; PAR-GLUC, glucuronide conjugate of PAR; PAR-NAC, mercapturic acid of PAR; PAR-SG, glutathione conjugate of PAR; PAR-SULP, sulphate conjugate of PAR; PGES, prostaglandin endoperoxide synthetase; ST, sulphotransferase; UDPGA = uridine diphosphoglucuronic acid (cofactor UDPGT); UDPGT = uridine diphosphoglucuronyl transferase

*Although mostly not indicated for sake of readability, in case of amino acids or amino acid like compounds, always the stereoisomer with the L-configuration is meant, unless stated otherwise.

PART I INTRODUCTION

I.1 Kinetics and biotransformation

Paracetamol (4'-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol, acetaminophen, PAR[¥]) is a widely used over-the-counter analgesic and antipyretic drug. In the UK, approximately 3.2 x 10^9 tablets of PAR are consumed every year, which is an average of 55 tablets/person (Jones, 1998). The pharmacological effects of PAR are generally considered to be based on inhibition of prostaglandin synthesis (Flower and Vane, 1972; Malmberg and Yaksh, 1982; Mattamal *et al.*, 1979). At therapeutic levels, PAR is considered to be safe for humans upon normal drug use (Thomas, 1993). The absorption of low therapeutic doses of PAR is usually rapid and complete, the systemic bioavailability and the plasma half-life being about 75% and 1.5-2.5 h, respectively. The toxicokinetics of PAR were reviewed in the past (Prescott, 1980) and investigated recently using a pharmacokinetic model (Tone *et al.*, 1990). As delineated from urinary metabolites and discussed in various review articles (Thomas, 1993), PAR is metabolised primarily by glucuronidation and sulphation (Fig. 1). These major conjugates PAR-sulphate (PAR-SULP) and PAR-glucuronide (PAR-GLUC), being more water-soluble than the parent compound, are eliminated from the liver and blood mainly via urine (both) and a little via bile (PAR-GLUC). About 30 and 55% of administered PAR is excreted in urine as PAR-SULP and PAR-GLUC, respectively (Howie *et al.*, 1977; Tone *et al.*, 1990). Like in various laboratory animals, a small amount of PAR is probably metabolised via a third metabolic pathway, i.e. oxidation by the microsomal cytochrome P450 (CYP)-containing mixed-function oxidase system (MFO) to NAPQI. A glutathione 1,4-Michael adduct of NAPQI and the corresponding cysteine conjugate and mercapturic acid breakdown products were found in human urine upon ingestion of PAR (Howie *et al.*, 1977; Knox and Jurand, 1977; Prescott, 1980). Although a minor oxidation reaction, hydroxylation of PAR to 3-hydroxyparacetamol (3-OH-PAR) is probably also occurring in man as methylated 3-hydroxyparacetamol has been found in urine of patients who had taken an overdose of PAR (Knox and Jurand, 1977). In addition, as shown for various laboratory animals, probably also human enzymes with peroxidase activity, like prostaglandin synthase and myeloperoxidase, have the ability to catalyse the metabolism of PAR. The enzyme-catalysed bioactivation by cytochrome P450 as well as peroxidase-like enzymes is discussed in Part III.

Most of the large amount of investigations regarding PAR-induced toxicity have of course been performed in (laboratory) animals. In either one or more of the species rat, mouse, hamster, and dog, besides the human metabolites, several other metabolites were found in urine, i.e. 3-thiomethylparacetamol (including its sulphate, its glucuronide and its sulfone derivative), *p*-aminophenol and the (pre)mercapturic acid conjugates of *p*-benzoquinone (Betowski *et al.*, 1987; Gemborys and Mudge, 1981; Hart *et al.*, 1982; Howie *et al.*, 1977; Knox and Jurand, 1977; Lubek *et al.*, 1988; To and Wells, 1985; Wong *et al.*, 1976). A composition of the metabolic pathways of PAR in the various species is depicted in Fig. 1.

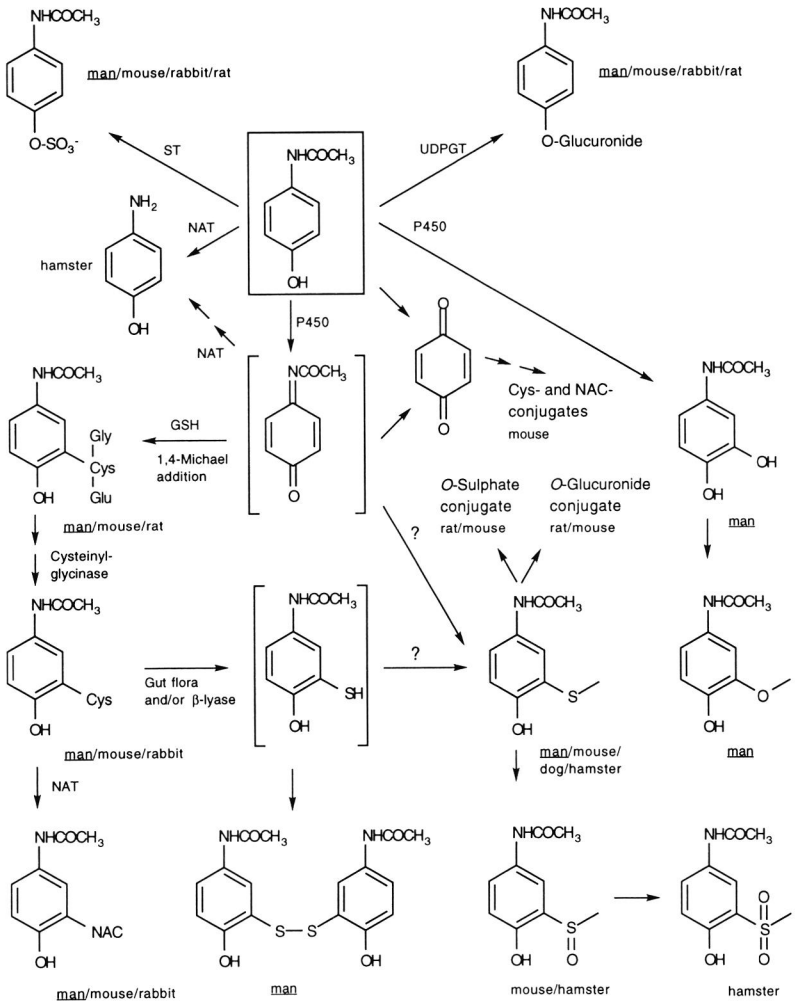


FIG. 1 Proposed metabolic pathways of PAR in man and experimental animals The individual metabolites were all detected in urine of organisms that ingested PAR in therapeutic quantities (man) or at overdose (see text for references). The structures between brackets are logical intermediates that have however not been detected in urine as such. The formation of the thiomethylconjugate of PAR could either occur via the thiol intermediate or via direct attack of NAPQI by *S*-methylaminoacids

I.2 Toxicodynamics

Upon intake of a toxic dose, PAR mainly causes P450-dependent centrilobular hepatotoxicity in man and various laboratory animals (Hinson, 1980; Mitchell *et al.*, 1973), as observed by the release of serum alanine aminotransferase (ALT) into the

serum. Serum ALT is often used as monitoring parameter for hepatic damage. Much evidence has been presented for *N*-acetyl-*p*-benzoquinone imine (NAPQI) to be the reactive electrophilic intermediate responsible for the observed toxicity (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b), although an as yet elusive *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) was proposed as well (Bessems *et al.*, 1998; De Vries, 1981; Potter and Hinson, 1989). In addition, the nontoxic catechol 3-hydroxyparacetamol (3-OH-PAR) is formed be it as a minor metabolite (Forte *et al.*, 1984; Harvison *et al.*, 1988b; Hinson *et al.*, 1980). At normal doses of PAR, in most species including man, only a trace amount of the reactive intermediate NAPQI is formed. In the presence of reduced glutathione (GSH), e.g. in isolated rat hepatocytes, NAPQI can either be reduced back to PAR or covalently linked to GSH to form a 3-glutathione-*S*-yl-paracetamol conjugate (PAR-SG) (Moldéus, 1978; Van de Straat *et al.*, 1986) without displaying significant adverse effects. After an overdose, however, or when specific microsomal P450s are increased, hepatic GSH is depleted more extensively and can no longer compensate for a massive production of NAPQI. Especially, depletion of mitochondrial GSH is correlated with hepatic toxicity (Vendemiale *et al.*, 1996). Paracetamol, most likely via NAPQI, can form adducts with proteins (Hinson *et al.*, 1995; Holtzman, 1995; Jollow *et al.*, 1973; Nelson, 1995), oxidise protein sulfhydryls (Birge *et al.*, 1988; Tirmenstein and Nelson, 1990), covalently bind to liver (as well as renal) DNA (Hongslo *et al.*, 1994) and eventually disrupt cellular homeostasis. A short overview mainly on the most common liver effects but also some other effects of acute and high ingestion of PAR is depicted in Table 1.

Thus, as will be described more in detail in Part IV, in mice, rats and humans, an overdose of PAR may result in severe centrilobular hepatic necrosis (Hinson *et al.*, 1981; Hinson *et al.*, 1990; Prescott, 1983; Vermeulen *et al.*, 1992). However, also renal tubular necrosis (Part V) (Björck *et al.*, 1988; Cobden *et al.*, 1982; Emeigh Hart *et al.*, 1991a; Emeigh Hart *et al.*, 1991b; Emeigh Hart *et al.*, 1996; Hoivik *et al.*, 1995; Möller-Hartmann and Siegers, 1991; Newton *et al.*, 1985) may develop. Although uncommon, acute renal failure due to a large PAR overdose may occur in the absence of fulminant hepatic failure (Eguia and Materson, 1997). Moreover, in mice pulmonary nonciliated bronchiolar epithelial (Clara cell) necrosis (Jeffery and Haschek, 1988), covalent protein adduct formation in lungs (Bartolone *et al.*, 1989) and strain specifically, cataractogenecity (Lubek *et al.*, 1988; Wells *et al.*, 1995) were reported to occur. In man, deleterious effects on blood platelets were found upon acute ingestion of large amounts of PAR (Fischereder and Jaffe, 1994). Furthermore, it is indicated that long term exposure of humans to high but still therapeutic doses of PAR is correlated with increased risk of chronic renal disease (Sandler *et al.*, 1989). PAR-induced liver tumours in mice (Flaks and Flaks, 1983) and bladder carcinomas in rats (Flaks *et al.*, 1985) have been reported as well. Genotoxic effects of PAR have been found *in vitro* (Brunborg *et al.*, 1995), in various laboratory animals (Hongslo *et al.*, 1994), and in man (Rannug *et al.*, 1995). Recently, even adverse as well as positive effects on estrogen-related physiologic processes, such as proliferation of cultured breast cancer cells but, although preliminary, an epidemiological finding of a

decrease of ovarian cancer, were reported (Cramer *et al.*, 1998; Harnagea-Theophilus *et al.*, 1999; Miller *et al.*, 1999).

TABLE 1 Subcellular effects and physiological changes caused by paracetamol

Parameters/mechanism	Species/Test system	Reference
GSH depletion	many species	(Mitchell *et al.*, 1973; Vendemiale *et al.*, 1996)
GSH oxidation	many species	(Albano *et al.*, 1985)
protein thiol depletion:	many species	
-liver proteins	-various species	(Roberts *et al.*, 1987)-
-plasma membrane proteins	-rat	(Tsokos-Kuhn *et al.*, 1988)
-hemoglobin	-mouse	(Axworthy *et al.*, 1988)
protein thiol oxidation	many species	(Albano *et al.*, 1985; Kyle *et al.*, 1990)
LPO	many species	(Wendel *et al.*, 1979)
production hydroperoxides	mouse, hepatocytes	(Adamson and Harman, 1989)
incr. malondialdehyde	rat liver, *in vivo*	(Vendemiale *et al.*, 1996)
mitochondrial membrane potential; change within 30 min	rat, liver slices	(Nazareth *et al.*, 1991)
plasma membrane potential; change after 4 hours	rat, liver slices	(Nazareth *et al.*, 1991)
decr. Na^+/K^+-ATPase activity; protein arylation	mouse / rat, liver plasma-membrane	(Corcoran *et al.*, 1987a; Corcoran *et al.*, 1987b; Corcoran *et al.*, 1988)
decr. Ca^{2+}/Mg^{2+}-ATPase activity; protein arylation; no support ox stress hypothesis	rat , liver plasma membrane	(Tsokos-Kuhn *et al.*, 1988)
membrane blebbing	rat, hepatocytes	(Moore *et al.*, 1985)
inhibition carbamyl phosphate synthetase-I	mice, *in vivo*	(Gupta *et al.*, 1997)
inhibition glutamine synthetase	mice, *in vivo*	(Gupta *et al.*, 1997)
incr. mitochondrial respiration	rat liver, *ex vivo*	(Vendemiale *et al.*, 1996)
liver necrosis	many species	many references (see text)
renal tubular necrosis	many species	many references (see text)
cataracts	susceptible mice strains	(Lubek *et al.*, 1988a; Lubek *et al.*, 1988b; Wells *et al.*, 1995)
incr. nuclear Ca^{2+}; within 2-6 h	mouse, *in vivo*	(Ray *et al.*, 1990; Ray *et al.*, 1991)
inhibition of replicative DNA synthesis	Chines hamster V79 cells	(Richard *et al.*, 1991)
inhibition of DNA synthesis	rat, *in vivo*	(Lister and McLean, 1997)
loss of large genomic DNA; within 2-6 h	mouse, *in vivo*	(Ray *et al.*, 1990; Ray *et al.*, 1991)
inhibition of cell cycling	HL-60 cells	(Wiger *et al.*, 1997)
induction of apoptosis	HL-60 cells	(Wiger *et al.*, 1997)

Significant differences exist with regard to the susceptibility of various species and even the various strains of laboratory animals to the deleterious effect of PAR upon a toxic dose (Gregus *et al.*, 1988; Hinson, 1980; Ioannides *et al.*, 1983; Lubek *et al.*, 1988). In general, hamsters and mice are sensitive whereas rats, rabbits, and guinea

pigs are relatively resistant to PAR-induced liver injury. These species differences (toxicodynamic and/or toxicokinetic) should be kept in mind, especially when one realises that the choice of the species is not always a scientific choice but an economical or a practical one. Many *in vivo* investigations are performed with mice whereas for many *in vitro* experiments the rat is chosen. Important differences in conjugation and deconjugation pathways between species and between organs (which is further discussed in Part II) may be involved in this differential sensitivity to PAR-toxicity (Miller *et al.*, 1993). In Table 2 a short overview is presented of interspecies and intraspecies/interindividual differences - genotype (inbred strains) as well as phenotype (including environmental factors) - in the susceptibility to PAR-toxicity.

I.3 Chemoprotection and structural modification

A tool in biomedical and toxicological research

The past 10 to 15 years, remarkably, PAR has become a model toxin and a tool in biochemical and clinical toxicological research, i.e. PAR has developed into a model compound for examination of the similarities and differences in toxicity mechanism between PAR and other xenobiotics, either with a similar structure (regioisomers and analogues or derivatives) or with similar toxicological features, such as pulegone and bromobenzene (Manautou *et al.*, 1995). Also, PAR is used as model toxin for establishing the usefulness of *in vitro* models such as liver slices in investigation of e.g. species differences in centrilobular damage (Miller *et al.*, 1993). With the elucidation of the many stages in the toxicity mechanism of PAR, an ever increasing number of chemical substances has been examined for potential chemoprotective properties (mechanism-based testing of chemoprotective properties of many substances (Chanda *et al.*, 1995; Mourelle *et al.*, 1990)), all targeted at a relatively specific biochemical action/lesion (Part VI) and investigated in various test systems, from reconstituted enzyme systems, microsomal incubations, via isolated hepatocytes, liver slices and perfused livers to whole animals. E.g. upon coincubation of PAR in rat liver slices with a suitable inhibitor of cytochrome P450, cell injury is prevented whereas addition after 2 h of incubation with PAR is ineffective. Then, however, treatment with the antioxidant dichlorophenol indophenol (DCPIP) is effective (Mourelle *et al.*, 1990). Some more important compounds examined and biochemical features studied as possible target site in chemoprotection are mentioned here: addition of GSH status recovering compounds like cysteine, clofibrate (Manautou *et al.*, 1996), addition of reducing compounds like ascorbic acid (De Vries, 1981), addition of antioxidant drugs like lobenzarit (anti-rheumatic) and curcumin (Donatus *et al.*, 1990; Remirez *et al.*, 1995), modulation of the conjugation with uridine-diphosphoglucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) (Fayz *et al.*, 1984), administration of garlic and related organosulfur compounds (Wang *et al.*, 1996), the antidotal effect of *N*-acetylcysteine (Corcoran *et al.*, 1985; Prescott, 1983), agents that protect against oxidative stress and lipid peroxidation (LPO) (Dai and Cederbaum, 1995; Harman, 1985; Harman and Fischer, 1983), compounds with glutathione peroxidase-like activity (Li *et al.*, 1994a), a

TABLE 2 Interspecies, interstrain and interindividual differences with respect to paracetamol-induced toxicity or biotransformation of paracetamol

Factor	Mechanism of action	Toxic effect	Reference
Interspecies differences			
hamster = mouse > guinea pig > rat	incr. bioactivation of PAR	incr. protein arylation	(Ioannides *et al.*, 1983)
hamster = mouse > guinea pig > rat	ratio toxication/detoxication pathways	incr. susceptibility	(Gregus *et al.*, 1988)
hamster > rat	ratio toxication/detoxication pathways	incr. susceptibility	(Miller *et al.*, 1993)
Interstrain/ethnic differences			
rat, Sprague-Dawleyvs Long Evans Hooded	incr. glucuronidation	decr. hepatotoxicity	(Price and Jollow, 1986)
rat, Fisher 344 vs Sprague-Dawley	yes and no deacetylation to *p*-aminophenol	Fisher 344 rats are susceptible to renal toxicity	(Newton *et al.*, 1985)
rat, hepatocytes (Sprague-Dawley vs Fischer344)	[GSH] in Fisher 344 higher than Sprague-Dawley rats	still, Fisher 344 rats are more susceptible to hepatotoxicity	(Willson *et al.*, 1991)
mouse, C57BL/6 vs DBA/2 strain	inducibility vs non-inducibility of CYP1A	susceptible vs resistent to cataractogenecity	(Lubek *et al.*, 1988a; Lubek *et al.*, 1988b; Wells *et al.*, 1995)
man, *in vivo*	genetic or environmental factors	2-fold inter-ethnic and 60-fold inter-subject variation in mercapturic acid excretion	(Critchley *et al.*, 1986)
Genetic 'deficiencies'			
man, hepatoma line	lack of CYP2E1 expression	no cytotoxicity	(Dai and Cederbaum, 1995)
man, 'Gilbert's syndrome'	lack of billirubin UDPGT [1]; decreased glucuronidatioon	incr. ratio urinary toxication/detoxication metabolites	(De Morais *et al.*, 1992)
Interindividual differences/lifestyle factors			
ageing as shown in mouse kidney, *in vivo*	incr. GSH and cysteine depletion	incr. susceptibility with age	(Richie *et al.*, 1992)
certain medicinal drugs	incr. glucuronidation	incr. excretion of glucuronide	references in (Burchell and Coughtrie, 1997)
diabetes (rat)	incr. UDPGA[2], incr. glucuronidation	decr. hepatotoxicity	(Price and Jollow, 1986)
diet (cruciferous vegetables) in man	incr. glucuronidation due to indoles	incr. excretion of glucuronide	referenced in (Burchell and Coughtrie, 1997)
fasting (rat)	altered carbohydrate metabolism	decr. glucuronidation (UDPGA[2])	(Price and Jollow, 1988)
obesity (rat)	incr. glucuronidation / decr. sulphation	incr. hepatic and renal toxicity	(Corcoran and Wong, 1987)
5-oxoprolinuria shown in human lymphocytes + activating system	GSH-synthetase deficiency	incr. cytotoxicity	(Spielberg and Gordon, 1981)
pregnancy in man	incr. glucuronidation and oxidation	incr. oral clearance / decr. elimination half-life	(Miners *et al.*, 1986)
sulfur deficiency (rat)	decr. sulphation	incr. hepatic necrosis	(Price and Jollow, 1989)

1 UDPGT = uridine diphosphoglucuronosyltransferase
2 UDPGA = uridine diphosphoglucuronic acid

compound with possible GSH reductase stimulating effect (Remirez *et al.*, 1995), inhibitors of P450 (Alexidis *et al.*, 1996; Li *et al.*, 1994b; Snawder *et al.*, 1993). The result of this type of research is also an increased understanding of the mechanisms of cytoprotective effects as such, i.e. independent of the primary damaging agent.

Structural modification

Besides efforts to modulate the PAR-toxicity or to understand the toxicity mechanism(s), important progress has been made as well by modifying the molecular structure of PAR itself. Regiosomers (congeners, e.g. 2'- and 3'-hydroxyacetanilide) and substituted analogues (derivatives) of PAR were synthesized and used in elucidating the relevance and the molecular mechanism of oxidative biotransformation in the observed toxicity of PAR (Barnard *et al.*, 1993a; Bessems *et al.*, 1996; Bessems *et al.*, 1997; Holme *et al.*, 1991; Van de Straat *et al.*, 1987b; Van de Straat *et al.*, 1986). Also, regioisomers and substituted analogues were studied for possible improvement of the analgesic properties and diminution of the toxic properties of PAR (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b; Bessems *et al.*, 1995; Harvison *et al.*, 1988a; Harvison *et al.*, 1986b; Holme *et al.*, 1991; Nelson *et al.*, 1978; Ramsay *et al.*, 1989; Rundgren *et al.*, 1988; Van de Straat *et al.*, 1987b; Van de Straat *et al.*, 1986; Weis *et al.*, 1996). These aspects are discussed in detail in Part VII. Notwithstanding the advantages of these more fundamental aspects of this research, the clinical relevance remains high as in 1992, in England and Wales, out of 1951 deaths due do overdoses of medicines, still 144 deaths were due to poisoning with PAR (Spooner, 1995).

I.4 Summary

Improvement of the therapeutic index of PAR could result from increase of the intrinsic analgesic activity (thereby lowering the therapeutic dose and possibly the size of a package), from decreased production of the toxic intermediate(s), from increased capacity to detoxify the toxic intermediate(s), from increased ability of tissue to withstand or even repair the molecular damage caused by toxic species and from modification of the chemical structure of PAR. This paper aims at reviewing studies that deal with the molecular, biochemical, and cellular aspects of the analgesic and, more importantly in this context, the toxic properties of PAR. Furthermore, the role of biotransformation in the activation and detoxification of PAR and some of the tools available for the protection against PAR toxicity will be discussed (chemoprevention and/or -protection). Special emphasis will be laid on the recent findings on arylation by PAR of a number of cytosolic, mitochondrial, microsomal and nuclear proteins and the consequences of modification of its molecular structure.

PART II BIOTRANSFORMATION - PHASE II

II.1 Bioinactivation and species differences

As indicated in the introduction, a large portion of ingested PAR is directly conjugated in detoxifying phase II reactions by sulphation and glucuronidation in most laboratory species. In humans, about 30 and 55% of administered PAR is excreted in urine as PAR-SULP and PAR-GLUC, respectively, whereas PAR-Cys and PAR-NAC each account for some 4% of the dose. At elevated though still therapeutic doses (1.5 g), sulphate conjugation becomes saturated with less than 20% and more than 75% being excreted as PAR-SULP and PAR-GLUC, respectively (Howie *et al.*, 1977; Tone *et al.*, 1990).

These findings are in line with general findings regarding sulphotransferases and UDP-glucuronosyltransferases (Mulder, 1990) and substantiated by data from rat. In liver perfusion experiments (via the portal vein), in contrast to PAR-SULP, recovery of PAR-GLUC in the perfusate and bile increased more than three-fold upon increase of the dose, indicating a higher capacity for glucuronidation than for sulphation, but a higher affinity for sulphation (Fayz *et al.*, 1984; Mitchell *et al.*, 1989). Similar results were obtained in rat hepatocyte incubations, where V_{max} values for sulphation and glucuronidation were comparable (1.1 and 1.3 nmol/10^6 cells/min, respectively) whereas K_M values were significantly different (0.03 and 2.1 mM, respectiely) (Mizuma *et al.*, 1985).

As mentioned in Part I, significant differences exist with regard to the susceptibility of various species to the hepatotoxic effect of PAR (Gregus *et al.*, 1988; Hinson, 1980; Ioannides *et al.*, 1983). Although even strain specificies exist, in general hamsters and mice are most sensitive, whereas rats, rabbits, and guinea pigs are relatively resistant to PAR-induced liver injury. Upon i.v. administration of 1 mmol/kg to the susceptible animal species, 27 to 42% of the dose is excreted as toxication pathway-related metabolites (PAR-SG and its hydrolysis breakdown products) in contrast to only 5 to 7% for the less sensitive species. In the sensitive species hamsters and mice, only 12 and 41%, respectively, of the dose are excreted as metabolites of the detoxifying route (PAR-GLUC and PAR-SULP) whereas in rats, rabbits, and guinea pigs these percentages are 62%, 27%, and 74%, respectively. Furthermore, hamsters and mice excreted mainly PAR-SG as such via bile (low γ-glutamyltranspeptidase (γGT) activity), whereas rabbits and guinea pigs excreted significant amounts of PAR-SG hydrolysis products via bile (high γGT activity) (Gregus *et al.*, 1988). These *in vivo* differences with respect to the ratio of toxication/inactivation in relation to centrilobular damage have been substantiated for rat and hamster in liver slices (Miller *et al.*, 1993). Therefore, it seems conceivable that the ratio between excretion (via bile) of PAR-SG (including breakdown products) and the combined excretion of PAR-GLUC and PAR-SULP, to some extent predict the toxicity of PAR in a specific species or even strain. The tendency of mice and hamsters to excrete more toxication pathway-related metabolites compared to rats and rabbits appears from Table 3.

TABLE 3 Metabolites of paracetamol found in excreta of species more and less sensitive to paracetamol-poisoning [1]

	Mouse		Hamster		Rat		Rabbit	1.5 g Man	>20 g
	Plasma	Urine	Bile	Urine	Bile	Urine	Urine	Urine	Urine
PAR	++	++		+			+	+	+
PAR-SULP	++	+	+ -	+	+	+++	+	++	+++
PAR-GLUC	++	++	+ -	++	+++	++	+++++	+++++	++++
3-OH-PAR [2]		+		_				+	
3-OCH_3-PAR [2]		+		+				+	+
PAR-SG		+	+++++		++		+		
PAR-Cys	+	++	+ -	+		-	+	+ -	++
PAR-NAC	+	++		++++		+		+	+
PAR-SCH_3		++		+++		+			
PAR-$SOCH_3$		++		++		+			
HQ-Cys		+							
HQ-NAC		+							
PAP				+					
References [3]	a	b	c	d	e	f	g	h	i

[1] Most abbreviations are self-explaining (see also text, mostly Part I), except maybe the following: HQ-Cys and HQ-NAC for the cysteine conjugate and mercapturic acid of 1,4-hydroquinone, respectively, and PAP for *p*-aminophenol. Furthermore, a (-)-sign means that analysis was amongst others aimed at that specific metabolite. A blank cell in the table means that the specific metabolite was just not encountered.

[2] Only data for the conjugated (GLUC and SULP) of PAR are indicated separately. Data for those conjugates of primary and secondary metabolites of PAR are included in the quantities of the corresponding aglycone.

[#] (a) (To and Wells, 1985), (b) (Hart *et al.*, 1982; Forte *et al.*, 1984; To and Wells, 1985; Pascoe *et al.*, 1988; Wang *et al.*, 1996), (c) (Madhu and Klaassen, 1991), (d) (Gemborys and Mudge, 1981; Warrander *et al.*, 1985), (e) (Mitchell *et al.*, 1989), (f) (Hart *et al.*, 1982; Price and Jollow, 1982; Corcoran and Wong, 1987), (g) (Lubek *et al.*, 1988), (h) (Howie *et al.*, 1977), (i) (Mrochek *et al.*, 1974; Knox and Jurand, 1977; Slattery *et al.*, 1987)

II.2 Interorgan transport of metabolites

Two major organs, the kidneys and intestine, have been implicated in the metabolism of sulfur-containing metabolites of PAR. Both organs contain γGT and dipeptidase for the breakdown of PAR-SG to PAR-CG and PAR-Cys. The kidneys play a major role in the disposition of sulfur-containing metabolites of PAR, either by direct excretion (glomerular filtration of PAR-Cys and probenecid-sensitive active transport of PAR-NAC) or by further biotransformation with subsequent renal excretion. The hamster urinary metabolites PAR-SCH_3 and PAR-$SOCH_3$ are derived

from PAR-SG breakdown products within the enterohepatic circulation (Gemborys and Mudge, 1981; Newton *et al.*, 1986). The fate and behaviour of glutathione- and cysteine-conjugates in general were reviewed (Commandeur *et al.*, 1995).

Collection of bile from PAR-dosed mice, containing mainly the GSH conjugate of PAR, reduced the urinary excretion of the pre-mercapturate (cysteine conjugate) and the mercapturate by >70%, indicating that these urinary metabolites originated from the biliary GSH-conjugate. However, ligation of the common bile duct did not alter this urinary excretion, indicating that enteroheptic circulation is not obligatory for the appearance of the (pre)mercapturates of PAR in urine. Intravenous administration of purified PAR-SG conjugate did not result in biliary excretion of the parent conjugate but in urinary excretion of primarily the PAR-Cys conjugate. Together with other results, these findings suggest that if the PAR-SG leaves the liver (via the blood), it can rapidly be converted to the PAR-Cys conjugate by γGT and dipeptidase, which appear in the intestine as well as the kidneys (Fischer *et al.*, 1985a). However, γGT-dependent breakdown may also occur in the bile duct and gallbladder.

It was suggested that in species such as guinea pig and perhaps also in humans, the liver and the bile duct play a more important role in breakdown of GSH-conjugates than in rat and mouse, two species that have been used extensively in research on PAR-dependent toxicity (Hinchman and Ballatori, 1990). As in liver of rat and mouse γGT-activity is very low relative to that of kidneys (Hinchman and Ballatori, 1990), the importance of the extrarenal breakdown of PAR-SG in humans may have been widely underestimated. Moreover, also the absence of a gallbladder in rat, a site where significant γGT-activity is localised in other species, may have contributed to this underestimation. In macaque relative to rat, the γGT-activity (per mg protein) in kidneys and liver is about eightfold lower and two- to threefold higher, respectively, making the ratio of γGT-activities between liver and kidneys almost twentyfold higher in macaques relative to rats. In addition, the γGT-activity in gallbladder (which is absent in rats) in macaque is even a little higher than in liver (Hinchman and Ballatori, 1990).

II.3 Summary

The species-specific susceptibility to PAR-dependent toxixity seems to be quite accurately reflected by the urinary metabolites. The susceptible species mice and hamster mainly excrete toxification pathway-related metabolites (PAR-SG and breakdown products), whereas the relatively insensitive species guinea pig, rat and rabbit excrete much more detoxication pathway-related products, such as PAR-SULP and PAR-GLUC. The site of breakdown of PAR-SG starting with activity of γGT may be also important as PAR-Cys, although a breakdown product of PAR-SG, may still possess hazardous properties. Some species dispose mainly PAR-SG via bile and/or hepatic vein, whereas others dispose mainly hydrolysis products of PAR-SG. It should be noted that these differences are caused not solely by species variance in phase II biotransformation enzymes but also by differences in the activity of phase I enzymes (see Part III).

The complex pattern of the primary as well as secondary biotransformation of PAR in man, including the distribution of PAR and metabolites, is presented in Fig. 2. The most striking interspecies differences with respect to metabolite formation, especially for detoxification versus toxification pathway-related metabolites, are presented in Table 3.

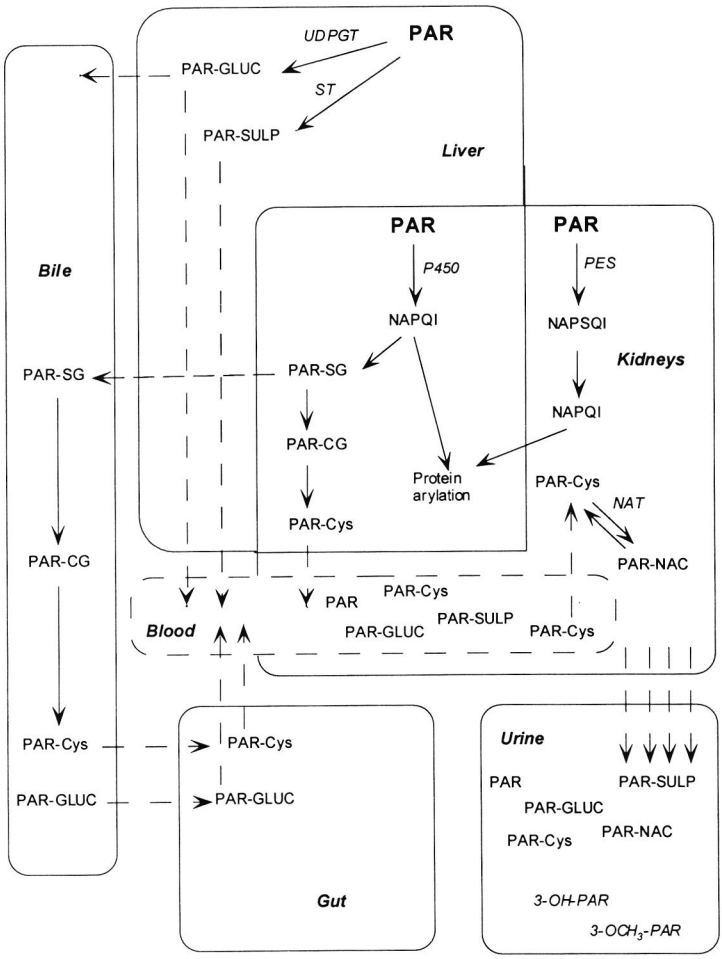


FIG. 2 Disposition of paracetamol in humans
Paracetamol disposition (distribution and metabolism) in human serum, urine and feces (see text for references). The disposition of the minor urinary metabolites 3-OH-PAR and 3-OCH_3-PAR is not indicated but suggested to occur via hepatic formation and renal excretion.

PART III BIOACTIVATION - PHASE I

III.1 Cytochrome P450

Mechanism of oxidation

P450 enzymes are the most predominant drug metabolising enzymes in the liver and are also present in most other tissues of the body. Thus, it is not unexpected that the cytochrome P450 mediated oxidative bioactivation of PAR was the subject of several extensive reviews (Hinson *et al.*, 1995; Nelson, 1995; Vermeulen *et al.*, 1992). There is general consensus now that *N*-acetyl-*p*-benzoquinone imine is the main electrophilic reactive metabolite formed in the oxidative biotransformation of PAR *in vitro* and *in vivo* (Hinson *et al.*, 1981; Miner and Kissinger, 1979). Although its direct detection in *in vitro* systems is very difficult (Dahlin *et al.*, 1984), and the exact mechanism of formation is still not unequivocally identified (Bessems *et al.*, 1998; Koymans *et al.*, 1989; Myers *et al.*, 1994), NAPQI has been detected as its glutathione conjugate in numerous *in vitro* and *in vivo* systems (Bessems *et al.*, 1997; Harvison *et al.*, 1988b; Hinson *et al.*, 1982; Newton *et al.*, 1986; Van de Straat *et al.*, 1986).

Originally, *N*-hydroxylation or 3,4-epoxidation was postulated to be the first step in P450 catalysed oxidation of PAR followed by dehydration to the electrophile NAPQI. However, upon synthesis, the hydroxamic acid (hydroxylamine derivative) that would be formed upon *N*-hydroxylation, exhibited a reported half-life of about 15-80 minutes, but was never detected in oxidative microsomal systems. Therefore *N*-hydroxylation was ruled out as the mechanism of bioactivation of PAR by P450 (Calder *et al.*, 1981; Gemborys and Mudge, 1981; Hinson *et al.*, 1979; Hinson *et al.*, 1980). A second postulated mechanism, i.e. the 3,4-epoxidation in the aromatic ring, was rejected by using $^{18}O_2$ and epoxide hydrolase in a rat liver microsomal incubation (Hinson *et al.*, 1980).

As a third mechanism, oxidation of PAR to NAPQI via the free radical species *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) was proposed (De Vries, 1981). Shortly thereafter, computational data were presented for two sequential hydrogen abstractions leading to NAPQI (designated as the peroxidase pathway), being thermodynamically favoured over oxygenation with hydroxylamine formation (*N*-hydroxy metabolite) (Loew and Goldblum, 1985). However, no important differences were predicted by the semi-empirical model of Loew and Goldblum for the first hydrogen abstraction being abstracted from the phenolic oxygen of PAR or from the acetylamino-nitrogen (Loew and Goldblum, 1985). By using an improved computational method (*ab initio*), Koymans *et al.* argued that primary hydrogen abstraction from the phenolic oxygen was energetically favoured over an abstraction from the acetylamino-nitrogen. In their model, this was predicted to be followed by either a second hydrogen abstraction, leading to NAPQI, or hydroxyl radical recombinations, leading to 3-hydroxyparactemol (3-OH-PAR) or an *ipso*-adduct intermediate, the latter giving rise to *p*-benzoquinone (PBQ) and acetamide. Also NAPQI was proposed to be able to hydrolyse to pBQ and acetamide (Koymans *et al.*, 1989). This mechanism is depicted in Fig. 3.

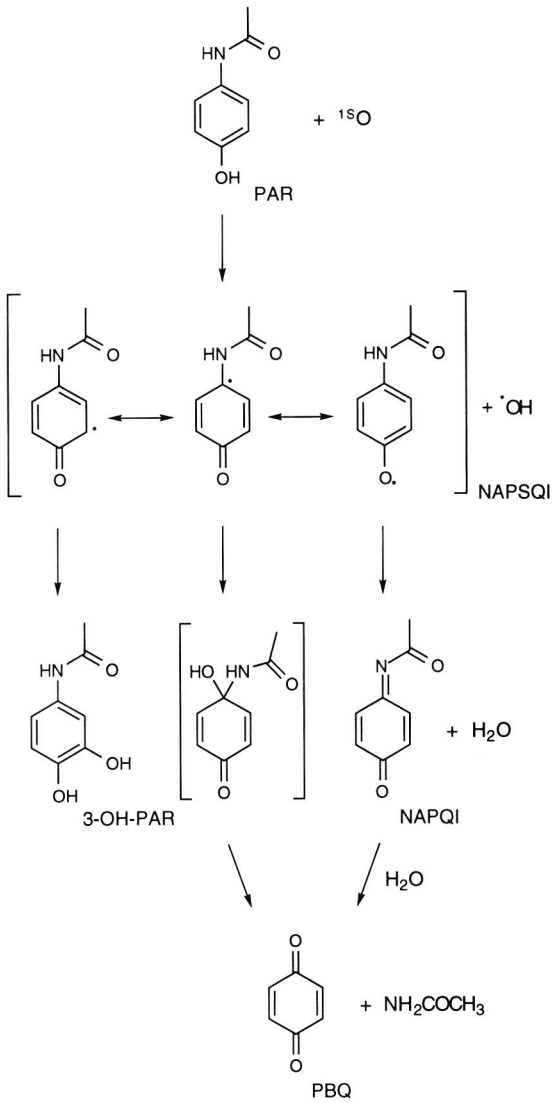


FIG. 3 Hypothesized phenoxy radical pathway.
Hypothesized oxidation of PAR by singlet oxygen, a substitute for cytochrome P450. An initial hydrogen abstraction occurs at the phenolic hydroxyl group of PAR and is followed by delocalisation of the radical. Recombination of the hydroxyl radical, formed in the active site of P450 from singlet oxygen, can give rise to 3-hydroxy-paracetamol (3-OH-PAR), *p*-benzoquinone (PBQ) plus acetamide and NAPQI, which have been found as three minor and one major P450-dependent metabolites of PAR, respectively. Modified from Koymans *et al.* (1989).

Moreover, the experimental finding of 3-OH-PAR, PBQ and acetamide, next to NAPQI as a P450 dependent metabolites of PAR in the past (Corcoran *et al.*, 1980; Forte *et al.*, 1984; Hinson *et al.*, 1982; Hinson *et al.*, 1980; Miner and Kissinger, 1979), combined with NMR-relaxation studies on the binding of PAR in the active site of different P450 enzymes (Myers *et al.*, 1994; Van de Straat *et al.*, 1987a), prompted several authors to support this mechanistic interpretation (Hoffmann *et al.*, 1990; Myers *et al.*, 1994). Interestingly, NAPSQI was detected indirectly (a melanine-like signal was actually observed) with ESR in a reductive mixed-function oxidase catalysed reaction (Van de Straat *et al.*, 1987d) upon the addition of NAPQI to an anaerobic, reconstituted system containing P450 and P450-RED. The absence of oxygen, as indicated by the authors, might imply that the reduction of NAPQI was due to the electron-donating activity of P450-RED only or of P450 + P450-RED in combination, but without involvement of oxygen as shown also under anaerobic conditions for 2,3,5,6-tetramethylbenzoquinone (Goeptar *et al.*, 1992).

However, it cannot be fully excluded that reduction of NAPQI was due to the oxygen reductase activity of P450 in the presence of small quantities of oxygen, as reviewed recently by Goeptar *et al.* (Goeptar *et al.*, 1995). As suggested here this could imply that the thus formed superoxide anion radical could be responsible for the reduction of NAPQI to NAPSQI with concomitant oxidation of the superoxide anion radical to molecular oxygen. Also, the NAPSQI could oxidise NADPH to NADP$^{\bullet}$. The NADP$^{\bullet}$ thus formed might react with O_2 to produce the superoxide anion $O_2^{-\bullet}$ again as was suggested earlier (Keller and Hinson, 1991). An 'all in mechanism' as proposed here is depicted in (Fig. 4). Moreover, so far still no direct evidence has been found for the existence of such semiquinone imine radical intermediates during their P450 catalysed oxidation of PAR and substituted analogues beyond the active site of the P450 enzymes (Bessems *et al.*, 1998). Therefore, as yet any discussion with respect to the relevance of NAPSQI in the mechanism of P450-dependent toxicity of PAR remains speculative.

It cannot be excluded that recently found differences in kinetics between cysteine conjugation and liver microsomal protein binding of PAR should be seen in the context of the P450-based mechanism of formation of NAPQI from PAR. After an initial linear increase in both reactions with increase of NADPH concentrations, further increase of this cofactor significantly decreased cysteine conjugation while the rate of protein binding plateaued. Furthermore, NADH and NADPH reacted fairly different upon addition to incubations with PAR concerning modulation of cysteine conjugation and protein binding. Also, ethanol feeding of mice (with probably CYP2E1 induction) before isolation of the microsomes, increased protein binding by about 97%, but cysteine conjugation by only 33% (Zhou *et al.*, 1997b). Although the absolute rates of formation were in the range of pmol/mg and nmol/mg microsomal protein for protein binding and cysteine conjugation, respectively, these differences might be due to some formation of NAPSQI by CYP2E1. Protein binding of NAPSQI could be favoured over cysteine conjugation.

P450s involved

Incubations with purified and reconstituted rat liver microsomal cytochrome P450 and PAR showed that, from 9 purified P450 enzymes studied, the constitutive and male-specific CYP2C11 (cytochrome $P450_{UT-A}$) exhibited the highest rate of formation of NAPQI (Table 4). The next highest was the ßNF-inducible CYP1A1 (cytochrome $P450_{ßNF-B}$). In addition, almost all of these rat P450s exhibited significant formation of 3-hydroxyparacetamol (3-OH-PAR). Moreover, the phenobarbital (PB)-inducible CYP2B1 (cytochrome $P450_{PB-B}$) primarily formed (3-OH-PAR) (Harvison *et al.*, 1988b). Except that species differences exist with respect to the expression of specific P450s, e.g. humans do not constitutively express CYP1A1 (Berthou *et al.*, 1992), it must be kept in mind, however, that results from reconstituted enzyme incubations may be less predictive for the *in vivo* situtation compared to microsomal incubations. In microsomal incubations, rat liver CYP1A2, CYP2E1, CYP3A1, CYP3A2 and the human liver CYP3A4 catalyse the oxidation of PAR to NAPQI (Patten *et al.*, 1993; Thummel *et al.*, 1993). In contrast to recent studies where PAR metabolites were not detected in human Hep G2 and lymphoblast cell lines transfected with P450 2A6 DNA (Patten *et al.*, 1993), baculovirus-expressed and purified human CYP2A6 (as well as CYP2E1) was shown to oxidise PAR to NAPQI as well as the nontoxic 3-OH-PAR (Chen *et al.*, 1998). And albeit at toxic doses of PAR, CYP2E1 was found to be the more efficient catalyst for the bioactivation to NAPQI (relative ratio NAPQI:3-OH-PAR formation was approximately 6:1), CYP2A6 also can contribute significantly to NAPQI formation (relative ratio 1:3) (Chen *et al.*, 1998). In addition, strong *in vitro* evidence was obtained from microsomal incubations retrieved from a transfected human lymphoblast cell line, that also human CYP2D6 is involved in oxidation of PAR (Zhou *et al.*, 1997a). For an overview, see Table 4.

In mice, where CYP3A4 has not been identified (Zaher *et al.*, 1998), CYP2E1 is probably the most important hepatic P450 enzyme involved in the bioactivation of PAR at low doses with little additional contribution at the high dose, whereas CYP1A2, probalbly exhibiting a higher K_M, contributes more to the bioactivation and toxicity of PAR at high doses (Hu *et al.*, 1993; Snawder *et al.*, 1994). Findings of resistance, only slight resistance and high resistance against PAR-mediated toxicitywith *cyp2e1* knock-out mice (CYP2E1 null phenotype), CYP1A2 null mice and mice being double-null for CYP1A2 and CYP2E1, respectively, provided definite proof for the dose-dependent involvement of CYP2E1 and CYP1A2 in bioactivation of PAR (Lee *et al.*, 1996; Tonge *et al.*, 1998; Zaher *et al.*, 1998). In human *in vitro* systems, CYP1A2 also exhibits high K_M kinetics (low affinity; only effective at high concentrations) whereas CYP2E1 - and actually CYP3A4 even more - displays low K_M kinetics (high affinity; already effective at low concentrations) (Patten *et al.*, 1993; Raucy *et al.*, 1989; Thummel *et al.*, 1993). The human CYP2C8 and CYP2C9 exhibited only negligible activity (Raucy *et al.*, 1989). The major P450 enzymes in the Caucasian as well as the Japanese population are those of the CYP3A (about 30% of total P450) and CYP2C (about 20%) subfamily, followed by CYP1A2 (about 13%), CYP2E1 (about 7%), CYP2A6 (about 4%), CYP2D6 (about 2%) and CYP2B6 (< 1%) (Shimada *et al.*, 1994). Thus, CYP3A4 is probably the most important P450 at therapeutic

TABLE 4 Role of different P450 enzymes (CYP) from liver in deactivation and activation of paracetamol

CYP	Mouse		Hamster		Rat (#)		Rab	Man		
	(1)	(2)	(3)*	*in vivo*	(4)	(5)*	(6)	(7)*	(8)*	(9)
1A1					++++ (#)		+			
1A2	++	+	++	++	++	++++	+	++++	+	
2A1					+ (#)					
2A6										+
2B1					+					
2B2					+ -					
2C3							-			
2C6					+ -					
2C8								+ -		
2C9								+ -		
2C11					+++++ (1)					
2C12					+ - (#)					
2E1	++ !	++	+			++++	+	+++++	+++	++
2D6									++	
3A?				++						
3A1						+				
3A2					+ -	+				
3A4										
3A6							-	++ ($)	+	
Ref.	a	b	c	d	e	f	g	h		i

(1)! Reconstituted CYP2E1 at low concentrations; CYP1A2 at high concentrations
(2) CYP1A2 null mice were only slightly resistant to PAR-mediated toxicity at relatively low dose; CYP1A2 and CYP2E1 ouble-null mice were highly resistant to PAR-induced toxicity
(3) Microsomal incubations.
(4) Reconstituted systems. In the original reference, the following nomenclature of the CYP enzymes was used: ßNF-B (CYP1A1), ISF-G (CYP1A2), UT-F (CYP2A1), PB-B (CYP2B1), PB-D (CYP2B2), PB-C (CYP2C6), UT-A (CYP2C11),
UT-I (CYP2C12), PCN-E (CYP3A2). For a large part of the enzymes, also significant amounts of 3-OH-PAR were formed.
(5) Control rat male microsomes.
(6) Reconstituted systems. In the original reference, the following nomenclature of the P450s was used: 6 (CYP1A1), 4 (CYP1A2), 3b (CYP2C3), 3a (CYP2E1), 3c (CYP3A6)
(7) Levels from microsomal incubations using monoclonal antibodies are presented. However, also reconstituted systems were used. Activities covaried with the specific content of the P450s in human liver microsomal samples.
(8) Incubations using microsomes from human lymphoblasts that were transfected with human P450s.
(9) Reconstituted systems from baculovirus-expressed and purified P450s

(#) In rat, the formation of 3-OH-PAR was investigated and appeared to be substantial or even outreached the NAPQI formation, measured as the GSH-conjugate
($) It should be noted that the levels presented are for microsomal incubations. However, the KM observed corresponds to the KM for CYP3A4 (0.15 mM), suggesting that at therapeutic concentrations in humans, CYP3A4 is the most important P450 involved in bioactivation.
* Microsomal incubatios are more relevant for estimation of the relative contribution of the various P450 enzymes

References: (a) (Snawder *et al.*, 1994), (b) (Tonge *et al.*, 1998; Zaher *et al.*, 1998), (c) (Madhu *et al.*, 1989; Raucy *et al.*, 1989), (d) (Madhu *et al.*, 1989), (e) (Harvison *et al.*, 1988), (f) (Patten *et al.*, 1993), (g) (Morgan *et al.*, 1983; Jeffery *et al.*, 1991), (h) (Raucy *et al.*, 1989; Thummel *et al.*, 1993), (i) (Zhou *et al.*, 1997), (j) (Chen *et al.*, 1998)

concentrations whereas CYP2E1 and CYP1A2 becoming significantly involved at high plasma levels and at serious intoxication, respectively.

However, even incubations using human liver microsomes are a flattered mirror imaging of actual situations in which humans may be exposed to PAR overdose situations. Scaling to whole organ situations by using Michaelis Menten kinetic parameters like K_M (Michaelis constant) and V_{max} (as determined in microsomal incubations or reconstituted enzyme systems) might therefore be an important tool in finding out the practical relevance of the various P450 enzymes. Another tool for *in vivo* investigations of agents that are known to specifically modify one or more enzymatic biotransformation steps *in vitro*, is pharmacokinetic analysis of human plasma and urine levels of PAR and various metabolites. Using a simple descriptive combined one compartment pharmacokinetic model (first-order absorption and elimination of PAR combined with first-order formation and elimination of its metabolites), the human pharmacokinetics of PAR were studied. In addition, fits of plasma and urine data clearly demonstrated the inhibitory effect of intake of watercress (which probably contains a precursor of the CYP2E1 inhibitor phenetyl isothiocyanate) on *in vivo* oxidative metabolism of PAR (leading to PAR-Cys and PAR-NAC) (Chen *et al.*, 1996). Even more sophisticated is the integration of *in vitro* biotransformation data on e.g. P450 inhibitors in predictive physiologically based models that can be used to predict the effects of various modulators. Such PBPK (physiologically based pharmacokinetic) models have been set up for e.g. for dibromoethane (Ploemen *et al.*, 1997) and offer the possibility to incorporate and modulate other relevant biotransformation (e.g. conjugating) enzymes and other relevant organs. Also, other process than biotransformation only, absorption, distribution and excretion, could be incorporated in this way (Tone *et al.*, 1990).

As mentioned above, in mice, rats as well as humans exposed to ethanol, next to CYP2E1, CYP3A is an important subfamily of P450 enzymes involved in the bioactivation and consequently the hepatotoxicity of PAR. Interestingly, CYP2E1 as well as enzymes of the CYP3A subfamily are very likely suicidally inactivated by PAR (Kostrubsky *et al.*, 1997b). This supports the ambiguous results that have been presented in the past regarding the protecting or activating role of CYP2E1 ligands (Anundi *et al.*, 1993; Burk *et al.*, 1990). Many inducers act by stabilization of the CYP2E1 protein (Eliasson *et al.*, 1992) but at the same time, are competitive inhibitors. The balance between stabilization and competetitive inhibition of PAR bioactivation will determine the observed effect (Dai and Cederbaum, 1995). In humans, isoniazid inhibited oxidation of PAR, measured as urinary excretion of the thioether of PAR and 3-OH-PAR, when both drugs were present at the same time, but 1 day after isoniazid was discontinued, bioactivation of PAR increased (Zand *et al.*, 1993). In addition to the importancy in hepatic biotransformation, CYP2E1 is

responsible for about 50% of renal bioactivation of PAR in mice. Interestingly, the oxidative metabolism of APAP in control male mouse kidney microsomes displayed an apparent low K_M of 43-45 μM and an apparent high K_M of 603-702 μM (Hu *et al.*, 1993).

The ratio between formation of NAPQI and 3-OH-PAR upon P450-dependent oxidation of PAR is not constant (Harvison *et al.*, 1988b; Zand *et al.*, 1993). The relative extent of NAPQI and 3-OH-PAR metabolite formation by different cytochrome P450 enzymes could be related to a P450 enzyme-specific orientation in the respective active sites (Myers *et al.*, 1994; Van de Straat *et al.*, 1987a). In humans, the relative contribution of the three main P450 enzymes involved (CYP1A2, CYP2E1, and CYP3A4) varies considerably and depends on various life stile factors and the use of pharmaceutical drugs (Guengerich, 1995; Raucy *et al.*, 1989). An overview of the P450 enzymes involved is presented in Table 4.

Recently, strong indications were found that also 3'-hydroxyacetanilide (3'-HAA), a nontoxic regioismer of PAR (4'-HAA), is a substrate of CYP2E1. Liver microsomal CYP2E1 enzyme activity was decreased and an anti-arylacetamide reactive protein adduct was detected in a protein that comigrated with CYP2E1 in mice treated with 3'-HAA. Also, incubation of 3'-HAA with hepatic microsomes resulted in a time dependent decrease in CYP2E1 enzyme activity whereas pre-incubation of microsomes with PAR did not result in covalent binding to or inhibition of CYP2E1 (Halmes *et al.*, 1998; Matthews *et al.*, 1997; Myers *et al.*, 1995; Salminen *et al.*, 1998).

III.2 Peroxidases

General

Although a wide range of xenobiotic substrates can be bioactivated by one or more P450 enzymes of the cytochrome P450 superfamily, also other enzymes may be relevant in the process of activation of PAR (phase I bioactivation). These are the peroxidase group of enzymes comprising myeloperoxidase, chloroperoxidase and lactoperoxidase (Nelson, 1981; Potter and Hinson, 1989; Potter *et al.*, 1986) but also prostaglandin *H* synthase (Harvison *et al.*, 1988a; Harvison *et al.*, 1986a; Potter and Hinson, 1987), which has been found in almost every mammalian tissue that has been investigated (Eling and Curtis, 1992). In white blood cells, for example, myeloperoxidase has been shown to bioactivate a wide range of drugs. In other tissues low in P450 activity, prostaglandin *H* synthase may also be responsible for bioactivation. For example in the kidneys, PAR-toxicity is thought to result from activation via this enzyme (Pirmohamed *et al.*, 1996). Horseradish peroxidase, as it is an enzyme isolated from a plant, has no direct relevance for these processes in mammals, although it has been used widely as a model enzyme system in mechanistic investigations into the bioactivation processes of xenobiotics (Bessems *et al.*, 1998; Josephy *et al.*, 1983; Metodiewa *et al.*, 1992; Potter *et al.*, 1986; Ross *et al.*, 1985).

Catalytic activities

It has to be noted that important differences exists between peroxidases in general and prostaglandin *H* synthase (PGHS) or prostaglandin synthase (PGS), which are

both synonymous for prostaglandin-endoperoxide synthase (PGES; which is the officicial name; EC 1.14.99.1). PGES exhibits two distinct activities of which one is exhibited only by PGES: the cyclooxygenase activity (synonymous to prostaglandin

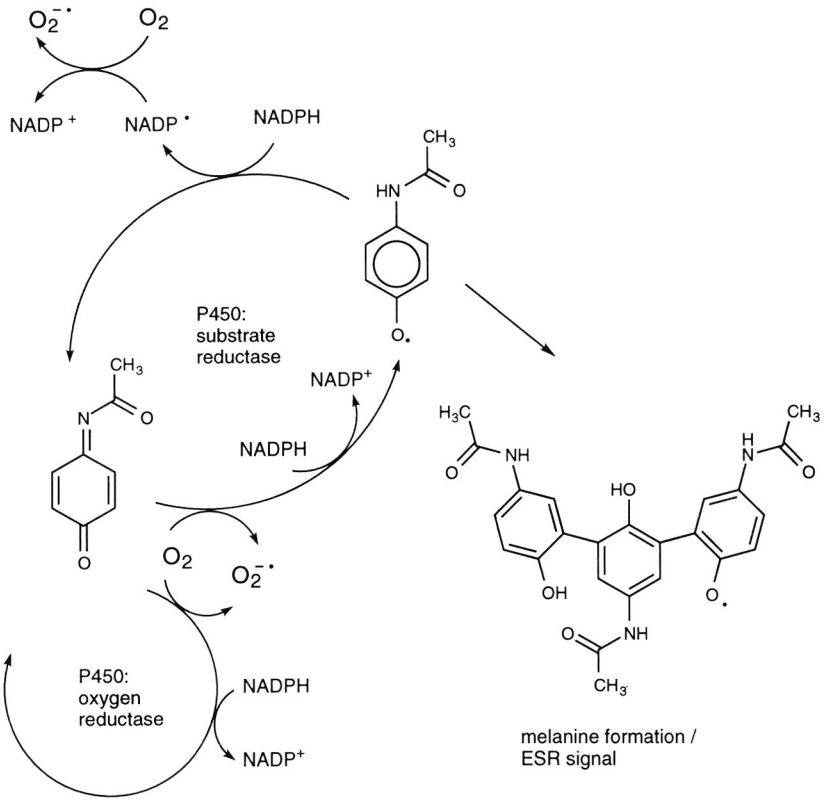


FIG. 4 Proposed 'all in mechanism' for P450 incubations with NAPQI
Composed from observations reported in Van de Straat *et al.*, 1987d and Keller and Hinson, 1991. See text for further explanations.

cyclooxygenase and fatty acid oxygenase) that catalyzes the oxygenation of arachidonic acid (AA) to its hydroperoxy endoperoxide (ROOH; PGG_2) with concomitant formation of water and consumption of two molecules of oxygen. The other one, the peroxidase or hydroperoxidase activity, that catalyzes the reduction of the hydroperoxy endoperoxide of arachidonic acid (PGG_2) to the hydroxy endoperoxide (ROH; PGH_2), is exhibited by all peroxidases (Kulmacz *et al.*, 1991; Kulmacz *et al.*, 1994; Moldéus *et al.*, 1982; Moldéus and Rahimtula, 1980). The therapeutic action of PAR is almost undoubtedly based on inhibition of the cyclooxygenase activity of PGES, which prevents prostaglandins from being formed thereby lowering body temperature from fever to more normal levels (antipyretic activity) and tempering the pain sensation (analgesic activity) (Flower and Vane, 1972; Harvison *et al.*, 1986a; Malmberg and Yaksh,

1982; Mattamal *et al.*, 1979). Although peroxidase activity is only one aspect of PGES, various laboratories have been trying to investigate the activation mechanism of PAR by using specific peroxidases, e.g. by studying possible structure activity relationships (oxidation potentials, coplanarity of *N*-acetyl side chain etc) for various PAR analogues (Barnard *et al.*, 1993b; Bessems *et al.*, 1998; Bessems *et al.*, 1995; Harvison *et al.*, 1988a; Harvison *et al.*, 1986a; Harvison *et al.*, 1986b; Park and Kitteringham, 1994).

Pharmacological cyclooxygenase-inhibition

The cyclooxygenase-inhibiting activity of PAR was suggested to be related to its capacity to quench the tyrosyl radical present in PGES (Kulmacz *et al.*, 1991). Even monomethylated analogues of PAR were found to be capable of inhibiting PGES (Harvison *et al.*, 1988a; Harvison *et al.*, 1986b). Also, PAR analogues with fluorine substitutions adjacent to the hydroxyl group, adjacent to the amide or in the acetamide group exhibited, although varying, *in vivo* analgesic and *in vitro* cyclooxygenase inhibiting capacities (Barnard *et al.*, 1993b; Bessems *et al.*, 1995; Park and Kitteringham, 1994). In general, coplanarity of the acetamide group with the phenyl ring is important in the cyclooxygenase inhibiting capacity of PAR analogues (Barnard *et al.*, 1993b; Bessems *et al.*, 1995).

Bioactivating activities

In contrast to PGES (see below), the peroxidase enzymes probably exhibit only one-electron oxidation activity towards PAR under physiological conditions (Potter and Hinson, 1989). PAR and several ring-alkylated and -halogenated analogues were shown to be liable to one-electron oxidative biotransformations by peroxidase enzymes to their respective alkylated and halogenated NAPSQI-analogues (Bessems *et al.*, 1998; Fischer and Mason, 1984; Fischer *et al.*, 1985b; Mason and Fischer, 1986). PAR could also substitute catalytic amounts of the cosubstrate serotonin in myeloperoxidase-oxidase reactions with cysteine as substrate. Eosinophil, lacto- and horseradish peroxidase could catalyse these reactions as well (Svensson, 1989). As myeloperoxidase is mainly present in polymorphonuclear leukocytes (PMNs) in humans (Chamulitrat *et al.*, 1991), this enzyme might be involved in the later stages of hepatic damage as observed upon PAR intoxication (see Part IV). A significant role for myeloperoxidase in the bioactivation process of PAR was suggested when protein binding and nucleic acid binding (DNA and RNA) of PAR was observed upon stimulation of the respiratory burst in neutrophylic type differentiated leukemic HL-60 cells. A mechanism was proposed requiring one-electron oxidation of PAR (Corbett *et al.*, 1989; Corbett *et al.*, 1992). Furthermore, metabolic activation by myeloperoxidase in neutrophils or stem cells, leading to free radical metabolite formation was suggested to be the cause of agranulocytosis, as observed rarely following PAR intake (Mason and Fischer, 1992).

In contrast, PGES is suggested to exhibit two activities with respect to PAR, similar to the dual activity in the synthesis of prostaglandins. Strong indications for one-electron oxidation as well as two-electron oxidation to NAPSQI and NAPQI,

respectively, have been obtained with *in vitro* experiments using microsomes from sheep seminal vesicles (Moldéus *et al.*, 1982; Moldéus and Rahimtula, 1980; Potter and Hinson, 1987; Potter and Hinson, 1989). Also, PGES from rabbit kidney inner medulla was suggested to exhibit metabolic oxidation of PAR, next to being inhibited by PAR (Mattamal *et al.*, 1979; Mohandas *et al.*, 1981). As mentioned above, the inhibition of PGES by PAR is the basis of the pharmacological properties of PAR which in its essence, are due to inhibiton of the prostaglandin synthesis (Harvison *et al.*, 1986a). Unfortunately however, it might be as well this PGES activity in the kidneys, as is hypothesized in this review, that is reponsible for the increased risk of chronic renal disease that has been ascribed to long-term use of PAR (Sandler *et al.*, 1989).

III.3 Summary

With respect to PAR-dependent hepatotoxicity it is generally accepted that P450-dependent bioactivation of PAR is a main cause for ultimate potentially fulminant hepatic necrosis upon administration or intake of a lethal dose of PAR. *N*-acetyl-*p*-benzoquinone imine (NAPQI) is presumed to be the ultimate metabolite causing depletion of GSH and protein thiols and arylation of of the latter. The exact catalytical mechanism of formation of NAPQI is as of yet unknown, be it a direct two-electron oxidation (abstraction of two hydrogen atoms) or a sequential mechansim of two successive single hydrogen atom abstractions with a free radical intermediate, i.e. *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI). Another, non-toxic P450-dependent metabolite is 3-hydroxyparacetamol (3-OH-PAR). In man, CYP3A4 is probably the most important P450 at therapeutic concentrations with CYP2E1 and CYP1A2 becoming significantly involved at higher concentrations and at serious intoxication, respectively. In mice and rats CYP2E1 and CYP1A2 are most important. Furthermore, the formation in significant amounts of the non-toxic 3-OH-PAR in rat by various of the P450 enzymes involved in the oxidation of PAR (Table 4), may be important in the low sensitivity of rat compared to mouse for PAR-dependent hepatotoxicity. In the less sensitive rabbit, P450 catalysed biotransformation may be at all of minor importance although few data were found on P450 activities with respect to PAR in this species.

The analgesic action of PAR is dependent on inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase (PGES) although the exact mechanism is not known. PGES is present in many tissues. For cyclooxygenase inhibitory activity, a *p*-hydroxyacetanilide structure is required although also analogues substituted *ortho*- to the phenolic hydroxyl group and analogues substituted in the methyl group of the acetyl moiety, such as *N*-trifluoromethylacetyl-*p*-aminophenol, still possess this potential. Importantly, the *N*-acetyl group should be coplanar.

Next to activity of the general peroxidases such as myeloperoxidase, the PGES activity may also be relevant for toxicity of PAR, especially in the kidneys at chronic low intake levels. Protein- but also RNA and DNA adducts may result from peroxidase-mediated bioactivation of PAR, maybe via formation of the radical *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI). At high concentrations in the kidneys, P450 is probably more important for bioactivation, i.e. CYP2E1. Renal CYP2E1-dependent bioactivation displayes species and even sex-differences.

PART IV MECHANISMS OF HEPATOTOXICITY

IV.1 General

Activation and inactivation

The hepatotoxicity of PAR is generally accepted to be primed by the formation of NAPQI, a metabolite formed during cytochrome P450 catalysed oxidation of PAR (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b). Hepatotoxic damage occurs mainly in the centrilobular (perivenous) zone (Anundi *et al.*, 1993). Recently, a model, filled with known published parameters on GSH synthesis, degradation, and transport, was developed to examine the bimolecular reaction of GSH conjugation with acceptor substrates. Simulations were performed to obtain the vascular and intracellular GSH concentrations in the absence and presence of PAR. The simulated results suggest that the average tissue GSH concentration as normally determined in liver homogenate and the formation of the PAR-SG conjugate are poor indices of the extent of toxic exposure. As the formation of NAPQI is regarded to be the rate-limiting step in the formation of the PAR-SG conjugate, the high concentration of cytochrome P450 enzymes in the perivenous region is probably more important for the observed zonal toxicity than the low GSH content (Chiba and Pang, 1995). It has to be noted, however, that also the zonal distribution of the primary phase II detoxication reactions, sulphation and glucuronidation, may be important in the observed zone-specific toxicity. Sulphation (which exhibits high affinity) is predominant in the periportal region (Pang, 1990), as shown specifically for PAR in the isolated perfused rat liver (Mitchell *et al.*, 1989). This finding was confirmed specifically for phenol sulphotransferase which exhibited a slight predominance in (isolated) perivenous hepatocytes (Tosh *et al.*, 1996). Glucuronidation was found to be active in both zones, however, at high concentrations, the high capacity of the glucuronidation seemed to be most predominant in the periportal zone (Mitchell *et al.*, 1989). This is probably due to the high capacity properties of UDP-glucuronosyltransferase (higher K_M and higher V_{max} than sulphotransferase). See also Part II for conjugating reactions.

Toxicity and defense mechanisms

In an attempt to enhance survival from noxious injury, organisms have developed several lines of defense mechanisms. One is represented by biochemical mechanisms which enable the organism to prevent injury after noxious insults, such as the early hepatic damage events oxidative stress and covalent protein binding. In the case of PAR-intoxication, the internal rescue mechanisms can be supported by increasing synthesis of GSH or possibly other sulfhydryl compounds by giving methionine or *N*-acetylcysteine (Mourelle *et al.*, 1990). The second class of defense mechanism is a biological response intended to overcome injury, by promoting tissue healing after the noxious insult (Mehendale, 1995). Establishing that the initial toxic or injurious events can be separated from the subsequent events that determine the ultimate outcome of injury offers promising opportunities for developing new avenues for

therapeutic intervention, with the aim of restoring and boosting the hormetic tissue repair mechanisms. In the late stages after PAR has been metabolized, patients often present many hours after taking an overdose, *S*-amino acids are no longer effective (Mourelle *et al.*, 1990). The initial (bio)chemical reactions between the reactive metabolite(s) and macromolecular cell components (proteins, lipids, DNA), were grouped and called Stage I, whereas subsequent processes of adaptation or failure of response to modification of essential cellular processes (such as energy supply and the protein machinery) were grouped in Stage II of toxicity (Chanda and Mehendale, 1996a; Chanda and Mehendale, 1996b). These potential causative events described shortly above (Stage I) will be reviewed in this Part (for a short overview of Stage I and Stage II, see Fig. 5). The Stage II processes will only be mentioned shortly, especially in the last paragraph of this Part.

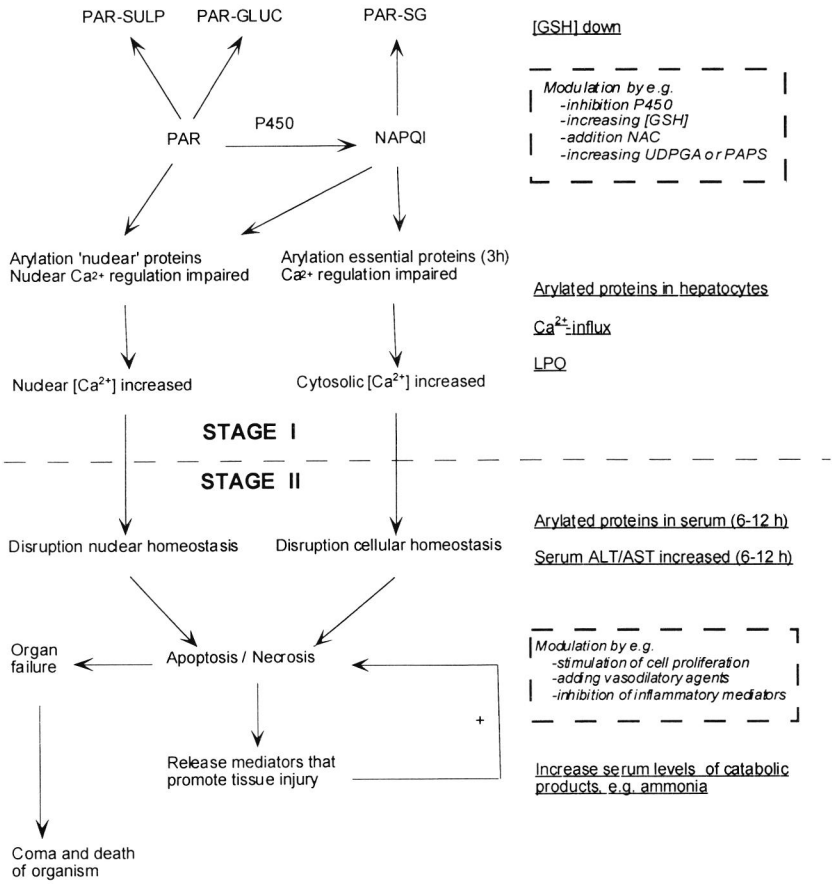


FIG. 5 Stage I and II in paracetamol-induced hepatotoxicity
Model of stages I and II that are discerned in the paracetamol-induced development of hepatotoxicity as deduced from various references (see text).

IV.2 Stage I - Initial events

Several hypotheses have been put forward in the past twenty years regarding the crucial early steps in the development of hepatic damage taking place directly after ingestion of PAR, once NAPQI is released in quantities that exhaust cellular GSH significantly. It must be stressed, however, that these hypotheses do not exclude each other, i.e. several mechanisms could contribute more or less to early hepatic damage. As reviewed by several authors (Hinson *et al.*, 1995; Holtzman, 1995; Nelson, 1995; Vermeulen *et al.*, 1992), one hypothesis is that oxidative stress, i.c. thiol oxidation, mediated by the oxidative capacities of NAPQI, is the main cause of hepatotoxicity. NAPQI can oxidise GSH, thereby lowering the GSH/GSSG status, and it can oxidise protein SH groups, leading to the formation of interstrand disulfide bridges, to interprotein crosslinking, or to mixed disulfides (between protein and glutathione). Another hypothesis is that oxidative stress accompanied often by lipid peroxidation (LPO) as caused by a redox cycling metabolite of PAR is the crucial step (Younes *et al.*, 1986). NAPQI was suggested to give rise to futile cycling of P450, using reducing equivalents of NADPH with concomitant reduction of molecular oxygen to the superoxide anion radical ($O_2^{-\bullet}$). The superoxide anion radical is enzymatically reduced to hydrogen peroxide (H_2O_2), which in turn may lead to hydroxyl free radical ($OH^\bullet$) formation in the presence of traces of metal ions in the Fenton reaction (Goeptar *et al.*, 1995). When reacting with lipids, these very reactive hydroxyl free radicals may initiate LPO (Bast, 1986). The third hypothesis is that covalent protein binding of NAPQI as an electrophile is the most important event, leading to disrupted homeostases once critical proteins have been modified (Cohen *et al.*, 1997; Gibson *et al.*, 1996; Pumford and Halmes, 1997). Not regarded as a very important event in the development of toxicity, a fourth event, i.e. covalent binding of NAPQI to lipids *in vitro* as well as *in vivo* has received some attention. With respect to hazard assessment of the use of PAR, more and more attention is paid to a fifth potential causative event for damage, i.e. the nuclear effects that are observed experimentally as well as epidemiologically (Bergman *et al.*, 1996). Mostly, low but chronic levels of exposure are studied with respect to potential nuclear effects of PAR. However, DNA effects as a result of high hepatotoxic doses of PAR, leading to apoptosis, have been receiving attention as well (Ray *et al.*, 1993; Ray *et al.*, 1996; Ray *et al.*, 1991). As apoptosis with respect to PAR-induced toxicity is a relatively new phenomenon, it remains to be established where in the Stage I or Stage II processes, apoptosis should be positioned. As the direct nuclear effects seem to be early processes, taking place within 6 h (Ray *et al.*, 1996), apoptosis is described here (although only briefly) under the nuclear effects in Stage I as well.

1. Oxidative stress and thiol oxidation

Several patho-physiological conditions may give rise to an unbalance between the production of and the protection against oxygen free radicals. This unbalance is called oxidative stress (Sies, 1986). This definition is often broadened to a condition of decreased reductive potential and an impaired capacity to cope with endogenous or exogenous oxidants. Oxidant stress mechanisms may be mediated either by reactive

oxygen species or by the direct oxidant action of a reactive metabolite in PAR-induced hepatotoxicity. This may be detectable as decreased ratios of NADPH/$NADP^+$ (Keller and Hinson, 1991), GSH/GSSG (Subrahmanyam *et al.*, 1987), ProtSH/ProtSSProt (Albano *et al.*, 1985). The latter two phenomena result from the fact that NAPQI can oxidise cysteine thiols in GSH, leading to GSSG, and in proteins, giving rise to protein disulfides and GSH-protein mixed disulfides (Albano *et al.*, 1985; Birge *et al.*, 1991a; Kyle *et al.*, 1990). Increased oxidation of protein thiol groups has been reported in hepatocytes to play a causal role in the observed PAR-mediated toxicity (Adamson and Harman, 1993). Oxidation of GSH by NAPQI may occur via the formation of a Meisenheimer complex via *ipso*-attack of GSH on the electrophylic C1-carbon (Fig. 6), as proposed by several authors for NAPQI as well as 2,6-diCH_3-NAPQI and 3,5-diCH_3-NAPQI (Coles *et al.*, 1988; Fernando *et al.*, 1980; Ketterer *et al.*, 1988; Nelson *et al.*, 1991; Rundgren *et al.*, 1988; Smith and Mitchell, 1985). In a similar bimolecular redox reaction, protein thiol oxidation may take place via the formation of unstable *ipso*-adducts, as proposed by Rundgren *et al.* upon investigating the effects of dithiothreitol on the metabolism, covalent protein binding, and cytotoxic effects of the quinone imines (Rundgren *et al.*, 1988). Moreover, even protein *S*-thiolation of proteins that are sensitive for such inactivation like glyceraldehyde-3-phosphate dehydrogenase could start by formation of an *ipso*-adduct of NAPQI as indicated, followed by displacement by GSH (Dietze *et al.*, 1997). It has to be noted that some oxidant stress phenomena could be Stage II events initiated by an increased role of Kupffer cells (hepatic macrophages) as described at the end of this Part (Hinson *et al.*, 1998).

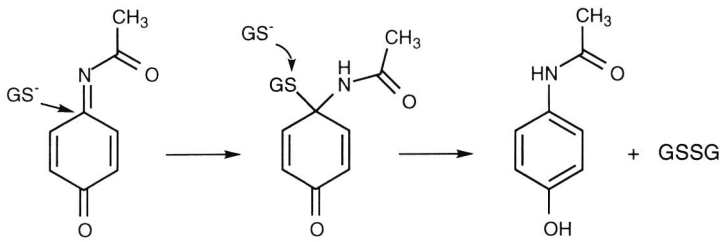


FIG. 6 Proposed pathway for *ipso*-attack of GSH with NAPQI
Proposed pathway of reduction of *N*-acetyl-*p*-benzoquinone imine via *ipso*-attack. Adapted from Ketterer and Hinson (1988)

Results from preliminary experiments with a PAR analogue in our laboratory suggesting oxidation followed by semi-permanent protein binding support this hypothesis. Briefly, in a buffered solution, the PAR analogue 3,5-diCH_3-PAR was oxidised with horseradish peroxidase and H_2O_2 with 3,5-diCH_3-NAPQI being formed as observed by UV-spectrophotometry (Bessems *et al.*, 1996). When microsomal protein was present during the oxidation, no formation of the 3,5-diCH_3-NAPQI was observed although 3,5-diCH_3-PAR seemed to disappear. Upon subsequent addition of GSH or

dithiothreitol, 3,5-diCH_3-PAR was observed again spectrophotometrically. This findings can be explained by the formation of a Meisenheimer-type complex between microsomal protein thiol groups and 3,5-diCH_3-NAPQI, being responsible for loss of π-conjugation and loss of absorbance at λ_{max}. Addition of an excess of GSH or dithiothreitol removes the semi-permanent adduct. The observed phenomena were much more significant when BSA was used instead of microsomal protein (unpublished observations). These findings are similar to those mentioned above (Rundgren *et al.*, 1988) and thus support the *ipso*-adduct hypothesis as formulated by the group of Nelson (Nelson *et al.*, 1991; Rundgren *et al.*, 1988).

Recently, the *in vivo* formation of hepatic protein aldehyde groups was used as a marker of oxidative damage upon the treatment of mice with $FeSO_4$, while increased serum levels of alanine aminotransferas (ALT; used as a marker for hepatic damage) were not yet observed (Gibson *et al.*, 1996). However, toxic doses of PAR did not result in protein aldehyde formation, whilst even the serum ALT levels were significantly increased. Moreover, combined treatment with $FeSO_4$ and PAR did not present protein aldehyde formation while serum ALT was increased compared to control mice, thus indicating antioxidant properties of PAR (Gibson *et al.*, 1996). The antioxidant properties of PAR were previously reported and confirmed (Van de Straat *et al.*, 1988a). Together with other findings, these data are consistent with the theory that PAR covalent binding is the primary mechanism of toxicity and argue against a major role for a-specific protein oxidation in PAR hepatotoxicity (Gibson *et al.*, 1996). This does not exclude, however, that thiol groups in some specific proteins become oxidised or glutathiolated (Birge *et al.*, 1991a), phenomena which in general must be regarded as adverse effects, but not necessarily as significant toxic reactions. It may well be that protein thiol oxidation and glutathiolation are protective mechanisms upon exposure to higher doses of PAR.

2. Oxidative stress and lipid peroxidation

As mentioned above, one of the phenomena often observed in combination with oxidative stress is lipid peroxidation (LPO). Reactive oxygen species (hydrogen peroxide, superoxide anions, and hydroxyl radicals) are required for its initiation as NAPQI is expected to be incapable of initiating a radical hydrogen abstraction from lipid molecules. However, reduction of NAPQI, which could occur in the presence of flavoproteins, followed by reoxidation by oxygen could give rise to superoxide anions with a consequent formation of reactive reduced oxygen species. Even protein bound NAPQI was suggested to be liable to one-electron reduction (Mourelle *et al.*, 1990). LPO has been regarded to be an important initiation event in the toxicity mechanism of PAR in the seventies and early eighties (Thelen and Wendel, 1983; Wendel *et al.*, 1982). Some dispute has existed since the late eighties, however. Hepatotoxic doses of PAR to Fisher 344 rats were not accompanied by increased biliary efflux of GSSG (Smith and Mitchell, 1985). Especially in isolated and cultured rat hepatocytes this phenomenon has been studied although results were not unambiguous (Albano *et al.*, 1983; Donatus *et al.*, 1990; Harman *et al.*, 1992; Van de Straat *et al.*, 1987b). A possible explanation for this ambuigity might be the increased sensitivity to oxidative

stress in hepatocytes that were isolated from fasted compared to fed rats, as fastening may lower the ATP content and thus the normal physiology of the cell. Moreover, fastening increases CYP2E1 activity substantially in rat (Hu *et al.*, 1995; Johansson *et al.*, 1988). Associated with the induction of CYP2E1 is an elevated production of reactive oxygen species (ROS) such as superoxide radicals and H_2O_2 in kidney and liver microsomes (Johansson *et al.*, 1988; Liu *et al.*, 1993b; Nordmann *et al.*, 1992; Rashba-Step *et al.*, 1993; Ueng *et al.*, 1993; Wu and Cederbaum, 1994). Furthermore, it has been concluded in various papers that LPO is not playing a causal role or only a minimal role in loss of cell viability induced by PAR (Donatus *et al.*, 1990; Garrido *et al.*, 1991; Kamiyama *et al.*, 1993; Mitchell *et al.*, 1985; Van de Straat *et al.*, 1988a; Younes *et al.*, 1988). The indigenous medicine curcumin for example was found to protect against PAR-induced LPO, without protecting against LDH leakage and GSH depletion (Donatus *et al.*, 1990). By showing that 3-mono-alkylation of PAR diminished LPO but not cytotoxicity in rat hepatocytes compared to unsubstituted PAR, Van de Straat *et al.* provided support for the hypothesis that LPO and cytotoxicity are not causally related in hepatocytes (Van de Straat *et al.*, 1988a).

The general question is whether PAR-induced LPO is a consequence of for example P450 oxidase activity or a consequence of other initial phenomena such as GSH depletion or oxidation of, and covalent adduct formation with proteins (Dai and Cederbaum, 1995). Also, it can not be excluded that on a limited scale in other organs, in e.g. the kidneys, local peroxidase activity leads to some LPO as it was shown *in vitro* that peroxidase catalysed oxidation of PAR in the presence of NADPH leading to, amongst others, polymers of PAR, very likely was accompanied by superoxide anion production (Fig. 7) (Keller and Hinson, 1991). Furthermore, LPO may well be a Stage II phenomenon instead of a Stage I phenomenon as discerned by Mehendale (Chanda and Mehendale, 1996b; Mehendale, 1991; Mehendale, 1995). Upon release of chemotactic and activating factors (such as interleukines and tumour necrosis factors), possibly following changes in DNA binding activities in transcription factors in hepatocytes that are damaged by PAR in Stage I of toxicity, Kupffer cells, peritoneal macrophages, polymorphonuclear leukocytes and eosinophils may be activated with concomitant release of myeloperoxidases, H_2O_2 and other activated oxygen species (Blazka *et al.*, 1996; Blazka *et al.*, 1995; O'Brien *et al.*, 1990). Very likely, this may result in LPO as well.

3. Covalent binding to proteins ('acetaminophen-binding proteins')

General

Just as NAPQI can oxidise GSH, it can covalently bind to GSH. Analogously, just as NAPQI can oxidise cysteine groups in proteins (Albano *et al.*, 1985; Birge *et al.*, 1991a; Kyle *et al.*, 1990) it can covalently bind to these amino acids in proteins leading to protein arylation *in vitro* and *in vivo* (Hoffmann *et al.*, 1985a; Hoffmann *et al.*, 1985b). Already in the seventies, covalent binding of radiolabeled PAR to proteins was described and suggested to play an important role in the toxicity mechanism of PAR (Potter *et al.*, 1973; Potter *et al.*, 1974). Since the identification of 3-cysteine-*S*-yl-4-hydroxyaniline (probably the *N*-acetyl moiety is lost during sample

preparation) as the major covalent adduct formed *in vitro* and *in vivo* between PAR and mouse liver proteins (Hoffmann *et al.*, 1985a), numerous papers appeared that attempted to elucidate which specific proteins became arylated (Bartolone *et al.*, 1989; Bartolone *et al.*, 1992; Halmes *et al.*, 1996; Hoivik *et al.*, 1996b; Pumford *et al.*, 1997; Pumford *et al.*, 1992; Zhou *et al.*, 1996). These attempts were triggered by the fact that covalent binding of the radiolabeled regioisomer 3'-hydroxyacetanilide (3'-HAA) was occurring without toxicity, although PAR toxicity was never observed without covalent binding (Halmes *et al.*, 1998; Salminen *et al.*, 1998; Tirmenstein and Nelson, 1991). For example recently, strong indications were obtained for the covalent binding of 3'-HAA to liver microsomal CYP2E1 with concomitant loss of activity (Halmes *et al.*, 1998; Salminen *et al.*, 1998).

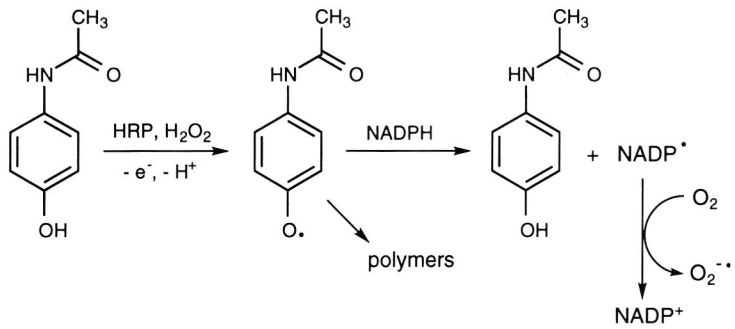


FIG. 7 Paracetamol- and peroxidase-dependent NADPH oxidation
Proposed mechanism of PAR-stimulated NADPH oxidation catalyzed by the peroxidase/H_2O_2 system. Adapted from Keller and Hinson (1991).

The search for critical proteins and the investigations into the time progression of adduct formation was accelerated when antibodies were raised against various epitopes of the 3'-(cystein-*S*-yl)-4'-hydroxyacetanilide adduct, in order to use them in ELISA and Western blot (immunoblot) analysis of protein adducts upon PAR administration (Bartolone *et al.*, 1988; Hinson *et al.*, 1996; Pumford *et al.*, 1989; Roberts *et al.*, 1987a). Proteins arylated by PAR were found in hepatic fractions as well as in serum of B6C3F1 mice that were administered PAR in a dose-range of non-toxic to toxic. The concentration- and time-dependent level of arylated proteins in serum closely parallelled serum ALT levels. Arylated proteins in liver of intoxicated mice peaked after 2 h whereas those in serum peaked 6-12 h after dosing (Pumford *et al.*, 1990b). The most intense immunostaining was found in the plasma membrane and the mitochondria whereas the most intense arylated individual protein appeared to be a 55 kDa cytosolic protein (Pumford *et al.*, 1990a; Pumford *et al.*, 1990b). In addition, the presence of 3-(cystein-*S*-yl)paracetamol adducts in liver proteins prior to hepatotoxicity suggests a threshold for adduct formation in the development of toxicity (Pumford *et al.*, 1990a). More and more, specific proteins were found to be

arylated in mice and man exposed to PAR, the extent of which for some proteins did and for others did not correlate with cellular damage (Birge *et al.*, 1990; Hinson *et al.*, 1995; Hinson *et al.*, 1990). An antiserum raised against a 4-acetamidobenzoic acid (antiarylacetamide) protein adduct detected the same primary PAR-protein adducts as an antiserum against a 3'-(cystein-*S*-yl)-4'-hydroxyacetanilide protein adduct (3-(cystein-*S*-yl)paracetamol protein adduct). However, minor differences were observed, indicating additional covalent protein binding to amino acids other than cysteine (Matthews *et al.*, 1996). For an overview of hepatic proteins in the various subcellular compartment being covalently modified by PAR ('acetaminophen-binding proteins'), and the effects of this modification on biochemical processes, see Table 5. For a visual perception, see Fig. 8. For another review on these aspects, including e.g. the quantification of concomitant enzyme inhibition, see Pumford and Halmes (Pumford and Halmes, 1997).

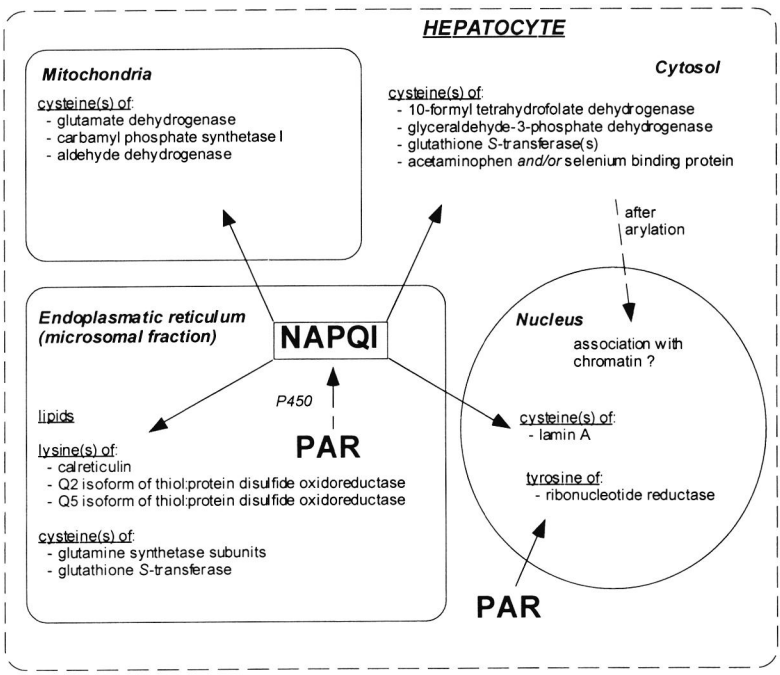


FIG. 8 Covalent adduct formation of macromolecules
Multiple proteins and other macromolecular structures covalently modified by high doses of PAR (mostly investigated in mice). Retrieved from various references (see text).

A concise description of the relationship between covalent binding of PAR to hepatic proteins and the development of hepatotoxicity in mice treated with a toxic dose of PAR (400 mg/kg) was presented by Hinson *et al.* (Hinson *et al.*, 1996), based on previous experiments (Roberts *et al.*, 1991). Adducts of PAR were observed

immunohistochemically in the innermost layers of cells surrounding the central hepatic vein as early as 15 minutes following a hepatotoxic dose. By 30 min there was a 90% depletion of hepatic glutathione and PAR-protein adducts were evident in the centrilobular area. By 1 h following this dose the protein adducts reached their maximum extent and were found exclusively in the centrilobular region of the liver. By 2 h, vacuolization and shrinking of hepatocytes were prominent. These events correlated with increases in serum ALT levels and PAR-protein adducts in serum which occurred as a result of hepatocyte lysis. By 6 h of treatment substantial loss of adducts from the necrotic cells was prominent (Hinson *et al.*, 1996). Moreover, in PAR overdose patients, a relation was found between plasma ALT and 3-(cystein-*S*-yl)paracetamol protein adducts in plasma. This highly suggests a dominant mechanistic role of this binding in humans and provides direct evidence of a similar mechanism of PAR-induced hepatic necrosis in man and in laboratory animals (Hinson *et al.*, 1990).

Cytosolic proteins

It has been known for quite some time that PAR becomes preferably covalently bound to a cytosolic GST upon a mouse liver homogenate incubation (Wendel and Cikryt, 1981). The first reported specific hepatic proteins in cytosol becoming arylated upon *in vivo* exposure of mice to hepatotoxic doses of PAR were a 44 kDa protein (Bartolone *et al.*, 1989; Birge *et al.*, 1991b), a 55 kDa protein (Pumford *et al.*, 1992) and a 58 kDa protein (Bartolone *et al.*, 1989; Bartolone *et al.*, 1992). The 58 kDa 'acetaminophen-binding protein' appeared to be native in many tissues although adduct formation was only found in tissues that were prone to PAR-based damage (Bartolone *et al.*, 1989). Antibodies raised against the 58 kDa PAR-arylated protein revealed a similar protein in the cytosol of a human liver specimen (Bartolone *et al.*, 1992). The 44 kDa 'acetaminophen-binding protein' showed high homology with a subunit of liver microsomal glutamine synthetase, indicating that it might be 'disrupted' from the endoplasmatic reticulum and becoming cytosolic upon arylation by PAR (Bulera *et al.*, 1995). Recently, two more cytosolic enzymes were shown to be modified upon PAR-intoxication in mice. *N*-10-formyltetrahydrofolate dehydrogenase was identified as a 100 kDa cytosolic target (Pumford *et al.*, 1997). A toxic dose of 400 mg/kg to mice resulted in a 25% decrease in cytosolic *N*-10-formyltetrahydrofolate dehydrogenase activity at 2 h already. Glyceraldehyde-3-phosphate dehydrogenase was identified as a 38 kDa subunit becoming covalently modified at the Cys-149 in the active site by NAPQI *in vitro*, as well as covalently modified and inhibited *in vivo* already within 2 h after PAR administration to mice (Dietze *et al.*, 1997). Recently, it was found that cytotoxic concentrations of PAR selectively inhibited protein phosphatase activity and altered the phosphorylation state of several cytosolic proteins in cultured mouse hepatocytes. As phosphatases exhibit crucial roles in the physiology of each cell, these alterations may have an as yet unforeseen role in the toxicity mechanism of PAR (Bruno *et al.*, 1998).

In the past ten years, numerous investigations were undertaken in order to elucidate the identity of the 55 kDa and the 58 kDa 'acetaminophen-binding proteins'. Partial internal peptide sequences of both the 55 kDa and the 58 kDa 'acetaminophen-binding

proteins' that become arylated by PAR present a high homology (87-100%) with the cDNA-deduced amino acid sequence of a cytosolic 56 kDa 'selenium-binding protein' (SP56), discovered two years before (Bansal *et al.*, 1990; Bartolone *et al.*, 1992; Cohen *et al.*, 1997; Pumford *et al.*, 1992). The average 97% overall homology of the 55 kDa 'acetaminophen-binding protein' with SP56 is based on seven peptide fragments of three to twenty five amino acids length (Cohen *et al.*, 1997; Pumford *et al.*, 1992). The 100% homology of the 58 kDa 'acetaminophen-binding protein' with SP56 is based on two peptide fragments of nine and eighteen amino acids, respectively (Bartolone *et al.*, 1992; Cohen *et al.*, 1997). By using a mouse genomic DNA library and a mouse liver cDNA library, one full-length cDNA, encoding the seven peptide fragments as found in the 55 kDa 'acetaminophen-binding protein' as well as the two fragments as found in the 58 kDa 'acetaminophen-binding protein', was picked up and cloned. Next, this full-length cDNA was used to deduce the full amino acid sequence of a virtual 56 kDa protein, designated as (deduced) AP56, and closely related to SP56, showing 100% homology in the total of nine peptide fragments used for screening. The cDNA deduced amino acid sequence of AP56 differs at only fourteen amino acids from the deduced sequence of SP56 (the 56 kDa 'selenium binding protein') (Lanfear *et al.*, 1993). The amino acid composition (in percentage of the total amino acids) of the real 58 kDa 'acetaminophen-binding protein' 58 ABP as isolated from mouse liver, appeared to be fairly similar with the calculated amino acid composition of SP56 (Bartolone *et al.*, 1992).

Although strong support was present that the 55 kDa and the 58 kDa 'acetaminophen-binding proteins' would be identical to deduced AP56, some findings deserve attention. Of all the fragments of the 55 kDa and 58 kDa PAR-arylated protein used for screening and homology-analysis, not one contains a cysteine residue, expected to be the residue where PAR is bound. It is possible, however, that PAR binds covalently to other amino acids such as lysine or histidine instead of cysteine (Fig. 9) (Streeter *et al.*, 1984b). This is not unlikely as a relatively low number of ten cysteine residues is present in deduced AP56 as well as SP56. Arylation of lysine residues in microsomal proteins has been found recently upon *in vitro* incubation (Holtzman, 1995). Sequencing of radioactive peptide fragments that had become ^{14}C-labeled upon incubation with ^{14}C-PAR would aid in the verification of the identity of ^{14}C-PAR-arylated proteins. Lastly, it has to be noted that the 58 kDa 'acetaminophen-binding protein' of Khairallah and Cohen's group, actually is a mixture of 4 isoforms, as revealed by two-dimensional gel electrophoresis (Bartolone *et al.*, 1992). Definitive conclusions on the 55 kDa, 56 kDa, or 58 kDa 'acetaminophen-binding proteins' being equal to SP56 and/or deduced AP56 may need analysis of covalently modified proteins by liquid chromatography/mass spectrometry (LC/MS).

TABLE 5 **(Covalent) protein modifications in subcellular compartments in various tissues observed upon incubation with paracetamol**

Protein	kDa	a.a.[1]	Time	Matrix	Effect (on)	Reference [2]
Microsomal (ER)						
calreticulin (a)		Lys		mouse liver *in vitro*	postranslational modifications	(Holtzman, 1995; Zhou *et al.*, 1996)
glutamine synthethase subunits (b)	44	Cys	30 min	mouse liver *in vivo*	synthesis of Gln from Glu and ammonia	(Bartolone *et al.*, 1989; Birge *et al.*, 1991; Bulera *et al.*, 1995)
glutathione *S*-transferase (c)	17	?		rat liver *in vivo* + *in vitro*	increased glutathione *S*-transferase activity	(Weis *et al.*, 1992; Yonamine *et al.*, 1996)
Q2 isoform of thiol:protein disulfide oxidoreductase (d)		Lys		mouse liver *in vitro*	postranslational modifications	(Holtzman, 1995; Zhou *et al.*, 1996)
Q5 isoform of thiol:protein disulfide oxidoreductase (e)		Lys		mouse liver *in vitro*	postranslational modifications	(Holtzman, 1995; Zhou *et al.*, 1996)
Mitochondrial						
aldehyde dehydrogenase (f)	54	Cys	1 h	mouse liver *in vivo*	oxidation of aldehydes; LPO	(Landin *et al.*, 1996)
carbamyl phosphate synthethase (g)		Cys	6 h	mouse liver *in vivo*	consumption of ammonia	(Gupta *et al.*, 1997)
glutamate dehydrogenase (h)	50	Cys	30 min	mouse liver *in vivo*	synthesis of Glu from α-ketoglutarate and ammonia	(Halmes *et al.*, 1996)
unknown (i)	67		30 min	mouse liver *in vivo*	unknown	(Halmes *et al.*, 1996)
Cytosolic						
acetaminophen and/or selenium binding protein (j)	55-58	Cys	30-60 min	mouse liver *in vivo*	scavenging of electrophiles	(Bartolone *et al.*, 1989; Pumford *et al.*, 1992; Hoivik *et al.*, 1996)
N-10-formyltetrahydrofolate dehydrogenase (k)	100	Cys	2 h	mouse liver *in vivo*	donation of one-carbon units in biosynthesis	(Pumford *et al.*, 1997)
glutamine synthethase subunits released from ER (l)			30 min	mouse liver *in vivo*	see microsomal proteins	(Bartolone *et al.*, 1989; Birge *et al.*, 1991; Bulera *et al.*, 1995)

glyceraldehyde-3-phosphate dehydrogenase	38	Cys	2 h	mouse liver *in vivo*	glycolytic pathway (ATP synthesis)	(Dietze *et al.*, 1997)
glutathione *S*-transferase (n)	25	?		mouse liver homogenate	not presented	(Wendel and Cikryt, 1981)
unknown (o)	33			mouse kidney *in vivo*	unknown	(Bartolone *et al.*, 1989)
Nuclear						
lamin A (p)	75	Cys	< 2 h		disruption of nuclear lamina	(Hong *et al.*, 1994; Khairallah *et al.*, 1995)
ribonucleotide reductase (q) [3]		Tyr		human mononuclear blood cells ; mouse mammary tumor cell line	quenching of tyrosyl; blocking replicative DNA synthesis	(Hongslo *et al.*, 1990; Richard *et al.*, 1991; Brunborg *et al.*, 1995)

(1) Amino acid most likely covalently modified.
(2) Specific enzymatic catalysis steps were retrieved from (Stryer, 1981)
(3) Actually this is not protein binding but quenching of the tyrosyl radical in the active site of ribonucleotide reductase.

(a) ε-Amino group of Lys-233 or Lys-23; possibly blocking of posttranslational modification of proteins
(b) see I.
(c) ε-Amino group of Lys-103; possibly blocking of posttranslational modification of protein
(d) ε-Amino group of Lys-202, Lys-209 or Lys-210 and Lys-354; possibly blocking of posttranslational modification of protein
(e) Unknown
(f) Probably cysteine; Cys-302 critical residue at active site; decreased oxidation of aldehydes, LPO
(g) Catalyses synthesis of carbamoyl phosphate (consumed in the urea production cycle) from ammonia and carbon dioxide
(h) Probably cysteine; 6 free reactive cysteines in protein; important in carbon and nitrogen metabolism
(i) Unknown
(j) Probably cysteine; 8 cysteines in protein; evidence to function as common target protein for toxicants
(k) Probably cysteine; Cys-707 essential cysteine at the active site; important enzyme in one carbon metabolism
(l) Probably cysteine; 11 cysteines of which 3 not buried inside the native protein; catalyses synthesis of glutamine from glutamate & ammonia
(m) Probably Cys-149 in the active site; at least *in vitro* reaction of NAPQI with this enzyme definitely caused Cys-149 adduct formation; probably also at lower rate other 2 cysteines in each subunit of glyceraldehyde-3-phosphate dehydrogenase
(n) Unknown
(o) Unknown
(p) Unknown
(q) Tyrosyl

Interestingly, Lanfear *et al.* recently showed that SP56 and deduced AP56 are different as they are encoded by two different genes. In addition, SP56 mRNA is highly expressed in liver, kidneys and, to a lesser extent, lung, whereas deduced AP56 mRNA is mainly expressed in liver (Lanfear *et al.*, 1993). The 58 kDa 'acetaminophen-binding protein' was shown to be constitutive in many tissues although it became covalently modified by PAR only in tissues sensitive to PAR-toxicity (Bartolone *et al.*, 1989). In addition, the 58 kDa 'acetaminophen-binding protein' probably is not native in plasma whereas PAR-bound 58 kDa 'acetaminophen-binding protein' was the most important modified protein found in plasma of PAR-intoxicated mice (Bartolone *et al.*, 1989). Although speculative, transport of the 58 kDa 'acetaminophen-binding protein' to extrahepatic tissues upon arylation by PAR is conceivable. NAPQI was postulated before to escape from hepatocytes, and participate in the arylation of protein thiols in erythrocytes, which has been oberved in mice that were administered PAR (Axworthy *et al.*, 1988).

Intermezzo

As mentioned, cysteine is not the only amino acid that becomes arylated upon NADPH-dependent microsomal oxidation of PAR. In mouse liver microsomal incubations, PAR-based radiolabel binding was found in ε-amino lysine groups in the intra-lumenal endoplasmatic reticulum proteins calreticulin and the Q2 and Q5 isoforms of the microsomal thiol:protein oxidoreductase (TPDO). No adducts of cysteine residues were found (Holtzman, 1995; Zhou *et al.*, 1996). Interestingly, a 'selenium binding protein' that was isolated from mouse kidneys showed 96.8 % homology with an isoenzyme of rat liver microsomal TPDO (Jamba *et al.*, 1996). Furthermore, the selenium is very likely bound by cysteine residues as this rat liver TPDO typically contains two typical bis (cysteinyl) sequence motives Cys-X-X-Cys (Jamba *et al.*, 1996). Other 'selenium binding proteins' that contain one to three of such Cys-X-X-Cys motive(s) are thioredoxin, endoplasmatic reticulum protein (ERp72), formate dehydrogenase, and, very interestingly, the 58 kDa 'acetaminophen-binding protein' (Jamba *et al.*, 1996). Now, if firstly, the TPDO isoforms Q2 and Q5 become covalently modified upon incubation with PAR, if secondly, no cysteine residues are present in the tryptic digests containing PAR-dependent radiolabel, if thirdly, these TPDO isoforms do contain Cys-X-X-Cys motives, and fourthly, if TPDO isoforms are 'selenium binding proteins', the most logical explanation is that it is just the presence of these Cys-X-X-Cys motives that prevents the cysteine residues from becoming covalently modified by the elusive NAPQI, just by binding selenium. In the past, covalent binding of PAR to 'selenium binding protein' was thought to occur analogously to cysteine binding (Pumford *et al.*, 1992). Analysis of hepatocyte incubations of PAR and PAR analogues for covalent protein binding by mass spectroscopy in our group, however, did not provide any indication for the formation of adducts to selenium containing amino acids such as selenocysteine (Li *et al.*, 1994a). It is therefore proposed that the cysteine residues in these selenium binding (but probably not selenium containing) proteins, are mainly active in reduction of NAPQI via *ipso*-attack (see the following Intermezzo in this Part).

Microsomal proteins

Typical with respect to covalent protein modification is the finding that liver microsomal glutathione *S*-transferase (GST) activity becomes seriously increased after administration of PAR to rats, while GSH content in the liver is markedly decreased (Yonamine *et al.*, 1996). Also, microsomal GSH peroxidase activity becomes

significantly enhanced within 3 h. The cytosolic GST activity, however, is decreased. NAPQI is known to become covalently bound (dithiothreitol insensitive) to the microsomal GST upon addition to rat liver hepatocytes (Weis *et al.*, 1992b). Although the effect on catalytic activity of cytosolic GST was not determined, it is known that PAR becomes preferably covalently bound to a cytosolic GST and to a lesser extent to microsomal GST upon a mouse liver (S9)-homogenate incubation (Wendel and Cikryt, 1981). As indicated in the Intermezzo, other microsomal proteins that were shown to become covalently bound to PAR at lysine moieties, albeit in mouse liver microsomal incubations, are calreticulin and the Q2 and Q5 isoforms of thiol:protein disulfide oxidoreductase (Holtzman, 1995; Zhou *et al.*, 1996). In addition, as found in and noted above for the cytosolic fraction, probably after disruption from the endoplasmatic reticulum, glutamine synthetase subunits were found to be covalently modified by PAR (Bulera *et al.*, 1995).

Mitochondrial proteins

Besides cytosolic and microsomal proteins, also mitochondrial proteins have recently been reported to become arylated upon a hepatotoxic dose of PAR to mice. Glutamate dehydrogenase (Halmes *et al.*, 1996), carbamyl phosphate synthetase I (Gupta *et al.*, 1997), and aldehyde dehydrogenase (Landin *et al.*, 1996) were identified as being adducted upon administration of hepatotoxic doses of PAR to mice. Covalent modification of these mitochondrial proteins may result in impaired functioning of these proteins which could give rise to impaired detoxification (oxidation) of aldehydes to acids, leading to LPO, and to impaired metabolism of ammonia (synthesis of glutamine). This could ultimately lead to mitochondrial damage as observed in hepatocytes as well as *in vivo* in mice as early as 1 h following PAR administration, hours before sincere toxicity emerges (Burcham and Harman, 1990; Burcham and Harman, 1991; Donnelly *et al.*, 1994; Meyers *et al.*, 1988; Ramsay *et al.*, 1989).

Concluding remarks

One protein that is returning in many reports that studied protein arylation by PAR, PAR-congeners (e.g. 3'-HAA), and other arylating toxicants (bromobenzene) during the past decade seems to be the cytosolic 58 kDa 'acetaminophen-binding protein' (which is not necessarily the same as the cDNA-deduced 56 AP). Although more extensive arylation of the 58 kDa 'acetaminophen-binding protein' does not seem to be correlated with a decrease in toxicity, it could be a preferential and common target for reactive metabolites and serve as electrophile scavenger, as with low 58 kDa 'acetaminophen-binding protein' there is increased arylation of other proteins (Hoivik *et al.*, 1996b). Moreover, an alternative role for the 58 kDa 'acetaminophen-binding protein' was suggested by indications that administration of PAR results in translocation of the arylated 58 kDa 'acetaminophen-binding protein' from hepatic cytosol into the nucleus (Hong *et al.*, 1994). This would be consistent with a role for this protein as electrophilic sensor such that translocation of arylated 58 kDa 'acetaminophen-binding protein' may be a signal to the nucleus for the presence of an

electrophile (Hoivik *et al.*, 1996b). Recently, even a nuclear protein was reported to become arylated upon PAR administration. Khairallah and Cohen reported covalent adduct formation of PAR to lamin A, one of the three intermediate filaments that form the nuclear lamina (Khairallah *et al.*, 1995). This phenomenon will be further discussed in the last paragraph of this Part.

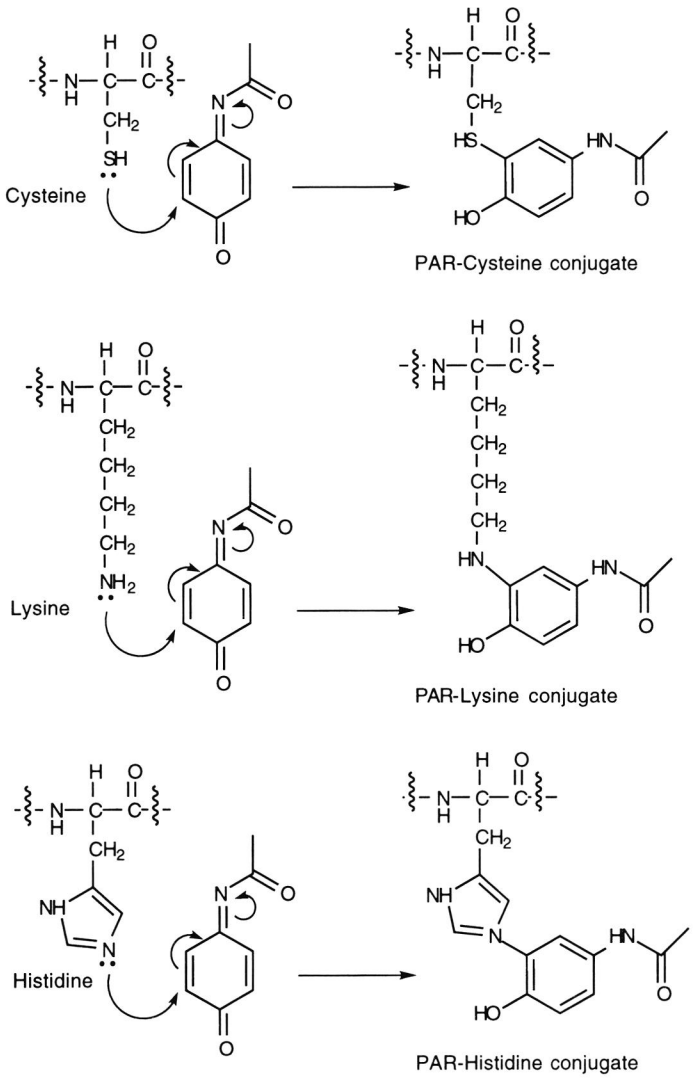


FIG. 9 Reactions of NAPQI with amino acids
Reactions of NAPQI with various protein amino acid monomers as suggested in various references (see text).

In conclusion, convincing evidence exists that protein arylation by PAR/NAPQI is a main trigger for processes that lead to disruption of cell homeostasis in hepatocytes. With about ten essential proteins in the cytosol and three main cell organelles (endoplasmatic reticulum, mitochondria, and nucleus) being struck by covalent protein modification, an additional role for protein thiol oxidation in the disruption of the cell physiology cannot be excluded, however, a prominent role is unlikely. In PAR overdose patients, the relation between plasma ALT and plasma 3-(cystein-*S*-yl)-paracetamol protein adducts, as found by an immunoassay, is highly suggestive of a dominant mechanistic role of protein arylation (Hinson *et al.*, 1990).

Intermezzo

The last proposal could be important when looking at the mechanisms underlying the extensive arylation of the so-called 'selenium binding protein', a 56-58 kDa cytosolic protein found in liver and kidneys in several species (Jamba *et al.*, 1996; Lanfear *et al.*, 1993; Pumford *et al.*, 1992). This protein is one of the most prominent proteins being arylated *in vivo* upon a (sub)toxic dose of PAR to mice. It was proposed that this 'selenium binding protein' would be arylated by reaction of NAPQI with the selenium analogue of the cysteinyl thiol group, the selenol or selenide (Pumford *et al.*, 1992). However, selenium containing protein adducts have never been identified. Furthermore, adminstration of sodium selenite to mice, prior to toxic doses of hepatotoxic doses of PAR, reduced the covalent binding of PAR to all cytosolic proteins, including the 56 kDa selenium-binding protein (Hinson *et al.*, 1996). While attempting to detect covalent protein binding of NAPQI in peroxidase systems, in rat liver microsomal and rat hepatocyte incubations with PAR and multiple substituted PAR analogues in our laboratory, selenium containing protein adducts were never found (unpublished results). Intensive investigations into the reactions between PAR, GSH, and ebselen, a potent anti-inflammatory selenium-containing drug reported to protect against PAR toxicity in hepatocyte incubations, did neither result in the detection of covalent binding of PAR to selenium containing amino acids (Li *et al.*, 1994a). The most likely explanation was the formation of ebselen selenol by GSH, followed by a fast reduction of NAPQI to PAR by the selenol, faster than the reduction by GSH. As studied by Baldew *et al.*, glutathionylselenol can be formed from selenite (SeO_3^{2-}) by GSH in a concerted reaction with glutathione reductase ((Vermeulen *et al.*, 1993) and references therein). *In vivo*, a protective action of a surplus selenite against PAR-induced toxicity has been shown (Schnell *et al.*, 1988). Analogously, an 'intraprotein cysteinylselenol' (ProtSeH) might be formed from a cysteine in a bis (cysteinyl) sequence motive inside a protein (Fig. 10).

Combining these findings and suggestions with the proposed mechanism of protein thiol oxidation (Nelson, 1995), it might be hypothesized that an 'intraprotein cysteinylselenol' (ProtSeH) moiety of the 'selenium binding protein' also forms a Meisenheimer complex with NAPQI, followed by attack of GSH and rearrangement, resulting in a 3'-arylated 'selenium binding protein'. This mechanism is theoretically possible since the 'selenium binding protein', unlike various GSH peroxidases, does not contain a selenocysteine encoded by an internal UGA codon (Lanfear *et al.*, 1993). Selenium is only bound and not incorporated. This hypothesis of *ipso*-attack awaits further investigation. One way to test this hypothesis might be to treat animals with ^{75}Se-sodium selenite (Jamba *et al.*, 1996) and ^{14}C-PAR after a few days, followed by investigation of individual amino acids of hepatic cytosolic proteins for ^{75}Se- and ^{14}C-label.

4. Covalent binding to lipids

Not regarded as a very important event in the development of toxicity, a fourth event, i.e. covalent binding of NAPQI to lipids *in vitro* as well as *in vivo* has received some attention. Covalent lipid binding (0.1 nmol/mg phosphoplipid) was about 3% of the covalent protein binding in incubations of PAR with microsomes from

3-methylcholanthrene-induced mice, which is equivalent to one modified phospholipid molecule out of 10^4. Covalent lipid binding upon horseradish peroxidase/H_2O_2 incubations in the presence of inactivated mouse liver microsomes amounted up to 10 nmol/mg phospholipid (Wendel and Hallbach, 1986). Although covalent lipid binding is also found after *in vivo* administration, it appears to be due to an electrophilic attack on nucleophilic centers in lipids rather than a radical reaction with electro-neutral alkyl residues of the phospholipids (Smith *et al.*, 1984).

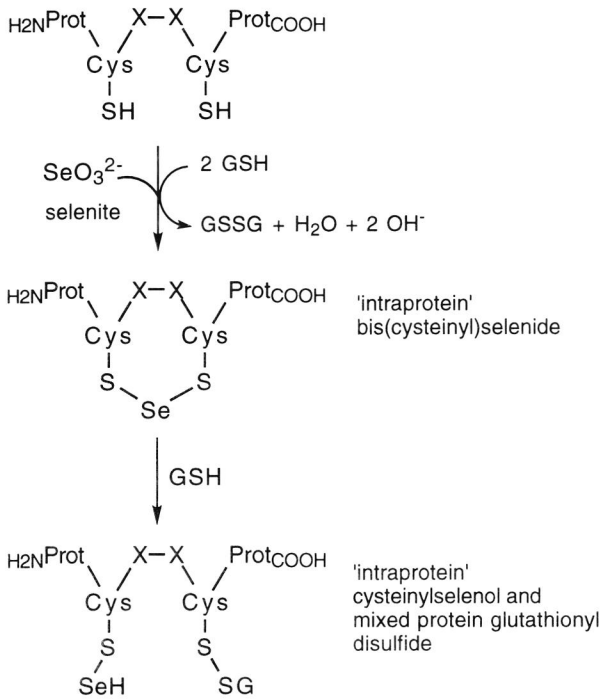


FIG. 10 Proposed mechanism for protection of protein thiols by selenite
Hypothetical reaction of selenite in the presence of GSH with a protein containing a Cys-X-X-Cys motif (e.g. the 58 kDa 'acetaminophen binding protein'), giving rise to a protein selenol. This reaction is analogous to the reaction of selenite with GSH as proposed by Vermeulen *et al.* (1993). Selenite is suggested to be converted into an 'intraprotein' bis(cysteinyl)selenide (ProtNH_2-Cys-Se-Cys-ProtCOOH), with concomitant consumption of two GSH equivalents. This bis(cysteinyl)selenide subsequently reacts with a third GSH equivalent to an 'intraprotein' cysteinylselenol (ProtNH_2-Cys-SeH) and three amino acids further to the carboxy terminus a mixed protein glutathionyl disulfide (X = amino acid).

5. Nuclear effects

The last hypothesis on causative events in PAR-induced hepatotoxicity that will be discussed is that of the nuclear mechanism. Ambiguity still exists as to possible genotoxic effects of PAR. Although genotoxic effects were reported to be found in liver

as well as other organs, the indicated mechanisms will probably be similar in liver and extrahepatic organs. Therefore, these effects will be discussed in this Part on hepatotoxicity. As reviewed recently, two studies indicate chromosomal damage in lymphocytes at therapeutic intake of PAR by human volunteers, whereas one study is negative (Rannug *et al.*, 1995). High doses of PAR have been reported to induce liver tumours in mice and bladder tumours in rats as has been reviewed in extenso recently (Flaks and Flaks, 1983; Flaks *et al.*, 1985; Rannug *et al.*, 1995). Mostly, however, low but chronic levels of exposure are studied with respect to carcinogenic effects of PAR. For these low levels, carcinogenicity studies were negative, and a recent review concluded that there was limited evidence for carcinogenicity of PAR in animals and inadequate evidence in humans when exposed to therapeutic levels (Bergman *et al.*, 1996).

In an *in vitro* study, covalent binding of DNA by a PAR metabolite was unequivocally proven upon incubations with microsomes from rat hepatic and renal tissues, supported by NADPH or cumene hydroperoxide, and upon incubation with HRP and H_2O_2. The binding levels in the peroxidase system were 200-fold greater than in the microsomal systems. The presence of cysteine or nuclear protein modulated the covalent binding in incubations containing radiolabeled NAPQI. Cysteine exhibited increased binding when present at 0.1 mM and decreased binding when present at 1.0 mM, both compared to incubations without cysteine. By combination of these *in vitro* results with *ex vivo* results, the authors suggested that DNA binding could occur at therapeutic doses in humans (Rogers *et al.*, 1997). Earlier, covalent binding of PAR to cellular nucleic acids (DNA as well as RNA) as the result of (provoked) respiratory bursts of granulocytes as well as neutrophilic type cells (differentiated from a leukemic cell line) was reported. Although the exact identity of the DNA adduct remained ambiguous, *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI), the one electron oxidation product of PAR was proposed (Corbett *et al.*, 1989; Corbett *et al.*, 1992). The *in vivo* relevance of these findings on direct DNA modification for humans taking PAR at therapeutic doses remains to be established. In addition and as mentioned above, it was found recently that following covalent binding of PAR to the 55 to 58 kDa 'acetaminophen-binding protein', this arylated protein is directed to the nucleus via transnuclear transport (Hong *et al.*, 1994). Although highly speculative, following this translocation, the nucleus might start Stage II of the toxicity process by signalling responses to neighbouring cells for promotion of tissue repair or by signalling macrophages inflicting release of inflammatory mediators (Cohen *et al.*, 1997; Hoivik *et al.*, 1996b; Mehendale *et al.*, 1994b).

In human volunteers, PAR decreased the unscheduled DNA synthesis (UDS) in peripheral lymphocytes and increased the frequency of micronucleated cells in the buccal mucosa. Concomitant intake of ascorbic acid did not decrease the observed genotoxic effects but extended the effects of PAR intake on UDS (Topinka *et al.*, 1989). Metabolic intermediates of high concentrations of phenacetin and PAR were suggested to induce a low frequency of non-neoplastic morphological transformations in a mouse embryo fibroblast cell line (C3H/10T1/2 clone 8) (Patierno, 1989). One of these metabolic intermediates of phenacetin as well as of PAR is *p*-benzoquinone (PBQ), a

hydrolysis product of and chemically very similar to NAPQI (Dahlin *et al.*, 1984; Koymans *et al.*, 1989; Miner and Kissinger, 1979).

This PBQ was reported recently to interact with microtubule proteins *in vitro*, thereby preventing microtubule formation. *In vivo* this may lead to interference with the formation of a functional spindle apparatus in the mitotic cell, thus leading to abnormal chromosome segregation and aneuploidy induction (Pfeiffer and Metzler, 1996). Aneuploidy is considered as a critical event in the multistep process of neoplastic cell transformation (Eastmond, 1993). Moreover, a nuclear protein was reported to become arylated upon PAR administration. Khairallah and Cohen reported covalent adduct formation of PAR to lamin A, one of the three intermediate filaments that form the nuclear lamina (Khairallah *et al.*, 1995). This finding is consistent with PAR-induced disruption of the nuclear lamina. Chromatin attachment to the inner nuclear membrane dropped within 2 h after treatment with PAR (Hong *et al.*, 1994). Moreover, unmetabolised PAR was reported to quench the tyrosyl radical in one subunit of the ribonucleotide reductase, thereby slowing the DNA polymerization necessary to fill gaps in DNA strands (Hongslo *et al.*, 1990; Hongslo *et al.*, 1994).

In support of the covalent binding theory, it was reported recently that reactive metabolites of PAR appeared to bind covalently not only to nuclear protein but also to hepatic and renal DNA from mice pretreated with diethylmaleate to deplete GSH and that measurable covalent binding to hepatic DNA was observed up to one week after PAR administration (Hongslo *et al.*, 1994). The effects of Ca^{2+}-endonuclease, DNA repair, and inhibitors of GSH depletion on DNA fragmentation and cell death upon cytotoxic doses of PAR in cultured mouse hepatocytes were described by Shen *et al.* As accumulation of Ca^{2+} in the nucleus, and fragmentation of DNA *in vitro* and *in vivo* (characteristic of Ca^{2+}-mediated endonuclease activation) are known to unfold well in advance of cytotoxicity and the development of necrosis, the authors suggested that unrepaired damage to DNA contributes to PAR-induced cell death and may play a role in necrosis *in vivo* (Shen *et al.*, 1992).

Recently, data were reported indicating that PAR interferes with nucleotide excision repair in several mammalian cell types, e.g keratinocytes (Skorpen *et al.*, 1998). This constitutes a mechanism by which paracetamol might contribute to genotoxicity in humans (Brunborg *et al.*, 1995). By electron paramagnetic resonance it was demonstrated that PAR added to crude cell extracts of a mammary tumour cell line of mouse destroyed a tyrosyl free radical of the small subunit of ribonucleotide reductase. These results show that PAR reduces DNA synthesis by specific inhibition of ribonucleotide reductase (Hongslo *et al.*, 1990). In a subsequent study, PAR treatment increased single strand breaks in nuclear DNA isolated from the liver but not from the kidneys, 2 h after i.p. injection of PAR at 600 mg/kg in male B6 mice. Only marginal DNA damage was noted at 300 mg/kg. Results also suggested that breaks were induced in DNA from a subpopulation of murine liver cells. Interestingly, the non-hepatotoxic PAR regioisomer (congener), 3'-hydroxyacetanilide (3'-HAA; 600 mg/kg) which also binds covalently to proteins, did not cause DNA single strand breaks. DNA polymerization, necessary to fill the gap in a DNA strand, was concluded to be blocked

by reversible inhibition of deoxyribonucleotide (dNTP) synthesis and may therefore also interfere with DNA repair (Hongslo *et al.*, 1994).

At 1 h following an oral dose of 1 g/kg bw to male Wistar rats, DNA synthesis was reported to be inhibited in various organs, i.e. spleen, testis, thymus, stomach, small intestine and bone marrow, but not in liver. This effect was shown to be transient and disappeared within 4 h, except in spleen. This transient inhibition was explained by a reversible inhibition of deoxyribonucleotide reductase (Lister and McLean, 1997). In addition, PAR was reported to inhibit replicative DNA synthesis in V79 Chinese hamster cells, probably by competitive reaction with a tyrosyl radical species involving the transfer of a hydrogen atom at the active site of ribonucleotide reductase (Richard *et al.*, 1991).

As mentioned earlier, mostly low but chronic levels of exposure were studied with respect to potential nuclear effects of PAR. However, DNA effects and an apoptotic-like mechanism in acute PAR-toxicity have been receiving attention as well (Ray *et al.*, 1993; Ray *et al.*, 1996; Ray *et al.*, 1991). In 1996, Ray *et al.* established for the first time that in addition to necrosis (toxic cell death), apoptosis (programmed cell death) may be involved in some stages of the highly integrated process of PAR-induced toxicity. PAR-induced cell death was preceded by massive elevation in serum ALT coupled with a rapid loss of genomic DNA (2-24 h), fragmentation of DNA (2-24 h), apoptotic nuclear condensation (2-6 h) followed by massive fragmentation and margination of heterochromatin at later hours (6-24 h) and a near total loss of glycogen in pericentral areas (Ray *et al.*, 1996). Although positioned here as a Stage I process, the later steps in apoptosis leading to cell death might as well be positioned in Stage II.

Intermediate between Stage I and Stage II might be the following effects that were found and seem to be independent of metabolic bioactivation. An almost equal antiproliferative effect was observed in a human hepatoma HepG2 subline expressing human CYP2E1 as well as in a comparable subline not expressing CYP2E1 (Dai and Cederbaum, 1995). From this early finding, new investigations emerged. PAR appeared to modulate serum growth factor signal transduction in Hepa 1-6 cells, thereby inhibiting cells from completing specific phases of cell division. PAR was found to inhibit *c-myc* expression, activation of NF-κB DNA binding and Raf kinase activity via serum growth factor. The serum growth factor appears to play an important role in counteracting the pro-apoptotic effects of transforming growth factor-ß (TGF-ß). The ability of PAR to inhibit cell division might interfere with organ regeneration and thus exacerbating acute liver damage caused by PAR (Boulares *et al.*, 1999). Another finding that might have direct consequences in the subsequent Stage II is the impairment of expression and secretion of tumour necrosis factor-α (TNF-α) in primary rat liver cell cultures treated with PAR. As TNF-α has both adverse (aggravation of primary damage to hepatocytes) and beneficial effects (stimulation of tissue repair) in toxic liver damage, the consequences of this finding remain to be established (Nastevska *et al.*, 1999).

IV.3 Stage II - Damage in hepatocytes and non-parenchymal cells

As mentioned briefly above, in the development of PAR-induced hepatic injury, several stages have been discerned (Fig. 5) (Chanda and Mehendale, 1996b; Mehendale, 1991; Mehendale, 1995; Mourelle *et al.*, 1990; Mourelle *et al.*, 1991). Stage I phenomena could be argued to collectively comprise all of the cellular damaging events but also the cytoprotective mechanisms (thus within the cell), i.e. selective protein modification, LPO and so on but also reduction of oxidative metabolites and oxidised protein thiols, superoxide dismutase activity etc. So called Stage II processes, observed shortly before and during tissue necrosis will be discussed here. At Stage II, processes spread from cell to cell in the extracellular matrix embedded in a tissue and from hepatocytes to nonparenchymal cells and even extrahepatic tissues. Simultaneously, in Stage II of toxicity, tissue-based protective response mechanisms (tissue repair) are launched (Laskin, 1994; Soni and Mehendale, 1998). Stage II toxicity comprises e.g. the release of a factor by hepatocytes that is chemotactic for Kupffer cells as well as blood monocytes. These cells can release reactive oxygen species (causing tissue damage and necrosis) and cytokines. A consequence of the latter might be a possible effect on the blood circulation, which may play an important additional role in the elimination of toxic quantities of PAR (Laskin, 1994; Skoglund *et al.*, 1987). An overview on the role of nonparenchymal cells and inflammatory mediators in hepatotoxicity in general has been published (Laskin, 1994).

As several of the covalently modified proteins are involved in Ca^{2+} sequestration in the endoplasmatic reticulum, impairment of the activities of these proteins might lead to disruption of cellular Ca^{2+} homeostasis and thereby cause cellular injury. In mouse hepatocytes, however, increase in cellular Ca^{2+} was determined to be a secondary event posterior to cytotoxicity and not occurring before 2.5 h after addition of PAR according to Grewal *et al.* (Grewal and Racz, 1993). A second mechanism for cellular injury could be impaired synthesis of plasma membrane proteins. As calreticulin and the Q5 thiol:protein oxidoreductase are involved in posttranslational modification of proteins, any toxin that inactivates these proteins could block the final synthesis of membrane proteins resulting in cytotoxicity (Holtzman, 1995; Zhou *et al.*, 1996).

Many reports described direct effects of NAPQI when added to hepatocytes or isolated mitochondria (Albano *et al.*, 1985; Weis *et al.*, 1992a; Weis *et al.*, 1992b). E.g. NAPQI can release Ca^{2+} from isolated mitochondria **via** pyridine nucleotide hydrolysis. The beneficial use of synthetical NAPQI as a tool in the investigation of subcellular (molecular/biochemical) processes in P450 and PAR dependent reactions is without doubt, its value in studying the PAR biotransformation-dependent effects in isolated cells or organs remains questionable, however. Due to the reactivity of NAPQI, being a relative strong electrophile as well as an oxidant, the rate and site of formation of NAPQI is of utmost importancy (Harman *et al.*, 1991). The incapability of BCNU to increase covalent protein binding in hepatocytes treated with NAPQI (Albano *et al.*, 1985) may be due to saturation of the hepatocyte system with an unrealistically high concentration of NAPQI used (250 μM).

Posttranslational modification of proteins can also be involved in the sequestration of NAPQI (Krishna and Wold, 1993). More likely, however, is altered protein

functioning triggered by modification (arylation, glutathiolation, protein thiol oxidation). One protein, the 55 to 58 kDa 'acetaminophen-binding protein', is sequestered into the nucleus, signalling the presence of a toxic compound (Cohen *et al.*, 1997). Interestingly, but beyond the system of Stage I and Stage II processses, bioactivation-independent effects on mitochondrial functioning in rat liver slices are observed within 30 min after PAR administration, i.e. long before cell damage is observed (Nazareth *et al.*, 1991). This can be an effect on mitochondrial DNA processing, a phenomenon similar to the inhibitory effect of PAR on ribonucleotide reductase (see below) where an essential tyrosyl residue was destroyed (Hongslo *et al.*, 1990; Hongslo *et al.*, 1994). One should be careful, however, in interpreting the relevance of these *in vitro* findings for the *in vivo* situation. In a comparative study, *in vivo* mitochondrial effects were found to be dependent on biotransformation whereas *in vitro* mitochondrial effects were not (Meyers *et al.*, 1988). Another, biotransformation independent observation is the complexation of PAR with human insulin (Smith and Ciszak, 1994). Whether this is relevant in an impaired potential of liver regeneration upon PAR intoxication remains to be established.

Most research in the seventies and eighties focussed on liver parenchymal cells, the hepatocytes. In the past ten years, however, more attention was paid to nonparenchymal cells such as Kupffer cells, the resident macrophages of the liver, which could be involved in the damage observed after 10 h in liver tissue (Koop *et al.*, 1991; Qu, 1992). About 1-6 h after PAR ingestion, damage in the hepatic tissue is observed that may be one of the indirect consequences of the early changes in the primary target cells, i.e. the hepatocytes. Solid evidence exists that as a result of signals from damaged hepatocytes, Kupffer cells and the mediators they release contribute to PAR-induced tissue injury (Laskin *et al.*, 1995; Mehendale *et al.*, 1994a). Release of mediators, however, might be also a consequence of early changes in Kupffer cells themselves at high dose administration. This could be due to the CYP2E1-based bioactivation of PAR in the Kupffer cells as these resident macrophages, in contrast to peritoneal macrophages, exhibit CYP2E1 activity. Although this activity is only about 20% of CYP2E1 activity in hepatocytes (Koop *et al.*, 1991), it is suggested here that CYP2E1-mediated bioactivation in Kupffer cells might contribute significantly to bioactivation of PAR at higher than therapeutic plasma levels. In addition, novel mechanisms in chemically induced hepatotoxicity (downregulation of subunit proteins [connexins], important in gap junction functioning), described by Mehendale *et al.* for CCl_4 (Mehendale *et al.*, 1994a), could be important in the toxicity mechanism of PAR.

Even a role for nitric oxide as a cytotoxic mediator in PAR-induced hepatotoxicity in the rat was demonstrated (Gardner *et al.*, 1998), a finding which was in line with a communication on nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of PAR in mice. Besides an increase in serum ALT, these effects were shown to be accompanied by increased serum levels of nitrate plus nitrite (a marker of nitric oxide synthesis). Tyrosine nitration occurs by peroxynitrite, a reactive intermediate formed by an extremely rapid reaction of nitric oxide and superoxide. Peroxynitrite has also hydroxyl radical-like activity. The involvement of

Kupffer cells (hepatic macrophages) in Stage II of PAR-toxicity, leading to increased synthesis of nitric oxide and superoxide presumably leading to peroxynitrite nitrotyrosine-protein adducts was hypothesized (Hinson *et al.*, 1998).

However, other phenomena that could be hypothesized to be related to Stage II of PAR-induced liver toxicity, such as adhesion and vascular plugging by neutrophils, were concluded not to be significant determinants of PAR-induced liver swelling and necrosis (Welty *et al.*, 1993). Maybe, for proper understanding of the PAR-toxicity mechanism Stage I and Stage II as discerned by Mehendale *et al.* should be further subdivided. E.g. a Stage IA could refer to the primary bioactivation process (the phase 1 biotransformation), whereas a Stage IB could encompass the adduct formation to GSH, proteins and potentially lipids (revolving phase II biotransformation enzymes and chemical reactions with macromolecules) (Chanda and Mehendale, 1996b; Mehendale, 1995; Mourelle *et al.*, 1990; Mourelle *et al.*, 1991).

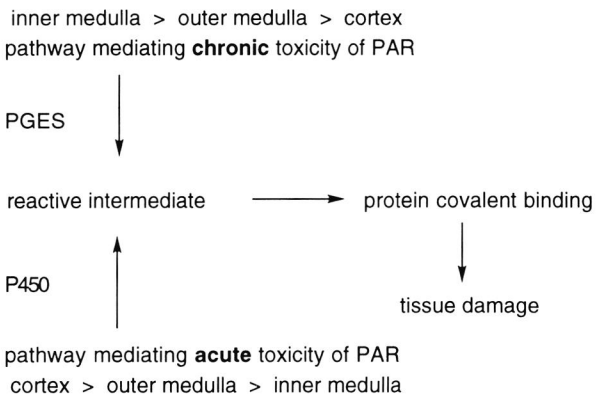


FIG. 11 Rabbit renal distribution of activities
Distribution in the differenct sections of the kidneys. Adapted from (Mohandas *et al.*, 1981).

Recently, the protection againts PAR and bromobenzene toxicity in mice by pretreatment (72 h) with a single dose of amphetamine was reported (Salminen *et al.*, 1997). Protection was proposed to be caused by induction of heat shock proteins (hsp) without decreased protein binding. Although not suggested in the original report, this might well be a Stage II phenomenon. Hsp-induction is normally a result of stressors such as severe hyperthermia and chemicals (references in (Salminen *et al.*, 1997)). A comparable protective mechanism might be working as suggested for reported autoprotection when stimulated tissue repair by a nonlethal dose of a toxicant protects against a subsequent, normally lethal dose, of the same chemical (Mehendale *et al.*, 1994b). That is to say that liver cells are prepared for a new noxious stimulus.

IV.4 Summary

Hepatotoxicity of PAR is the result of a cascade of interrelated biochemical events. Each of the eventualities, protein oxidation, covalent modification and inhibition of enzyme activity, lipid peroxidation, transnuclear transport of 'acetaminophen-binding proteins' and inhibition of protein phosphatase activity, although not all simultaneously nor of equal importance, probably have a role in the mechanism causing liver damage upon PAR-intoxication. A dominant mechanistic role is probably reserved for the formation of 3-(cystein-*S*-yl)paracetamol protein adducts. These adducts are found in liver fraction with concentration- and time-dependency upon administration of PAR to mice. Furthermore, the levels of arylated proteins in serum correlate with serum ALT levels. In addition, a threshold level is suggested for adduct formation in the development of toxicity as 3-(cystein-*S*-yl)paracetamol protein adducts appear in hepatic fractions prior (peak after 2 h) to serum (peak after 6-12 h). Moreover, these 3-(cystein-*S*-yl)paracetamol protein adducts are found in serum of PAR-intoxicated patients and correlate with serum ALT levels.

In the past decade, many protein targets of the reactive metabolite of PAR were identified, some of which as important enzymes. Plasma membrane and mitochondrial fractions appear to contain most covalently modified proteins whereas a 55 to 58 kDa cytosolic protein appears to be the most intense arylated individual protein. Scavenging of the reactive metabolite by proteins may, in some ways, be much more important and at least more complex than the scavenging by glutathione. The processes in which probably most effort was put into in the nineties, notably adduct formation of the 55 to 58 kDa 'acetaminophen-binding proteins', may not be a molecular toxicological endpoint but a protection mechanism. In addition, a role in signalling of electrophilic damage to the nucleus followed by provocation of tissue damage has been suggested. The mechanistic role of selenium-compounds and selenium binding proteins in the protection against PAR-hepatotoxicity also needs further investigation.

An emerging number of *in vitro* and *in vivo* results as well as epidemiological reports point to all kinds of nuclear effects. The most prominent findings are impaired DNA repair and even DNA-adduct formation at low target concentrations but also activation of Ca^{2+}-endonuclease at high concentrations. In addition to necrosis (toxic cell death), also apoptosis (programmed cell death) may be involved in some stages of the highly integrated process of PAR-induced toxicity. It remains to be established, however, what the relevance as well as the possible consequences of the experimental and epidemiological findings are for PAR-intoxicated patients as well as humans taking PAR at therapeutic levels.

Phenomena that also have retrieved much attention in the past decade and which may be related to the nuclear effects are intra- and intercellular signalling. After the cascade of noxious insults in the first hours after administration of a toxic dose of PAR, mainly in the hepatocytes (distinguished as Stage I), another gush of events commences, i.e. excretion by hepatocytes and non-parenchymal cells (e.g. Kupffer cells) of signalling factors, inflammatory mediators and reactive oxygen species such as superoxide anion radical, nitric oxide and peroxynitrite (distinguished as Stage II). Consequences of these actualities may be tissue damage but also tissue repair, the

balance of which determines recovery or death. These findings could provide important new leads for improvement of clinical treatment of PAR-intoxication.

PART V MECHANISMS OF RENAL TOXICITY

V.1 Introduction

Although the hepatotoxicity of PAR is generally accepted to be primed by the formation of NAPQI, a metabolite formed during cytochrome P450 catalysed oxidation of PAR (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b), the cause of renal toxicity is less clear. Prostaglandin endoperoxide synthase (PGES), *N*-acetyltransferase as well as P450 enzymes are known to be involved in PAR-dependent toxicity as investigated in one or more species. High doses of PAR result in renal cortical necrosis in man and the F344 rat. Cellular injury is primarily confined to the proximal tubule and significant reductions in glomerular filtration rate (Blantz, 1996; Hu *et al.*, 1993; Newton *et al.*, 1983; Trumper *et al.*, 1996). Like their hepatic counterpart, renal microsomes also metabolize PAR to an arylating intermediate via a P450 dependent mechanism. Thus, at least part of the acute PAR-dependent renal damage is probably due to a biochemical mechanism similar to that in liver. In addition, PAR is deacetylated to *p*-aminophenol in rat renal and hepatic cytosol and microsomes (Newton *et al.*, 1983).

In a recent short review article, various aspects related to PAR-dependent renal toxicity have been summed up. Factors that may influence renal toxicity include chronic liver disease, gender and conditions that alter the activity of P450-metabolizing enzyme systems (Blantz, 1996). In contrast to acute renal failure related to high dose intake of PAR, conflicting results have been presented with respect to the question whether chronic intake of PAR by humans contributes to chronic renal disease and analgesic nephropathy. An epidemiological report on this subject concludes that chronic use of PAR is related to chronic renal disease (Sandler *et al.*, 1989) whereas Blantz (Blantz, 1996) refers to another epidemiological investigation that failed to demonstrate a significant incidence of chronic renal disease in healthy individuals who use over-the-counter analgesics such as PAR. Unambiguous conclusions are probably hampered by the absence of a clear mechanism of analgesic nephropathy. Elucidation thereof has been hampered due to the lack of animal models that closely mimic the human disease as rodents do not appear to be an appropriate model (Schnellmann, 1998).

V.2 Prostaglandin synthase and *N*-acetyltransferase

High acute doses of PAR may result in hepatic centrilobular and renal cortical necrosis in man and the F344 rat (Newton *et al.*, 1983). Chronic exposure, however, is correlated much stronger to renal toxicity and probably depends on prostaglandin endoperoxide synthetase (PGES), as illustrated by results obtained in rabbit kidney microsomes. Covalent binding clearly correlated with the zone that microsomes were prepared from (Fig. 11) (Mohandas *et al.*, 1981). The very high affinity of PGES for

PAR indicates that even at therapeutic doses metabolic activation to nephrotoxic metabolites (presumably NAPQI and/or NAPSQI) may occur (Fig. 12) (Larsson *et al.*, 1985).

Human kidney medulla microsomes also catalysed the PGES-based metabolic activation of PAR at rates similar to those found in rabbit kidneys (Larsson *et al.*, 1985). Similar to protein arylation in liver, covalent binding to an 58 kDa protein preceeded nephrotoxicity in mice (Hoivik *et al.*, 1996a). Significant species-, strain-, as well as gender-specific variations in PAR induced renal toxicity were observed (Table 2) (Hoivik *et al.*, 1995; Hu *et al.*, 1993; Mugford and Tarloff, 1995; Newton *et al.*, 1985). An early finding of interest in the pathogenesis of analgesic nephropathy was the reporting of *p*-aminophenol, a well known nephrotoxic agent, in urine of hamsters that were administered PAR (Gemborys and Mudge, 1981). Oxidation of *p*-aminophenol, possibly via PGES, to the *p*-aminophenoxy free radical may be catalysed by PGES. Oxidation or disproportionation of this radical will form 1,4-benzoquinoneimine which can covalently bind to tissue macromolecules (Fischer *et al.*, 1985c; Fowler *et al.*, 1991; Newton *et al.*, 1983). NAPQI was shown to be converted to the *p*-aminophenoxy free radical in a microsomal incubation by combined *N*-acetyltransferase catalysis and reductive activity of the microsomal mixed-function oxidase system (Fischer *et al.*, 1985c). Subsequent reduction of the *p*-aminophenoxy free radical to *p*-aminophenol seems to be common sense, thus providing a mechanistic explanation for *p*-aminophenol as a urinary metabolite of PAR. Moreover, in F344-rats, deacetylation of PAR to *p*-aminophenol is regarded as a prerequisite to nephrotoxicity (Newton *et al.*, 1985) although it is suggested here that not PAR but NAPQI is the actual substrate for *N*-acetyltransferase. In contrast to F344-rats, Sprague-Dawley (SD) rats were susceptible to PAR-mediated renal toxicity, without the involvement of *N*-acetyltransferase (Fig. 12) (Mugford and Tarloff, 1995). Interestingly, in bile of Wistar rats, PAR-GLUC as well as PAR-SG were detected upon i.p. administration of *p*-aminophenol, supporting the close metabolic relation between PAR and *p*-aminophenol(Klos *et al.*, 1992).

Although based on a limited amount of data, covalent binding of PAR or a metabolite to DNA isolated from the kidneys of mice that were treated with PAR after fasting and depletion of GSH was also reported (Hongslo *et al.*, 1994). Stronger evidence for DNA binding was obtained recently in *ex vivo* ^{32}P-postlabeling experiments using renal DNA from PAR-treated mice as well as in *in vitro* metabolic activation experiments. Interestingly, in the *in vitro* experiments, horseradish peroxidase (HRP) and H_2O_2 produced binding levels 200-fold greater than microsomes with cumene hydroperoxide or NADPH. These data would further support the hypothesis that acetaminophen metabolites bind covalently to DNA in experimental animals *in vivo* at doses that mimic therapeutic doses in humans (Rogers *et al.*, 1997). These suggestions remain to be established, however. More information with respect to PAR-dependent processes in relation to DNA and the cell nucleus, which may be comparable in liver and kidneys, was presented in Part IV.

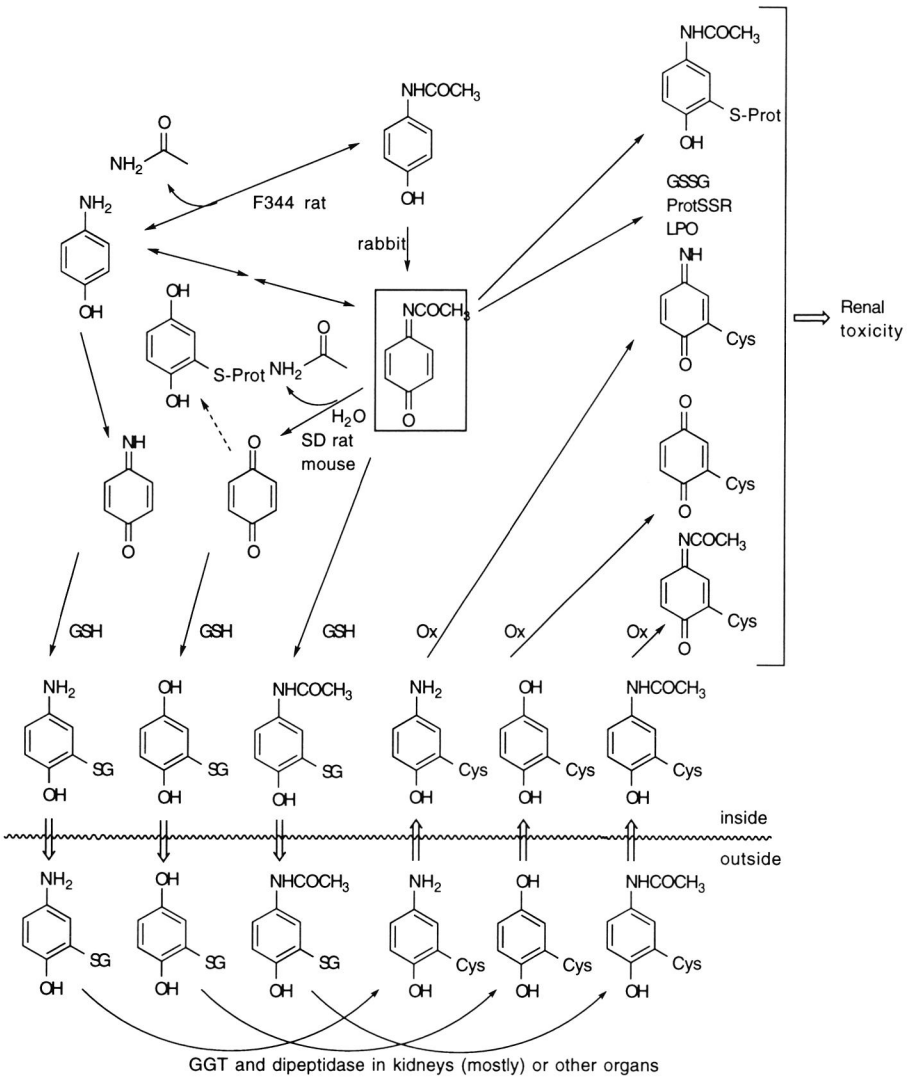


FIG. 12 Biotransformation of PAR in kidneys of various species
Biotransformation of PAR in the renal proximal tubule cell of the rabbit with P450 peroxidase as well as PGES activity and of the Fisher 344 rat with specific *N*-deacetylation favoured over CYP2E1 activity. The pathways between NAPQI, quinone imine and *p*-aminophenol may involve free radicals. The route releasing acetamide and leading to PBQ is also important in mice and Sprague Dawley rats. Composed from various references (see text).

V.3 Cytochrome P450

Although the cytochrome P450 enzymes differ somewhat in liver and kidneys, the consequences of exhaustive cytochrome P450 catalysed oxidation of PAR to NAPQI are

similar in both organs and are discussed in Part IV (protein and nonprotein thiol depletion followed by cellular dysfunction and organ failure). PAR-induced nephrotoxicity in CD-1 mice clearly differs from that described for Fischer rats (F344). The bioactivation mechanism in CD-1 mice is more similar to that in the SD-rat and likely involves renal cytochrome P450-dependent activation and subsequent covalent binding of a reactive metabolite without prior deacetylation (Emeigh Hart *et al.*, 1991a). Proteins of similar size as found in liver (44 and 58 kDa) became arylated in kidneys of mice upon administration of PAR. In addition, a 33 kDa protein became arylated. The severity of renal damage and the amount of adducts present could be significantly reduced with the P450 inhibitor piperonyl butoxide (Bartolone *et al.*, 1989). The development of PAR-toxicity in mouse kidney slices indicated the existence of an *in situ* activation system (Hoivik *et al.*, 1996a). Moreover, no differences were found in renal damage between bile-canulated and not bile-canulated rats, indicating the renal toxicity to be independent of hepatic biotransformation (Trumper *et al.*, 1996). Vice versa, hepatic damage was not altered when rats were pretreated with acivicin (AT-125), an inhibitor of γGT (Trumper *et al.*, 1996). In fact, the renal PAR-based protein arylation and toxicity is caused primarily by renal CYP2E1 catalysed biotransformation. The increase in PAR-dependent renal toxicity in female CD-1 and C3H/HeJ mice by testosterone-pretreatment correlated well to the inducibility of renal CYP2E1 by testosterone (Hoivik *et al.*, 1995; Hu *et al.*, 1993).

The significant sex-related difference observed in the NADPH-dependent bioactivation of PAR in renal microsomes from C3H/HeJ mice further supports the important role of CYP2E1. Moreover, it stresses the importancy of gender differences in P450-based bioactivation of PAR (Hu *et al.*, 1993). Summarising, in CD-1 and C3H/HeJ mice the mechanisms contributing to renal and hepatic toxicity are similar, although independent, and involve P450-dependent bioactivation of PAR. And in contrast to bioactivation in liver, important gender differences are observed in renal (CYP2E1-based) bioactivation and toxicity (Hu *et al.*, 1993). See Fig. 13 for an overview on interaction of hepatic and renal biotransformation steps via interorgan transport of PAR and metabolites.

In a recent study with renal (as well as hepatic) S9-incubations (cytosolic and microsomal fraction) from male and female SD-rats covalent protein binding of [^{14}C-phenyl]-PAR was reduced more than 85% by omission of NADPH. 1-Aminobenzotriazole (ABT; a suicide inhibitor of P450) reduced covalent binding by only 50% and bis(*p*-nitrophenyl) phosphate (BNPP; a reversible inhibitor of P450) had no effect. From these data the authors concluded that there must be other NADPH-requiring enzymes, not destroyed by ABT or inhibited by BNPP, that participate in the covalent binding of PAR (Mugford and Tarloff, 1995). It is possible, however, that NADPH is consumed without an NADPH-based enzyme. In a radical propagation reaction, PAR is likely oxidised by HRP/H_2O_2 to the phenoxy radical NAPSQI, which then can abstract a hydrogen atom from NADPH. The NADP$^{\bullet}$ thus formed may react with O_2 to produce the superoxide anion $O_2^{-\bullet}$ (Fig. 7) (Keller and Hinson, 1991).

V.4 Glutathione *S*-conjugation

It has been hypothesized recently that a GSH-conjugate of PAR/NAPQI or of a secondary metabolite is involved in renal toxicity of PAR in CD-1 mice (Emeigh Hart *et al.*, 1996). *In vivo* inhibition of γGT and organic anion transport (by AT-125 and probenecid) significantly decreased renal toxicity upon administration of PAR (Fig. 13). An extensive review was presented (Commandeur *et al.*, 1995). The observed decrease in PAR nephrotoxicity after inhibition of γGT (preventing the formation of the PAR-Cys) together with the complete prevention of damage after inhibition of the organic-anion transport with concomitant accumulation of substrates (PAR-Cys and PAR-NAC but not PAR-SG) are indicative for PAR-Cys or PAR-NAC contributing to the observed renal toxicity in CD-1 mice. As (1) PAR-Cys, and not PAR-NAC, is concentrated in mouse kidneys, (2) PAR-Cys is the predominant urinary metabolite, (3) inhibition of *N*-deacetylation does not alter nephrotoxicity (references in (Emeigh Hart *et al.*, 1996)), and (4) inhibition of ß-lyase activity by aminooxyacetic acid did not alter renal toxicity, it is most likely that PAR-Cys but not the mercaptan PAR-SH, plays a crucial role in the toxicity.

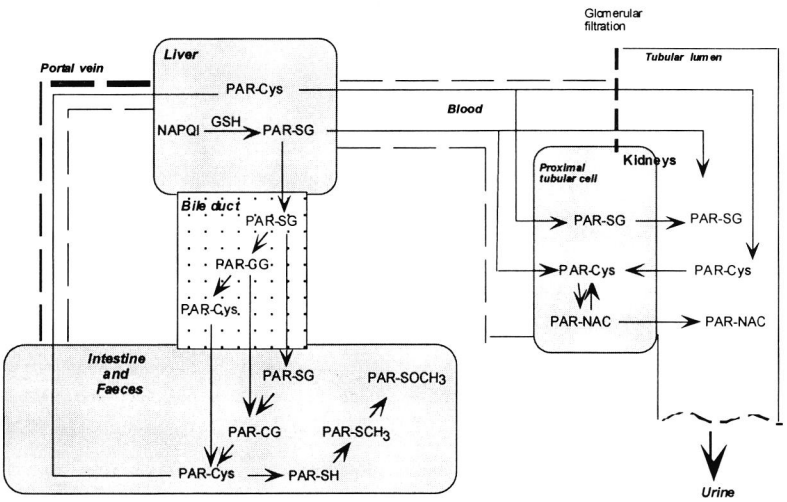


FIG. 13 Interorgan transport of paracetamol and its conjugates
Adapted and modified from Commandeur *et al.*, 1995

The PAR-dependent GSH-conjugate referred to above may be the 3-glutathione-*S*-yl-paracetamol conjugate but also a GSH-conjugate of *p*-aminophenol, being suggested as a secondary metabolite of PAR. In addition, another P450-based PAR-metabolite, *p*-benzoquinone (PBQ), being a hydrolysis metabolite of NAPQI, or its GSH-conjugates could contribute to the renal toxicity (Emeigh Hart *et al.*, 1996). Species differences as reported for PAR-based renal toxicity might be related to differences in GSH-

conjugation and protein-alkylation. As *p*-benzoquinone is known as a minor metabolite of PAR, mono-, di-and even tri(glutathione-*S*-yl)hydroquinone conjugates thereof formed in liver could be transported to the kidneys. E.g. upon administration of tri(glutathione-*S*-yl)hydroquinone, qualitative species differences have been reported for nephrotoxicity, correlating with protein alkylation of a 34 kDa in the S_3 segment of the renal proximal tubule (sensitive male Fisher 344 rats versus resistant BALB/c and B6C3F_1 mice) (Kleiner *et al.*, 1998).

V.5 Summary

Prostaglandin endoperoxide synthase (PGES), *N*-acetyltransferases as well as P450s are involved in the renal toxicity upon administration or intake of a high dose of PAR. Although important species and gender differences exist, acute renal toxicity may be observed next to hepatotoxicity. In mice and the SD-rat, the mechanisms contributing to renal toxicity of PAR involve P450-dependent bioactivation of PAR. In contrast to bioactivation in liver, important gender differences are observed in renal (CYP2E1-based) bioactivation and toxicity. In addition, F344 rats and hamsters exhibit renal deacetylation of PAR to *p*-aminophenol, followed by PGES-dependent formation of the phenoxy radical of *p*-aminophenol. In addition, species differences in the renal disposition of the cysteine- and the *N*-acetylcysteine conjugates may be relevant although their importance remains to be investigated.

Upon chronic intake of low doses of PAR, the kidney rather than the liver may be the target organ. Activation by PGES, which exhibits high affinity, may be responsible for chronic renal disease although epidemiological results are still controversial and rodents don't seem to be appropriate animal models. Rabbits might be better animal models for investigating PAR-dependent chronic renal disease. Recently, findings in mice that suggested covalent binding of PAR to DNA were supported by *in vitro* findings. The clinical relevance of these findings at therapeutic doses remains to be established, however. At the moment, epidemiological findings are ambiguous.

PART VI TOOLS TO MODULATE TOXICITY

VI.1 *N*-acetylcysteine

The most important antidote used in the clinic since long is *N*-acetylcysteine (NAC; Fluimicil™), the second one probably methionine (Jones, 1998; Prescott, 1983; Thomas, 1993). Both compounds are good examples of mechanism-based chemoprotection as they are GSH precursors and are preferred over GSH since this tripeptide is not absorbed as such by cells but only after breakdown into the corresponding amino acids. However, in human hepatocytes contrasting results were obtained with respect to methionine. As in human hepatocytes several sulfhydryl compounds increase intracellular GSH levels, in the order *N*-acetylcysteine > thioproline > cysteine > 2-oxo-4-thiazolidine carboxylic acid > methionine, only *N*-acetylcysteine, thioproline, and cysteine substantially counteracted PAR-based GSH-depletion (Larrauri *et al.*, 1987). Recently, combined treatment of intoxicated

mice with NAC and cimetidine (inhibitor of P450-dependent metabolic oxidation), 2 h after PAR administration, was reported to provide a 100% survival rate and a marked reduction in plasma ALT and AST levels. In comparison, single administration of either NAC or cimetidine caused only a partial improvement of these parameters (Al-Mustafa *et al.*, 1997). These authors referred to a paper that reported the absence of additive effects of NAC and cimetidine in PAR-intoxicated patients. This was suggested (Al-Mustafa *et al.*, 1997) to be due to a different protocol used (initiation of cimetidine treatment 8 h post-PAR overdose) and stresses the importance of the time-schedule in these treatments. Furthermore, the combined treatment might be useful as lower plasma levels of NAC could be sufficient, thereby minimising the risk for potential adverse reactions to NAC such as listed (Al-Mustafa *et al.*, 1997; Thomas, 1993). It should be stressed that NAC appears to be effective only when given within a few hours after PAR poisoning (Thomas, 1993). This period seems to correlate with the Stage I toxicity events as described in Part IV. This appears plausible since NAC is presumed to be mainly active via repletion of GSH and to some extent via repletion of the cofactor for sulphate conjugation but not via intercellular processes that are designated as Stage II hepatotoxicity. An overview of a selection of modulating agents, which are only partially described in the text, see Table 6.

VI.2 Other GSH-precursors

An interesting idea has been since long to formulate antidotes together with PAR in order to prevent PAR-mediated toxicity (chemoprotection), but at the same time retain analgesic activity. It was found that the addition of methionine to a PAR formulation (PAR/methionine 5/1) did not affect the analgesic potency of PAR in rats. Besides, methionine reduced the acute toxicity (LD_{50}) of paracetamol by 50% in mice (Ponsoda *et al.*, 1991). Moreover, in ten human volunteers the pharmacokinetics of paracetamol (1500 mg) were not affected by methionine (300 mg) (Ponsoda *et al.*, 1991). Addition of methionine to PAR formulations or even esterifying methionine to PAR, thus delivering methionine simultaneously with potential overdosing, is an option that has been suggested in the past as a simple way to reduce the high mortality upon paracetamol overdosage (Neuvonen *et al.*, 1985; Skoglund *et al.*, 1986). Although no information is available regarding current registration, actually, in the United Kingdom a PAR-preparation containing methionine, named Pameton, has been available (Thomas, 1993).

Further investigations into chemoprotective agents that may be clinically relevant and result in better antidotes than *N*-acetylcysteine (as mostly not indicated in this review for amino acids and derivatives, *N*-acetyl-L-cysteine is meant) have been performed also. Based on the knowledge on the protective effects of methionine when administered beforehand or simultaneously to toxic doses of PAR, it was found that incubation of human hepatocytes with the methionine containing endogenous compound *S*-adenosylmethionine attenuated the GSH depletion of human hepatocytes incubated with toxic concentrations of paracetamol (Ponsoda *et al.*, 1991). Moreover, *in vivo* in mice, *S*-adenosylmethionine significantly decreased the number of PAR-based deaths when administered 2-5 h after PAR (Bray *et al.*, 1992). Thus, *S*-adenosylmethionine

might prove to be suitable to improve clinical antidote therapy compared to methionine or *N*-acetylcysteine or to be fitting as antidote in combination with one or both of these.

Cysteine (actually L-cysteine), the limiting reagent for GSH-biosynthesis, has been investigated as a potential antidote to be formulated together with PAR or to be given as an antidote after intoxication. The administration of cysteine is known to increase GSH-levels, but the amino acid can be neurotoxic and even mutagenic at therapeutic doses (references in (Roberts *et al.*, 1998)). Thiazolidine prodrugs of cysteine, constructed from the amino acid and a variety of alkyl or aryl aldehydes or ketones, such as aldose monosaccharides, have shown protective activity against PAR-dependent hepatotoxicity and function as a slow release of the precursor for the GSH-biosynthesis (Nagasawa *et al.*, 1984; Roberts *et al.*, 1992; Roberts *et al.*, 1987b). Recently, even attempts were described to construct prodrugs of cysteine containing disaccharides in order to obtain analogues with a pendant cyclic sugar moiety that may allow selective delivery to carbohydrate receptors, such as the asialoglycoprotein receptor of hepatocyts (Roberts *et al.*, 1998).

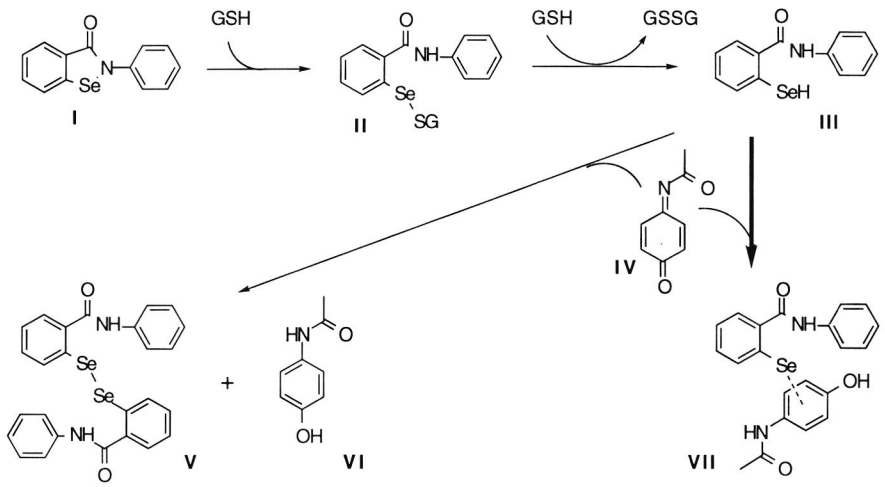


FIG. 14 Proposed mechanism for protection by ebselen
Proposed mechanism for protection by ebselen againts PAR-mediated cytotoxicity (Adapted from Li *et al.*, 1994a)

VI.3 Selenium compounds

Another mechanism-based compound that has been investigated more recently for its chemoprotective properties in relation to PAR-mediated hepatotoxicity is ebselen, an anti-inflammatory agent (Harman *et al.*, 1992; Li *et al.*, 1994a). Ebselen protected against PAR cytotoxicity when co-incubated in freshly isolated hepatocytes. The protective effect of ebselen was probably not caused by direct reaction with PAR or

inhibition of P450 but by reduction of NAPQI by selenol (Li *et al.*, 1994a). It was suggested that the selenol of ebselen, formed upon redox reactions with GSH (Cotgreave *et al.*, 1992; Haenen *et al.*, 1989), was much more a reductant than a nucleophile towards NAPQI when compared with GSH as no indication has been found for the formation of a nucleophilic substitution product between NAPQI and ebselen (Fig. 14) (Li *et al.*, 1994a). In addition, peroxidase-like activity of ebselen, protection of ebselen as a direct, thiol-independent antioxidant or radical scavenger could add to the protection of ebselen against PAR-induced cytotoxicity (Li *et al.*, 1994a).

It is known that administration of other selenium compounds, such as sodium selenite (Na_2SeO_3), to mice prior to PAR administration, decreases the hepatotoxicity of PAR as well. Concomitantly, inhibition of the PAR-induced hepatic GSH-depletion and covalent binding to hepatic protein was observed. It was suggested that sodium selenite protected via enhanced glucuronidation of PAR thereby diverting the amount of PAR converted to NAPQI (Schnell *et al.*, 1988). However, another mechanism, analogous to the mechanism noted later for ebselen, might also be responsible for the observed protection by sodium selenite against PAR-induced hepatotoxicity in mice. The protective effect of sodium selenite against the renal toxicity of another compound, cisplatin, was suggested to be caused by methylselenol as well as glutathionylselenol, a new metabolite of selenite (Baldew *et al.*, 1992; Vermeulen *et al.*, 1993) (see also Part IV on hepatotoxicity mechanisms). With coadministration of PAR and ebselen to hepatocytes, glutathionylselenol thus formed might effectively reduce NAPQI, thereby lowering the NAPQI-based covalent modification of sensitive proteins. In the case of administration of sodium selenite to protect against PAR-dependent toxicity (Schnell *et al.*, 1988), selenite is likely converted to glutathionylselenol by a GSH-dependent mechanism while significantly depleting GSH. The fall in the level of GSH could trigger an increase in activities of γ-glutamylcysteine synthetase (γ-GT) as actually observed by Schnell *et al.* (Schnell *et al.*, 1988). This might well increase GSH-levels again.

This suggestion is substantiated by the recent observation of a decrease in the PAR-based arylation of the 56 kDa selenium binding protein (SP56; see also Part IV on hepatotoxicity) in mice upon preadministration of sodium selenite, prior to marginally toxic doses of PAR, in comparison to administration of PAR only. The extent of decrease of binding to this SP56 was, however, not different from the decrease in covalent binding to other proteins (Hinson *et al.*, 1996). A prior interaction of selenium with the selenium binding protein should have resulted in a selective decrease in the available binding sites of this protein for NAPQI. The absence of such selectivity in the decrease of covalent binding as found by Hinson *et al.* (Hinson *et al.*, 1996) is suggested here to be based on the same mechanism as explained in the paragraphs above for protection of PAR-cytotoxicity by ebselen. This is, protection by sodium selenite against PAR-induced covalent binding and hepatotoxicity was caused by a decrease in the level of NAPQI and a decrease in the level of general protein modification, due to blocking of cysteine thiols by selenium thus forming thioselenols ($R\text{-}CH_2\text{-}S\text{-}SeH$). Thioselenols in turn, would reduce NAPQI to PAR. See also Fig. 10 in Part IV. Probably many proteins that have free accessible cysteines are chemically prone to attack by selenite and could thus be called selenium binding proteins.

Importantly, these are different from selenoproteins that have specifically one or more selenocysteine(s) incorporated in their amino acid backbone.

VI.4 Various modulating agents

Treatment of mice for 10 days with the peroxisome proliferating agent clofibrate (500 mg/kg bw/day; i.p.) protected against PAR induced hepatotoxicity upon a PAR challenge after an overnight fast (Manautou *et al.*, 1994; Nicholls-Grzemski *et al.*, 1992). Although clofibrate pretreatment has no effect on urinary excretion of PAR-GLUC, PAR-SULP, PAR-Cys, nor PAR-NAC, elimination of PAR from plasma and liver and urinary excretion of the unchanged PAR was faster in clofibrate-pretreated mice. This was accompanied by elevated biliary excretion of PAR-SG at 2 h after PAR dosing, and by increased urinary PAR excretion (Manautou *et al.*, 1996). Total covalent binding to hepatic proteins was diminished significantly, however, the level of a 58 kDa PAR binding protein was not diminished by clofibrate pretreament. Furthermore, no effect of clofibrate pretreatment on microsomal PAR bioactivation to NAPQI was observed. Hepatic nonprotein sulfhydryl levels were increased, leading to the hypothesis of clofibrate protection by increase in hepatic GSH levels (Manautou *et al.*, 1994; Manautou *et al.*, 1996).

Recently, protection against PAR-dependent hepatotoxicity in mice by pretreatment (chemoprevention) with a single dose of amphetamine 72 h before PAR, was suggested to be caused by induction of heat shock proteins (hsp) (Salminen *et al.*, 1997). Although it was suggested in Part IV that hepatocytes might be protected by amphetamine pretreatment by becoming prepared to cope with stressors, future studies to better characterize the relationship between hsp induction and susceptibility to toxicity are warranted.

Many investigations have resulted in protective agents that were effective in *in vitro* systems or in test animals when added before (chemoprevention) or concomitantly with (chemoprotection) addition of or treatment with PAR (like clofibrate or amphetamine), respectively. Therefor, for clinical use, these protective agents are rather useless. However, many of them have been proven to be advantageous as tools in the elucidation of the toxicity mechanism of PAR. For example ascorbic acid reduced the NAPSQI radical formed upon peroxidase-catalysed oxidation much more efficiently than GSH, thus suggesting that the endogenous ascorbate might be more important in the detoxification of the PAR phenoxy radical than high concentrations of GSH in tissues with high peroxidase activity (Ramakrishna Rao *et al.*, 1990). Also, ascorbate added to hamster liver microsomal incubations with PAR inhibited covalent binding of PAR. However, administration of ascorbate (that rises hepatic ascorbate levels rapidly in control rats) immediately after PAR, did not decrease covalent binding nor hepatotoxicity in hamsters (Miller and Jollow, 1984).

A completely different mechanism which has not been resolved unambiguously yet is probably responsible for the chemoprotective properties of oleanolic acid, a triterpene, against PAR and a number of other hepatotoxicants. Oleanolic acid, used in China to treat hepatitis, strongly protects against PAR induced hepatotoxicity in mice. The mechanism of this protection against PAR-induced hepatotoxicity, at least in part,

appears to be due to the decreased formation of toxic metabolites of PAR by CYP1A and CYP2A, as well as increased detoxication by enhanced glucuronidation of PAR (Liu *et al.*, 1993a; Liu *et al.*, 1995). In addition, compounds such as phenetyl isothiocyanate (or phenetyl isothiocyanate containing watercress), propylene glycol, taxol and triacetyloleandomycine were found to inhibit or induce CYP1A2, CYP2E1 and/or CYP3A with concomitant decrease or increase of hepatotoxicity (Chen *et al.*, 1996; Kostrubsky *et al.*, 1997a; Kostrubsky *et al.*, 1997b; Li *et al.*, 1997; Snawder *et al.*, 1993; Thomsen *et al.*, 1995). An overview of modulating agents for which sufficient evidence was present to be active via enhancement or inhibition of bioactivating and detoxifying enzymes and that were reviewed earlier (Vermeulen *et al.*, 1992) is presented in Table 6 without further discussion.

TABLE 6 *In vitro* and *in vivo* modulation of paracetamol-induced renal and hepatic effects

Protective agent	Species/Test system	Mechanism	Effect	Reference
At the level of glucuronidation and sulphation				
oleanolic acid	mice, *in vivo*	incr. glucuronidation	decr. hepatotoxicity	(Liu *et al.*, 1993; Liu *et al.*, 1995b)
pregnenolone-16α-carbonitrile	hamster, *in vivo*	incr. UDPGT / increase UDPGA	decr. hepatotoxicity	(Madhu and Klaassen, 1991)
ranitidine	rat, *in vivo*	inhibition conjugative route	incr. hepatotoxicity	(Leonard *et al.*, 1985)
ranitidine	rat (Fischer 344), *in vivo*	inhibition of glucuronidation	incr. hepatotoxicity	(Rogers *et al.*, 1988)
selenite	rat (male SD)	incr. total glucuronidation (maybe preceded by reduction of NAPQI to PAR) [1]	decr. hepatotoxicity	(Schnell *et al.*, 1988)
At the level of deacetylation				
bis(*p*-nitrophenyl) phosphate (BNPP)	rat (F344); *in vivo* and *in vitro*	inhibition PAR deacetylation to PAP	decr. protein arylation; decr. renal toxicity	(Newton *et al.*, 1983; Newton *et al.*, 1985a)
bis(*p*-nitrophenyl) phosphate (BNPP)	mouse (CD-1)	inhibition deacetylation	no effect	(Emeigh Hart *et al.*, 1991)
tri-*o*-tolyl-phosphate (TOTP)	mouse (CD-1)	inhibition deacetylation	no effect	(Emeigh Hart *et al.*, 1991)
At the level of PGES bioactivation				
indomethacin	rabbit, kidney microsomes	inhibition PGES	decr. protein arylation	(Mohandas *et al.*, 1981)
At the level of P450 bioactivation (unspecified)				
caffeine	rat, *in vivo*	stimulation P450	incr. hepatotoxicity	(Sato and Izumi, 1989)
carbamazepine	man, *in vivo*	induction P450	incr. metabolism PAR	(Smith *et al.*, 1986)

chlordecone	mouse, *in vivo*	induction P450	incr. hepatotoxicity	(Fouse and Hodgson, 1987)
chlordecone	mouse, hepatocytes	induction P450	incr. cytotoxicity	(Fouse and Hodgson, 1987)
cimetidine	man/mouse/rat, *in vivo*	inhibition P450	decr. hepatotoxicity	(Leonard *et al.*, 1985; Kadri *et al.*, 1988)
cimetidine (+ *N*-acetylcysteine)	mouse, *in vivo*	inhibition P450	decr. hepatotoxicity	(Al-Mustafa *et al.*, 1997)
cobaltous chloride	hamster, *in vivo*	destruction P450	decr. biliary PAR-SG excretion	(Madhu *et al.*, 1989)
cobaltous chloride	mouse, *in vivo* treated not with PAR but with 3'HAA	suppression P450	decr. covalent binding	(Salminen *et al.*, 1998)
disulfiram	rat, *in vivo*	inhibition P450	decr. hepatotoxicity	(Jörgensen *et al.*, 1988)
ethanol	mouse, *in vivo*	induction P450	incr. hepatotoxicity	(Carter, 1987)
ethanol	mouse, *in vivo*	inhibition P450	decr. hepatotoxicity	(Thummel *et al.*, 1989)
ethanol	man, *in vivo*	depletion of NADPH	decr. hepatotoxicity	(Thummel *et al.*, 1989)
4-methylpyrazole	rat, *in vivo*	inhibition P450	decr. hepatotoxicity	(Burk *et al.*, 1990)
methylxanthines	rat (SD), *in vivo*	inhib/activation P450's	protection/potentiation	(Kalhorn *et al.*, 1990)
metyrapone	hamster, *in vivo*	inhibition P450	decr. biliary PAR-SG	(Madhu *et al.*, 1989)
Mirex	mouse, *in vivo*	induction P450	incr. hepatotoxicity	(Fouse and Hodgson, 1987)
Mirex	mouse, hepatocytes	induction P450	incr. cytotoxicity	(Fouse and Hodgson, 1987)
β-naphthoflavone	rat, hepatocytes	inhibition P450	decr. cytotoxicity	(Kyle *et al.*, 1987)
phenobarbital	rat, hepatocytes	induction P450	incr. cytotoxicity	(Devalia *et al.*, 1982)
piperonyl butoxide	mouse (male CD-1), *in vivo*	inhibition P450	decr. protein arylation and tissue damage in liver and kidneys	(Bartolone *et al.*, 1989)
piperonyl butoxide	mouse (male CD-1), *in vivo*	inhibition P450	decr. PAR-SG;; decr. protein arylation; decr. hepatoxicity	(Emeigh Hart *et al.*, 1991)
piperonyl butoxide	mouse (male CD-1), *in vivo*	inhibition P450	decr. renal PAR toxicity; however no effect on PAP toxicity	(Emeigh Hart *et al.*, 1991)
piperonyl butoxide	hamster, *in vivo*	no effect on hamster P450?	no influence on PAR-SG excretion	(Madhu *et al.*, 1989)
pregnenolone-16α-carbonitrile	hamster, liver microsomes	modulation P450(s)	decr. NAPQI formed	(Madhu and Klaassen, 1991)
propylene glycol	mouse, *in vivo*	inhibition P450	decr. hepatotoxicity	(Hughes *et al.*, 1991)
ranitidine	rat , *in vivo*	inhibition P450	decr. hepatotoxicity	(Leonard *et al.*, 1985)
SKF525A	rat liver slices	inhibition P450 only 0-2 h of incubation	decr. cytototoxicity	(Mourelle *et al.*, 1990)
At the level of specified CYP bioactivation				
α-naphthoflavone	hamster, *in vivo*	inhibition CYP1A	decr. biliary PAR-SG	(Madhu *et al.*, 1989)

alpha-hederin	mouse, *in vivo*	suppression CYP1A, 2A, 3A	decr. hepatotoxicity	(Liu *et al.*, 1995a)
caffeine	mouse, liver microsomes	stimulation CYP3A family	incr. PAR-SG	(Jaw and Jeffery, 1993)
clotrimazole	mouse, *in vivo*	inhibition CYP3A	decr. cov. binding and necrosis	(Salminen *et al.*, 1998)
CYP1A2 and CYP2E1 not expressed	CYP1A2 and CYP2E1 double-null mice,	no CYP1A2 and CYP2E1 expressed	decr. hepatotoxicity	(Zaher *et al.*, 1998)
dexamethasone	mouse, *in vivo*	induction CYP3A	incr. hepatotoxicity	(Madhu *et al.*, 1992)
diallylsulfide and other org. sulfur compounds	mouse, *in vivo*	inhibition CYP2E1	decr. cov. binding and hepatic necrosis	(Wang *et al.*, 1996; Salminen *et al.*, 1998)
diallysulfide	human cell-line ex-pressing hCYP2E1	inhbition CYP2E1	decr. cytotoxicity	(Dai and Cederbaum, 1995)
DMSO	mouse, *in vivo*	inhibition CYP2E1	decr. hepatotoxicity	(Jeffery and Haschek, 1988)
DMSO	mouse, *in vivo*	no CYP2E1 present	no decr. lung toxicity and nasal toxicity	(Jeffery and Haschek, 1988)
ethynylpyrene	rat , hepatocytes	inhibition CYP1A	decr. cytotoxicity	(Bessems *et al.*, 1997)
flavonoids	rat (SD), liver microsomes	modulation CYP 3A4	PAR-SG up or down	(Li *et al.*, 1994b)
fluvoxamine	mouse (male NMRI), *in vivo*	inhibitor CYP1A2	no effect	(Thomsen *et al.*, 1995)
isoniazid	rat, *in vivo*	induction CYP2E1	incr. hepatotoxicity	(Burk *et al.*, 1990)
3-methylcholanthrene	hamster, *in vivo*	induction CYP1A	incr. biliary PAR-SG	(Madhu *et al.*, 1989)
4-methylpyrazole	human cell-line ex-pressing hCYP2E1	inhbition CYP2E1	decr. cytotoxicity	(Dai and Cederbaum, 1995)
methylxanthines (e.g. caffeine)	rat, microsomes and *in vivo*	modulation NAPQI formation by CYP1A/CYP3A [2)]	modulation hepatotoxicity	(Lee *et al.*, 1991a; Lee *et al.*, 1991b; Lee *et al.*, 1996)
oleanolic acid	moude, *in vivo*	suppression CYP1A, 2A	decr. hepatotoxicity	(Liu *et al.*, 1993; Liu *et al.*, 1995b)
phenethyl isothiocyanate	mouse, *in vivo*	inhibition CYP1A2 + CYP2E1	decr. hepatotoxicity	(Li *et al.*, 1997)
phenethyl isothiocyanate	mouse, liver microsomes	inhibition CYP1A2 + CYP2E1	decr. PAR-SG	(Li *et al.*, 1997)
propylene glycol	mouse (maleB6C3F1), hepatic microsomes	inhibition CYP2E1 but not CYP1A2 (30% activ. remained)	decr. PAR-SG	(Snawder *et al.*, 1993)
propylene glycol	mouse (male NMRI), *in vivo*	inhibition CYP2E1	decr. hepatotoxicity	(Thomsen *et al.*, 1995)
taxol	rat, *in vivo*	induction CYP3A	incr. hepatotoxicity	(Kostrubsky *et al.*, 1997a)
TCDD	hamster, *in vivo*	induction CYP1A	incr. biliary PAR-SG	(Madhu *et al.*, 1989)
triacetyloleandomycine	rat, *in vivo*	inhibition CYP3A	decr. hepatotoxicity	(Kostrubsky *et al.*, 1997b)
watercress	man, *in vivo*	inhibition CYP2E1 suggested	decr. urinary PAR-Cys and PAR-NAC	(Chen *et al.*, 1996)
Modulation at the level of reduction of NAPQI				
acetylsalicylic acid	rabbit, kidney microsomes	inhibition PGES or reduction of NAPSQI	decr. protein arylation	(Mohandas *et al.*, 1981; Ramakrishna Rao *et al.*, 1990)

ascorbate	mouse, liver microsomes	reduction of NAPQI	decr. protein arylation	(Dahlin *et al.*, 1984)
ascorbate	hamster, hepatocytes	reduction of NAPQI (or NAPSQI as a comproportionation product?)	50% decr. protein arylation [3]	(Miller and Jollow, 1984)
ascorbate	hamster, *in vivo*	reduction of NAPQI	no decr. protein arylation or hepatotoxicity	(Miller and Jollow, 1984)
ascorbate	man, erythrocytes	antioxidant	protection hemoglobin and Na^+/K^+ATP-ase	(Tukel, 1995)
ebselen	rat (Wistar), hepatocytes	reduction of NAPQI to PAR	LDH leakage down; only by simultaneous or pre-treatment	(Li *et al.*, 1994a)
tocopherol	man, erythrocytes	antioxidant	protection hemoglobin and Na^+/K^+ATP-ase	(Tukel, 1995)
Modulation at the level of GSH synthesis or GSH-conjugation				
S-adenosyl-L-methionine	man, hepatocytes	elevation of GSH	protection against GSH depletion	(Ponsoda *et al.*, 1991)
N-acetylcysteine	mouse, *in vivo*	increased GSH synthesis	prevention hepatic damage	(Corcoran *et al.*, 1985)
N-acetylcysteine	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrauri *et al.*, 1987)
N-acetyl-L-cysteine	mouse, *in vivo*	increased GSH synthesis	decr. hepatotoxicity	(Corcoran and Wong, 1986)
N-acetylcysteine (+-cimetidine)	mice, *in vivo*	incr. hepatic GSH concentrations	decr. hepatotoxicity	(Al-Mustafa *et al.*, 1997)
AT-125 (acivicin)	mouse (male CD-1)	inhibition gGT	decr. renal toxicity	(Emeigh Hart *et al.*, 1996)
1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU)	mouse, hepatocytes	inhibition of GSH peroxidase and GSSG reductase	incr. membrane damage due to incr. ROOH	(Adamson and Harman, 1989)
buthionine sulfoximine	mice (male C3H)	inhibition γ-glutamylcysteine synthetase	depletion hepatic and renal GSH	(Drew and Miners, 1984)
buthionine sulfoximine	rat, hepatocytes	inhibition GSH-synthesis	depletion GSH	(Hue *et al.*, 1985)
buthionine sulfoximine	rat, *in vivo*	inhibition γ-glutamylcysteine synthetase	incr. clearance of PAR and partial clearance of PAR-SULP	(Galinsky, 1986)
buthionine sulfoximine	rat, *in vivo*	incr. UDPGT and GST	incr. plasma concetrations PAR-GLUC	(Manning *et al.*, 1991)
clofibrate	mouse (male CD-1)	incr. hepatic [GSH]	incr. biliary PAR-SG; decr. protein arylation	(Manautou *et al.*, 1994; Manautou *et al.*, 1996)
coenzym A	mouse, *in vivo*	decr. GSH depletion	decr. mortality	(Bertelli *et al.*, 1990)
cysteine	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrauri *et al.*, 1987)
cysteine-prodrugs				
2-oxo-4-thiazolidine carboxylate	man, hepatocytes	no decr. GSH depletion !	no decr. cytotoxicity	(Larrauri *et al.*, 1987)
L-2-oxothiazolidine-4-carboxylate	mouse, *in vivo*	converted to L-cysteine, stimulation of GSH synthesis	decr. toxicity	(Williamson *et al.*, 1982)
thiazolidine-4R-carboxilic acids	mouse, *in vivo*	stimulation of GSH resynthesis	decr. hepatotoxicity	(Nagasawa *et al.*, 1984)
thiazolidine-saccharides	mouse, *in vivo*	increase GSH biosynthesis; no GSH depletion	decr. hepatotoxicity	(Roberts *et al.*, 1987; Roberts *et al.*, 1992; Roberts *et al.*, 1998)

goldthioglucose	mouse, hepatocytes	inhibition of GSH peroxidase and GSSG reductase	incr. membrane damage due to incr. ROOH	(Adamson and Harman, 1989)
isopropylester of GSH	mouse, i.p. *in vivo*	increased GSH levels in various organs	decr. hepatic damage	(Uhlig and Wendel, 1990)
isaxonine	rat (SD); hepatocytes	probably GSH depletion	incr. cytotoxicity	(Shrivastava *et al.*, 1994)
methionine	mouse, *in vivo*	probable stimulation GSH synthesis	decr. hepatotoxicity	(Neuvonen *et al.*, 1985)
phenylpropanolamine	mouse, ICR	depletion of [GSH]; only when 3 hr pre-PAR	incr. hepatotoxicity	(James *et al.*, 1993)
prednisolone	mouse	repletion of GSH ?; pos. effect on GSH synthesis?	decr. hepatotoxicity	(Speck *et al.*, 1993)
thioproline	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrauri *et al.*, 1987)
Zn^{2+} (as zinc sulfate)	mouse, ICR	replenishment hepatic GSH	decr. ALT; decr. malondialdehyde	(Woo *et al.*, 1995)
Modulation at the level of oxidative stress				
allopurinol	mouse, *in vivo*	antioxidant	decr. hepatotoxicity	(Jaeschke, 1990)
1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU)	mouse, hepatocytes	inhibition GSSG-reductase and increased sensitivity to oxidative stress	incr. cytotoxicity	(Gerson *et al.*, 1985)
catalase	rat, hepatocytes (BCNU present)	scavenging H_2O_2	decr. cytotoxicity	(Kyle *et al.*, 1987)
curcumin (low concentration)	rat, hepatocytes	antioxidant	decr. cytotoxicity	(Donatus *et al.*, 1990)
desferrioxamine	mouse, hepatocytes	decr. sensitivity to oxidative stress	decr. cytotoxicity	(Gerson *et al.*, 1985)
deferrioxamine	mouse, *in vivo*	chelation of Fe^{3+} ions; inhibition LPO	no decr. hepatotoxicity !!	(Younes *et al.*, 1988)
dichlorophenol indophenol (DCPIP)	rat liver slices	antioxidant after 2 h of incubation	decr. cytotoxicity	(Mourelle *et al.*, 1990)
flavones	rat, hepatocytes	no mprotein arylation; antioxidant?	decr. cytotoxicity	(Devalia *et al.*, 1982)
lobenzarit	rat (Wistar), hepatocytes	probably AO effect and/or stimulation GSSG-RED	decr. LDH leakage	(Remirez *et al.*, 1995)
piperidine analogues	rat, hepatocytes	antioxidant; decr LPO	decr. cytotox	(Alexidis *et al.*, 1996)
propylgallate	human cell-line ex-pressing hCYP2E1	no inhibition LPO	no decr. cytotoxiticy	(Dai and Cederbaum, 1995)
superoxide dismutase	rat, hepatocytes (BCNU present)	scavenging O^{2-}	decr. cytotoxicity	(Kyle *et al.*, 1987)
zinc	mouse	induction of metallothionein	decr. Fe2 + decr LPO ?	(Chengelis *et al.*, 1986)
Various modulations at stage I of toxicity development				
ascorbate	hepatocytes	decr. protein arylation	no influence *in vivo* toxicity	(Miller and Jollow, 1984)
acetaylsalicylic acid	mouse / rat	unknown post-bioactivation process	decr. hepatoxicity	(Whitehouse *et al.*, 1976; De Vries *et al.*, 1984)
cyclohexamide	rat (SD)	inhibition of protein synthesis (incorporation of acetyl carbon in proteins)	decr. radiolabel in renal protein in [acetyl ^{14}C]-PAR dosed rats	(Newton *et al.*, 1985b)
PBN (N-*t*-butyl-α-phenylnitrone)	human cell-line ex-pressing hCYP2E1	radical trapping (spin-trapping)	decr. cytotoxicity	(Dai and Cederbaum, 1995)

probenecid	mouse (male CD-1)	inhibition organic-anion transport	decr. renal toxicity	(Emeigh Hart *et al.*, 1996)
Modulation at stage II of toxicity development				
amphetamine	mouse, *in vivo*	induction heat shock prtein in liver	decr. hepatotoxicity	(Salminen *et al.*, 1997)
chlorpromazine	mouse, *in vivo*	decr. nuclear Ca^{2+} increase and DNA fragmentation	decr. hepatic apoptosis/necrosis	(Ray *et al.*, 1993)
cholesteryl hemisuccinate	rat, *in vivo*	decr. apoptotis	decr. hepatic apoptosis/necrosis	(Ray *et al.*, 1996)
dextran sulfate	rat, *in vivo*	inhibition of macrophages	decr. hepatic necrosis	(Laskin *et al.*, 1995)
fructose	rat, liver slices	increased intracellular ATP in stage II of hepatotoxicity	prevention of damage	(Mourelle *et al.*, 1991; Martin and McLean, 1996)
gadolinium chloride	rat, *in vivo*	inhibition of macrophages	decr. hepatic necrosis	(Laskin *et al.*, 1995)
iodoacetate	rat, liver slices	inhibition glycolyse	incr. damage	(Martin and McLean, 1996)
lipopolysacharide	rat, *in vivo*	activation of macrophages	incr. hepatic necrosis	(Laskin *et al.*, 1995)
misoprostol	rat, *in vivo*	decr. microvascular injury	decr. hepatic necrosis	(Lim *et al.*, 1995)
thioacetamide	rat, *in vivo*	sustained tissue repair	decr. hepatic necrosis	(Chanda *et al.*, 1995; Chanda and Mehendale, 1996)
verapamil	mouse, *in vivo*	decr. nuclear Ca^{2+} increase and DNA fragmentation	decr. hepatic apoptosis/necrosis	(Ray *et al.*, 1993)

1) The mechanism depicted between brackets is hypothesized in this review. See Chapter III
2) Actual effect (stimulation or inhibition, dependent on previous induction or not and on the sort of induction (CYP1A or CYP3A)
3) Remaining 50% inhibitable by cysteine

Explanations:
1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) = a glutathione reductase inhibitor
bis(*p*-nitrophenyl) phosphate (BNPP) is an inhibitor of carboxylesterases and thus of *N*-deacetylation
tri-*o*-tolyl-phosphate (TOTP) is an inhibitor of carboxylesterases and thus of *N*-deacetylation

The question mark '?' is used when no straight explanation of a finding was presented in the reference but deduced in this review.

VI.5 Modulation of Stage II

Later on in the process of PAR intoxication, Kupffer cells become activated, a process generally associated with release of pro-inflammatory cytokines with a variety of pathophysiological responses (Blazka *et al.*, 1995). Antibodies against TNF-α and IL-1α appear to protect against PAR-induced hepatotoxicity in mice significantly. This suggests that TNF-α and IL-1α are released in response to PAR intoxication and are responsible for certain pathological manifestations of PAR induced hepatotoxicity (Blazka *et al.*, 1995). For example, the decrease in body temperature upon a dose of 500 mg/kg bw PAR was counteracted by the antibodies against TNF-α and IL-1α.

Recently, even a possible role of the source of cellular energy was hypothesized in the modulation of PAR-based hepatotoxicity. SD-rats that were fed a normal rodent chow supplied with 15% glucose in drinking water (as source of energy for the centrilobular hepatocytes) during 7 days suffered from increased lethality to PAR

(Chanda and Mehendale, 1996b). Also, a lowered ATP content of cells as found in fructose medium compared to glucose medium, rendered freshly isolated rat hepatocytes less vulnerable to quinone toxicants (Toxopeus *et al.*, 1994). It had been demonstrated before that aerobic oxidation of glucose is decreased in toxic liver diseases although glucose is often used as a ready source of energy for the patients with severe hepatic disorder (references in (Chanda and Mehendale, 1996b)). Thus, as glucose would be an inappropriate substrate to support a rapid and timely cell division and tissue repair and glucose might increase the toxicity of PAR by inhibition of hepatocellular regeneration and tissue repair (Chanda and Mehendale, 1996b), change of the sources of energy in clinical settings might improve the chances for recovery from a PAR intoxication.

VI.6 Summary

Despite numerous studies that have been performed with the main aim to find protective agents for PAR-induced hepatotoxicity, to be added before (chemoprevention), concomitantly or following PAR (chemoprotection), very few agents have been used succesfully in the clinic. Of the many agents that have been investigated in laboratory animals and were reviewed before, some were highlighted here because of their importance. In addition, most of the recent findings with respect to modulation of PAR-mediated toxicity were discussed. Modulation of PAR-mediated toxicity by structural modification of PAR will be discussed in detail in Part VII.

As became clear, adduct formation between NAPQI and numerous cellular proteins is of utmost importance in the hepatotoxicity mechanism of PAR. In the first thirty years since PAR became an over-the-counter analgesic, most efforts were put into prevention of primary damage (Stage I). Decreasing the reactive metabolite formation via inhibition of P450 and structural modification of PAR and increasing the efficiency of scavenging of the reactive metabolite via the *N*-acetylcysteine and GSH routes were important goals. A new lead in preventing further primary damage in liver might be protection of susceptible target sites in proteins, maybe via selenium containing compounds. Often however, most primary damage has taken place already upon admission to an emergency ward. Treatment with the most important mechanism-based antidote since long, *N*-acetylcysteine (a precursor of GSH which itself does not enter the cell), may be only partially effective upon progression of hepatic failure. Therefore, it seems to be clinically very relevant to aim additionally at the Stage II events of intra- and intercellular signalling as to influence the balance of tissue repair and damage in favour of repair.

PART VII STRUCTURAL MODIFICATION OF PARACETAMOL

VII.1 Introduction

Next to the many reports describing prevention of liver injury of PAR (1) by decreasing the production of toxic intermediate(s), (2) by increasing the capacity to detoxify the toxic intermediates(s), or (3) by increasing the ability of the tissue to

withstand or even repair the molecular damage caused by the toxic species (Corcoran *et al.*, 1985), several reports have been published on approaches (4) to modify PAR-induced organ toxicity by changes of its chemical structure. Two lines of research may be distinguished, the first (4a) aiming at modification of the structure of PAR in order to reduce its toxicity while conserving its pharmacological properties as much as possible, the second (4b) more mechanistically based and aiming at structural analogues and analysing its consequences for toxic properties. Thus, taking the presumed molecular mechanisms of analgesic activity as well as that of the hepatotoxicity of PAR into consideration, there have been several efforts to improve its analgesic activity while preventing its toxicity by modifying its structure (Fig. 15) (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b; Bessems *et al.*, 1995; Bessems *et al.*, 1997; Dearden *et al.*, 1980; Harvison *et al.*, 1988a; Harvison *et al.*, 1986b; Porubek *et al.*, 1987; Skoglund *et al.*, 1986; Skoglund *et al.*, 1988; Van de Straat *et al.*, 1987b; Van de Straat *et al.*, 1987c). Besides, several patents describe the synthesis of ring-substituted PAR derivatives and *N*-substituted *p*-amino-anilides with halogen substitution(s) *ortho* of the amino group. These patent applications included preliminary results on investigations into supposed analgetic, anti-pyretic and/or anti-inflammatory properties (Anonymous, 1966; Nickl *et al.*, 1988; Pieper *et al.*, 1987). However, up to our knowledge, thorough descriptions of analgesic and inflammatory properties are rare. Unfortunately, investigations on toxic properties are mostly limited to acute toxicity (LD_{50}).

VII.2 Prodrugs

A special class of PAR derivatives are the so called prodrugs. A prodrug can be converted in a biological system as to give one or more drugs. Benorilate (Fig. 15), an ester of acetylsalicylic acid and PAR, is such a prodrug and might be seen as an early attempt of line 1. By esterifying the hydroxyl group, a delay would be introduced in the speed at which the PAR concentration would build up after an overdose as activity of an esterase would be required. Benorilate, however, is relatively rapidly hydrolysed by esterases to its basic constituents, causing liver GSH depletion and liver necrosis (although somewhat delayed) due to the formation of PAR (De Vries, 1981; De Vries *et al.*, 1981). A second example of a prodrug for which some *in vitro* experiments have been performed with respect to biological system dependent formation of PAR is found in the group of *N*-(substituted 2-hydroxyphenyl and 2-hydroxypropyl)carbamates based on ring-opened derivatives of active benzoxazolones and oxazolidinones. This class of compounds contains compounds such as metaxalone and mephenoxalone (prodrugs of PAR and oxazolidones) and chlorzacetamol (a mutual prodrug of chlorzoxazone (a muscle relaxant) and PAR) (Vigroux *et al.*, 1995).

Several structural changes were directly aimed at prevention of hepatotoxicity by linking PAR covalently to compounds facilitating GSH resynthesis (line 2). It was shown that esterification of the phenolic group of PAR with *N*-acetyl-DL-methionate (Fig. 15) prevented both the hepatotoxicity of PAR in mice (Skoglund *et al.*, 1986; Skoglund *et al.*, 1988). Furthermore, replenishment of GSH-levels occurred

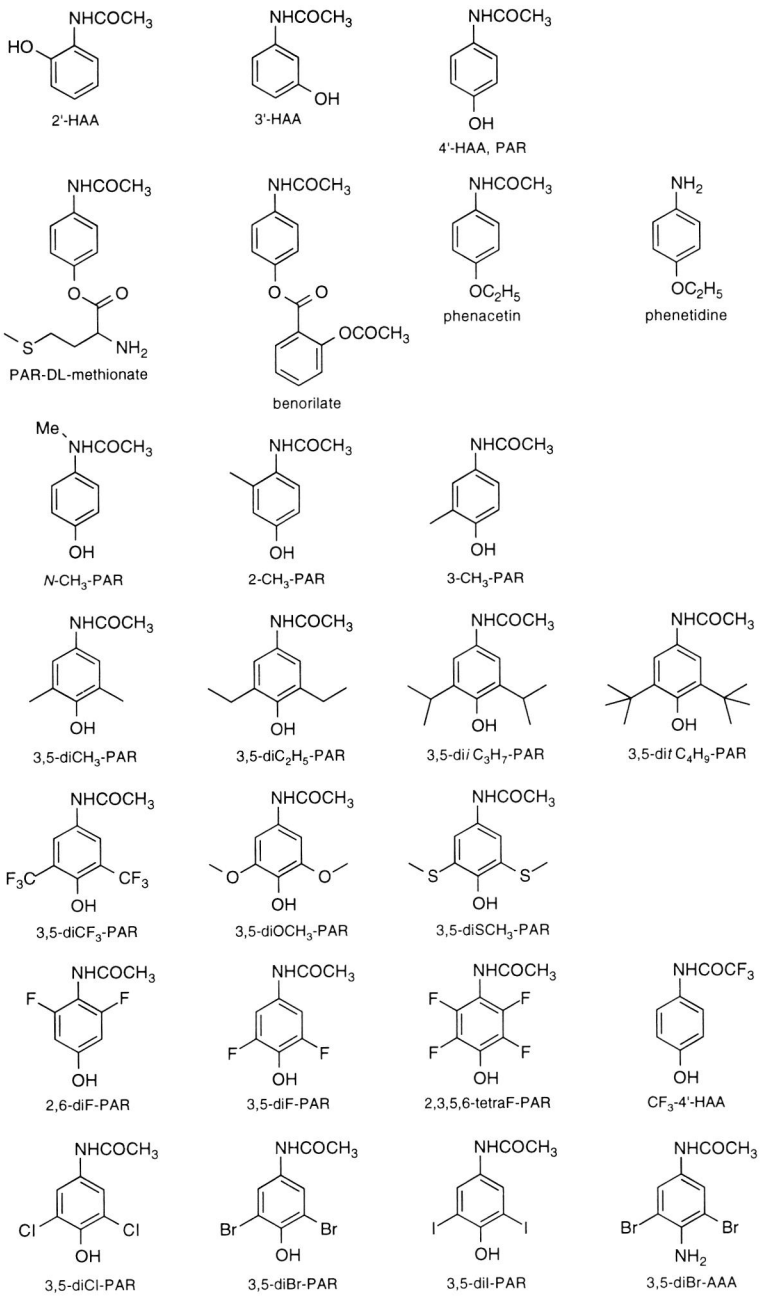


FIG. 15 Analogues of paracetamol
Analogues of paracetamol - Regioisomers, *O*-conjugates, an *O*-alkyl derivative, an *O*-alkylated *p*-aminophenol, ring-substituted analogues, *N*-acetyl-substituted analogues and a ring-substituted *N*-acetyl *p*-aminoaniline

significantly faster in the group treated with the PAR-ester compared to the PAR only-dosed group due to the availability of methionine as a precursor of GSH. Pharmacokinetics and pharmacodynamics of this PAR-methionine ester and PAR were similar (Skoglund *et al.*, 1986; Skoglund *et al.*, 1988). Interestingly, in the same experiments, simultaneously administered *N*-acetyl-cysteine was more effective in the prevention of GSH depletion 1 h after dosing than PAR-methionine or simultaneous dosing of PAR and free *N*-acetyl-DL-methionate. However, it was less effective in promoting *de novo* GSH synthesis towards 16 h. Lastly, there was no statistically significant difference between PAR-methionine ester and free *N*-acetyl-DL-methionate with respect to effects on GSH depletion or hepatic cell integrity (Skoglund *et al.*, 1986).

VII.3 Phenacetin

Before PAR was discovered, analgesic and antipyretic activity was found to be present in phenacetin, a PAR analogue in which the phenolic hydroxyl group of PAR was masked by an ethyl substituent. Phenacetin was used for many years as an analgesic and antipyretic drug until renal damage became epidemiologically associated with long-term therapeutic treatment (Sandler *et al.*, 1989). In contrast to what was found later for PAR, hepatotoxicity was no problem with phenacetin, which is further substantiating the pro-toxicant properties of the phenolic hydroxyl group. Actually, PAR was suggested to be responsible for the therapeutic effects of phenacetin and in such low concentrations was considered to be nontoxic for the kidneys. However, as phenacetin is almost completely metabolised in the liver while the toxic effects are expressed elsewhere, further activation of phenacetin metabolites, such as *p*-phenetidine (*N*-deacetylated phenacetin), PAR (*O*-dealkylated phenacetin) and *p*-aminophenol (*N*-deacetylated and *O*-dealkylated phenacetin) proximate to the site of toxicity is eligible. E.g. protein binding was observed upon activation of *p*-phenetidine by human kidney medulla microsomes (Larsson *et al.*, 1985).

VII.4 Regioisomers

Two regioisomers (congeners, positional isomers) of PAR (4'-hydroxyacetanilide; 4'HAA) studied are 2'-hydroxyacetanilide (2'-HAA) and 3'-hydroxyacetanilide (3'-HAA). The latter has been used frequently to study the role of covalent binding in cytotoxicity as 3'-HAA is non-hepatotoxic, nevertheless giving rise to reactive metabolites which arylate hepatic proteins in murine hepatocytes as well as *in vivo* in mice and hamsters (Holme *et al.*, 1991; Rashed *et al.*, 1990; Rashed and Nelson, 1989a; Roberts and Jollow, 1978; Roberts *et al.*, 1990; Tirmenstein and Nelson, 1989). Metabolism of 3'-HAA by rat liver P450 leads to the formations of two main products of aromatic hydroxylation which subsequently can be further oxidised to their respective *ortho*- and *para*-benzoquinone derivatives and form conjugates with GSH as illustrated in Fig. 16 (Rashed and Nelson, 1989a; Rashed and Nelson, 1989b; Streeter *et al.*, 1984a). Similarly 2'-HAA, that is metabolised to 2',5'-dihydroxyacetanilide, was found not to be hepatotoxic in mice (Hamilton and Kissinger, 1986; Roberts and Jollow, 1979; Roberts and Jollow, 1980). A review on

the P450 catalysed oxidation of a variety of PAR analogues has been published by Koymans *et al.* (Koymans *et al.*, 1993).

An apparently important phenomenon in the toxicity of PAR and PAR-analogues is the dual capacity of the once-formed oxidised reactive metabolites to both oxidise and covalently bind protein and nonprotein thiols. This is illustrated for example by the findings regarding the formation of specific glutathione conjugates and the differences in protein binding and hepatotoxicity observed between hepatotoxic PAR (4'-HAA) and non-hepatoxic 3'-hydroxyacetanilide (3'-HAA). As illustrated in Fig. 16 and Table 7, upon microsomal oxidation of 3'-HAA via 2',5'-HAA-, 3',4'-HAA and 2',3'-HAA, at least two acetamidobenzoquinones are likely formed, i.e. 2-acetamido-*p*-benzoquinone (2-APBQ), and 4-acetamido-*o*-benzoquinone (4-AOBQ). These benzoquinones are likely reactive soft electrophiles, which might give rise to less critical types of covalent binding when compared to NAPQI (Rashed and Nelson, 1989a; Rashed and Nelson, 1989b; Streeter *et al.*, 1984a). Once formed they react readily with soft nucleophiles like GSH (Rashed and Nelson, 1989a; Rashed and Nelson, 1989b) or protein thiols close to their site of formation. This was proposed by Nelson *et al.* (Nelson *et al.*, 1990) and substantiated by earlier findings. In mouse liver microsomal incubations, NADPH-dependent covalent binding of radioactivity from [^{14}C]3'-HAA compared to [^{14}C]-PAR to microsomal protein was almost four times as rapid (Streeter *et al.*, 1984a). This may prevent extensive cytosolic GSH depletion as observed in hamsters (Roberts *et al.*, 1990) and protect some critical target proteins in cytosol or mitochondria from damage (Rashed *et al.*, 1990). An overview of the biotransformation of the isomeric analogues of PAR was presented (Rashed *et al.*, 1990). In mice, although 3'-HAA treatment produces only slightly smaller levels of covalent binding to liver cytosolic and mitochondrial proteins than PAR, no inhibition of plasma membrane Ca^{2+}-ATPase in liver, less extensive mitochondrial GSH depletion and lesser decrease of cytosolic glyceraldehyde-3-phosphate dehydrogenase activity was observed (Dietze *et al.*, 1997; Myers *et al.*, 1995; Tirmenstein and Nelson, 1989).

A possible explanation for this disparity in protein binding and toxicity between PAR (4'-HAA) and 3'-HAA might be the remarkable difference between their oxidative metabolites, being a quinone imine (NAPQI) and benzoquinones (4-AOBQ and 2-APBQ), respectively. Although NAPQI as well as 4-AOBQ and 2-APBQ are reactive soft electrophiles, only NAPQI is capable of forming the special benzoquinone imine *ipso*-adduct (as depicted in Table 7) with soluble and nonsoluble thiols. In case of soluble thiols (GSH), formation of this *ipso*-adduct, could facilitate transport of this latent form of PAR away from the site of formation (microsomal environment). In this form, NAPQI might even escape from hepatocytes, where most of the oxidative biotransformation activity is located, and participate in the arylation of protein thiols in erythrocytes, which has been observed in mice that were administered PAR (Axworthy *et al.*, 1988).

Furthermore, glutathiolated hydroquinone structures and covalently bound hydroquinones may be formed which are labile to further (auto)oxidation processes causing formation of di-glutathiolated quinones, analogously to (halogenated)

hydroquinones and *tert*-butylhydroquinone (Lau *et al.*, 1996; Peters *et al.*, 1996). The finding of a di-GSH conjugate upon chemical reaction of the 3'-HAA metabolite 2-APBQ (Fig. 16 and Table 7) with GSH (Rashed and Nelson, 1989a) is only conceivable when one of the mono-GSH conjugates is autooxidised before a second GSH molecule is trapped. The differences between PAR and 3'-HAA in oxidative metabolite formation, GSH depletion, and protein arylation are excellently reviewed (Rashed *et al.*, 1990). Recently, some more investigations were undertaken to compare especially which proteins were covalently adducted/inhibited or not upon administration of PAR or 3'-HAA to mice (Dietze *et al.*, 1997; Matthews *et al.*, 1997; Myers *et al.*, 1995; Salminen *et al.*, 1998; Tirmenstein and Nelson, 1991). Treatment of mice with a large dose of radiolabelled 3'-HAA was shown to result in selective arylation of proteins in cytosol and microsomes but not in mitochondria. A major 3'-HAA protein adduct was observed in microsomes at 50 kDa with peak levels appearing at 1 h. Minor adducts were observed at 47 kDa in microsomes and 56 kDa in cytosol (Matthews *et al.*, 1997). Further *in vivo* and *in vitro* studies revealed the 50 kDa microsomal protein to be CYP2E1, based on comigration, immunoblotting, loss of catalytical CYP2E1 activity and inhibition of the 50 kDa binding by the CYP2E1 inhibitor diallysulfide, leading to the postulation that 3'-HAA is a suicide inactivator of CYP2E1 (Halmes *et al.*, 1998; Matthews *et al.*, 1997; Salminen *et al.*, 1998). Furthermore, the finding that some of the covalent protein binding of 3'-HAA to CYP2E1 is less stable (partial loss by ultrafiltration and electrophoresis) emerged the hypothesis that the heme as well as the apoprotein might be adducted (Myers *et al.*, 1995; Salminen *et al.*, 1998). It should be noted that the panlobular binding and some of the centrilobular binding (not and partially decreased by treatment of mice with diallylsulfide) in mice that were administered 3'-HAA could be due to bioactivation by non-CYP2E1 bioactivation as other enzymes, such as CYP1A2, CYP2A, CYP2B and CYP2C. These P450s have been shown, at least in humans, to be expressed uniformly throughout the liver acinus (reference in (Salminen *et al.*, 1998)). Other papers on 3'-HAA are referred to elsewhere in this review.

NAPQI, the reactive P450-dependent metabolite of PAR is an oxidant as well as an electrophile since PAR-SG and PAR and GSSG are formed upon chemical reaction of NAPQI with GSH in a ratio of 1:1:1 via 2:1:1 to 3:2:2, with increasing concentrations of NAPQI (Albano *et al.*, 1985). The conjugative mechanism is likely a Michael-type addition, whereas reduction of NAPQI by GSH is likely to occur via *ipso*-addition (Coles *et al.*, 1988; Ketterer *et al.*, 1988), resulting in a Meisenheimer complex, similar to the formation of a carbinolamide upon hydration of NAPQI (*ipso*-adduct) (Novak *et al.*, 1989; Novak *et al.*, 1986). A similar *ipso*-adduct of a thiol is formed upon reaction of ethanethiol with NAPQI (Fernando *et al.*, 1980). One possible explanation might be that the Michael adduct 3-glutathion-*S*-yl-paracetamol adduct is fairly stable and not (it has never been found) liable to further oxidation. As mentioned shortly above, the less stable *ipso*-adduct might diffuse to compartments other than the site of formation and subsequently form GSH and NAPQI in a reverse reaction, thus allowing NAPQI to react again with GSH or protein thiols distinct from the endoplasmatic reticulum. Both protein arylation further away from the site of reactive intermediate formation in the

case of PAR, e.g. in hemoglobin and blood plasma (Axworthy *et al.*, 1988; Bartolone *et al.*, 1989), and decreased stability of hepatic protein adducts in the case of 3'-HAA as reported recently (Matthews *et al.*, 1997; Myers *et al.*, 1995), are in line with this hypothesis.

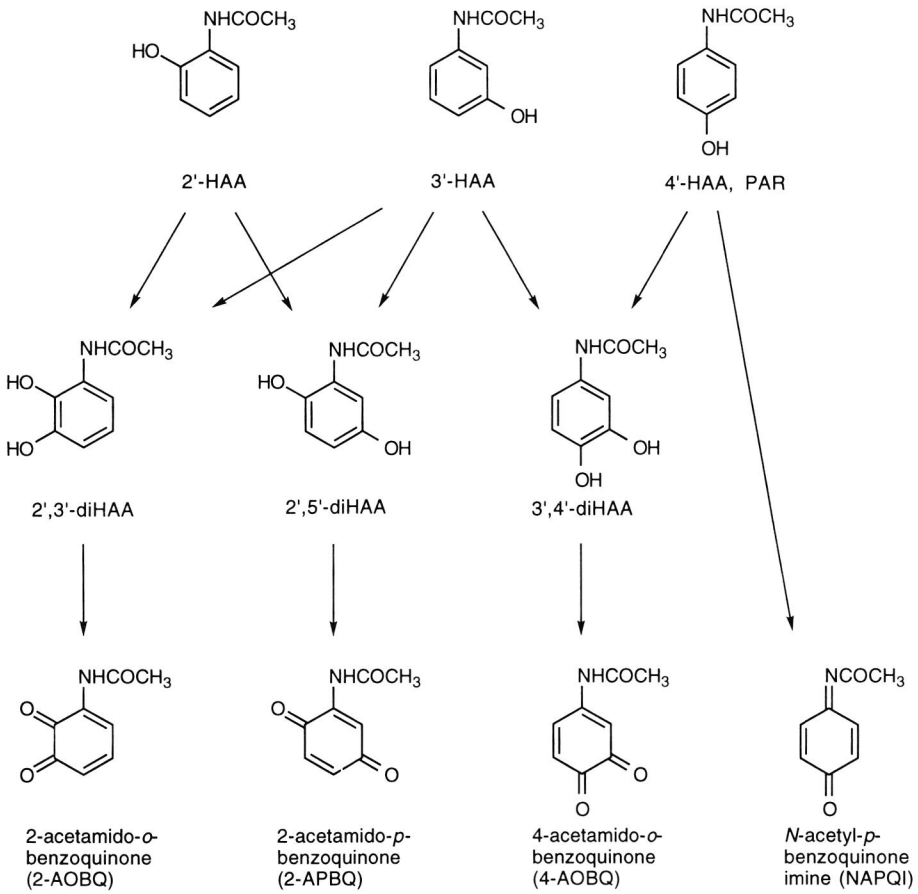


FIG. 16 Oxidative biotransformation of PAR and its regioisomers
A comparison. See text for references

VII.5 *N*-Methylparacetamol and 2,6-dimethylparacetamol

N-Methylparacetamol (*N*-methyl-PAR), synthesized on the basis of the expectation that *N*-oxidation would be hindered by *N*-methylation (Nelson *et al.*, 1978), indeed appeared to be not hepatotoxic. The prevention of toxicity was presumably due to the fact that *N*-methyl-PAR is a poor substrate for the hepatic P450 (Harvison *et al.*, 1986b). The same mechanism probably applies to another methylated analogue, viz.

2,6-dimethyl-PAR (Birge *et al.*, 1988; Fernando *et al.*, 1980; Porubek *et al.*, 1987). Torsion of the *N*-acetyl group out of plane is suggested to prevent either the positioning in the P450 active site or the second hydrogen abstraction from the nitrogen. This suggestion is supported by the finding in cultures of mouse hepatocytes, where 2,6-dimethyl-PAR, in contrast to PAR and 3,5-dimethyl-PAR, did not impel changes in synthesis of two specific proteins (Bruno *et al.*, 1992). A similar mechanistic reason was suggested (next to the oxidation potential) for 2,6-difluorinated PAR which will be discussed further on in this Part (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b). These findings were substantiated when chemically synthesized 2,6-dimethyl-NAPQI appeared to be an efficient inhibitor of calmodulin-activated Ca^{2+}-pump ATPase activity, basal (calmodulin-independent) Ca^{2+}-pump ATPase activity, as well as Na^{+},K^{+}-pump ATPase activity (Nicotera *et al.*, 1990) whereas 3,5-dimethyl-NAPQI, which primarily oxidizes protein thiols, caused selective inhibition of only the calmodulin-activated Ca^{2+} pump ATPase activity. The hypothetical formation of 2,6-dimethyl-NAPQI *in situ* in hepatocytes or *in vivo* would certainly exhibit more dramatic toxicity effects than observed. Both oxidation and arylation of protein thiols can alter the functional properties of important proteins. Of the two reactions, arylation (NAPQI and 2,6-dimethyl-NAPQI) appeared to be the less specific and more damaging event (Nicotera *et al.*, 1990). Another analogue of PAR, methylated *ortho* towards the *N*-acetyl group is 2-methyl-PAR. It was calculated that *ortho*-methylation will cause some torsion of the *N*-acetyl group, although not enough to prevent P450 binding that much as was observed with the 2,6-dimethyl PAR analogue (unpublished observations). In line with at least some P450 catalysed oxidation of 2-methyl-PAR, some hepatotoxicity was observed with 2-methyl-PAR although significantly less compared to PAR (Harvison *et al.*, 1986b).

VII.6 3,5-Dialkylated paracetamol analogues

As another possibility for prevention of PAR-induced hepatotoxicity, notably monosubstitution at the 3-position or disubstitution at the 3-and 5-position of the aromatic nucleus of PAR by alkyl groups, was evaluated. In view of the essential role of irreversible GSH depletion and the covalent binding to critical protein nucleophilic groups such as thiols in the hepatotoxity of PAR, it has been proposed that protecting of the electrophilic 3- and 5-positions in NAPQI would prevent PAR toxicity (Van de Straat *et al.*, 1986). As expected, 3,5-dialkyl substitution, in contrast to 3-monoalkyl substitution, efficiently prevented GSH depletion, lipid peroxidation and the toxicity of PAR, measured as LDH leakage in rat hepatocytes. Hepatotoxicity was also absent upon administration of 3,5-dialkylated PAR analogues to mice, in contrast to PAR and 3-monoalkylated PAR (Van de Straat *et al.*, 1987c). The P450-catalysed oxidation of the mono- and dialkylated PAR-analogues to their corresponding NAPQI-analogues did not appear to be significantly influenced by this mono- or dialkyl substitution, but only 3,5-dialkylation was found to prevent the conjugation of the respective quinone imines with GSH. Oxidation of GSH to GSSG, was still found to occur in microsomal incubations with the 3,5-dialkyl derivatives (Van de Straat *et al.*,

1986). This reaction is apparently less critical to the hepatocyte, since GSSG is normally reduced back again to GSH rapidly by GSSG reductase.

The toxic potential of PAR was not completely eliminated by 3,5-dialkylation, however, as synthetical 3,5-diCH_3-NAPQI appeared to inhibit calmodulin activated Ca^{2+}-pump ATPase activity in red blood cells, probably due to its oxidant properties (Nicotera *et al.*, 1990). Furthermore, 3,5-diCH_3-NAPQI was found to be deacetylated to a dimethylated benzoquinone imine, thereby re-establishing conjugative properties (Rossi *et al.*, 1988). Moreover, 2,6-diCH_3-PAR as well as 3,5-diCH_3-PAR exhibited cytotoxic properties concomitantly with covalent protein binding in hepatocytes (Porubek *et al.*, 1987). The cytotoxic properties of 2,6-diCH_3-PAR are likely due to electrophilic as well as oxidant properties of a NAPQI-like metabolite, whereas those of 3,5-diCH_3-PAR should be likely attributed to the oxidant properties of a NAPQI-analogue. The 3,5-diCH_3-PAR associated covalent protein binding could be due to hydrolytic removal of the complete *N*-acetyl group, as well as deacetylation of the reactive metabolite 3,5-diCH_3-NAPQI, resulting in a dimethylated quinone or a dimethylated quinone imine, respectively. Both compounds possess covalent binding properties towards thiol groups. The increased selective protein arylation to a cytosolic 58 kDa protein by 2,6-diCH_3-PAR in cultured hepatocytes from phenobarbital induced compared to uninduced mice (Birge *et al.*, 1989) is in line with the fact that the active site of phenobarbital inducible P450 is wider than that of ßNF-inducible P450 (Lewis *et al.*, 1987). Since in 2,6-diCH_3-PAR, the *N*-acetyl group is not coplanar (unpublished data from our group), it is not expected to fit into the active site of CYP1A1.

Differences were observed with respect to the time-course of protein adduct formation, the susceptibilities of the modification of cysteine residues by 3,5-diCH_3-NAPQI or NAPQI upon incubation in freshly isolated hepatocytes (Weis *et al.*, 1992b). Comparable results were obtained upon *in situ* formation of these quinone imines by co-incubation of hepatocytes with 3,5-diCH_3-PAR or PAR with a peroxidase model system (Weis *et al.*, 1996).

As a variation on substitution of the positions *ortho* to the phenolic hydroxyl group, disubstitution with the electron donating methylether- and methylthioether-substituents R = -OCH_3 and R = -SCH_3 (Fig. 15) resulted in better cyclooxygenase inhibiting properties (an *in vitro* test for analgesic activity) than PAR, which was most likely due to their lower oxidation potentials than PAR (Bessems *et al.*, 1995). Analogous to 3,5-diCH_3-PAR, these compounds displayed lower cytotoxicity than PAR which is probably due to their blockade of the aromatic positions, normally prone to *S*-glutathiolation. Interestingly, structural modulation by various alkyl-, methylether- and methylthioether substituents (R = -CH_3, -C_2H_5, -tC_4H_9, -OCH_3, -SCH_3) did not block the phase I bioactivation in an *in vitro* peroxidase system as revealed by ESR-detection of their respective phenoxy free radicals (Bessems *et al.*, 1998). This substantiates the general assumption that cyclooxygenase inhibition is dependent on one-electron oxidation of phenolic compounds. The formation of the respective phenoxy free radicals was in line with *ab initio* calculations that showed that hydrogen abstraction from the phenolic hydroxyl group of the PAR-analogues used

in the calculations (R = $-CH_3$ and R = $-OCH_3$) is energetically more favourable than hydrogen abstraction from the acetylamino nitrogen (Bessems *et al.*, 1998).

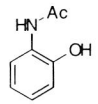
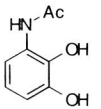
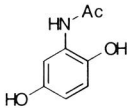
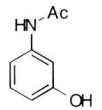
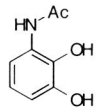
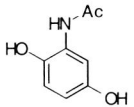
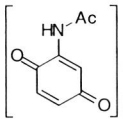
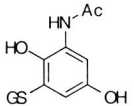
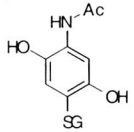
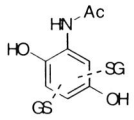
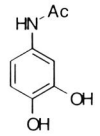
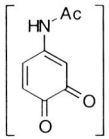
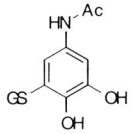
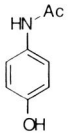
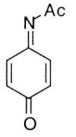
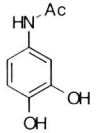
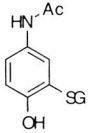
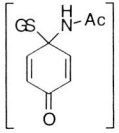
VII.7 3,5-Dihalogenated and other dihalogenated paracetamol analogues

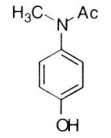
Another group of PAR analogues is formed by halogenated compounds. E.g. 4-hydroxytrifluoroacetanilide (Fig. 15) was analgesically inactive, probably because of the relative instability due to rapid hydrolysis to *p*-aminophenol (Aboul-Enein *et al.*, 1982). However trifluoroacetanilide and 4-ethoxytrifluoroacetanilide (the trifluoro analogue of phenacetin) both exhibited analgesic activity. Trifluoroacetanilide might be analgesically active due to metabolic hydroxylation to 4-hydroxytrifluoroacetanilide which acts on the site of action, thus by-passing its hydrolysis to *p*-aminophenol (Aboul-Enein *et al.*, 1982). Unfortunately, no information was presented at that moment on the toxicity of these trifluorinated analogues.

More information regarding the analgesic activity and toxicity of trifluorinated and fluorinated analogues of PAR is retrieved from more recent literature. 2,6-Difluorination of PAR prevented PAR-induced hepatotoxicity when administered to mice. Furthermore, no thioether metabolites were found in urine (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b). The reduced hepatotoxicity is probably due to impaired oxidation by P450, due to non-coplanarity of the *N*-acetyl group in 2,6-diF-PAR, together with an increased oxidation potential of two electron withdrawing fluorine atoms at *meta* positions of the phenolic hydroxyl group (Barnard *et al.*, 1993a). Trifluormethylation of the *N*-acetyl group of PAR caused such an increase in lipophilicity of PAR, that a shift in toxicity from the liver to the central nervous system was observed combined with markedly different routes of metabolism, e.g. involving extensive *N*-detrifluoroacetylation to *p*-aminophenol (Barnard *et al.*, 1993a). A small disparity is observed between the results with 3,5-difluorinated PAR as presented by Bessems *et al.* and those presented by Barnard *et al.* In rat hepatocyte incubations, 3,5-diF-PAR appeared to be approximately as toxic as PAR (Bessems *et al.*, 1997), whereas *in vivo* in mice, 3,5-diF-PAR was less toxic than PAR (Barnard *et al.*, 1993a). However, the *in vivo* differences in LD_{50}, serum ALT, and hepatic GSH between PAR and 3,5-diF-PAR are not more than twofold (Barnard *et al.*, 1993a), which is relatively small for *in vivo* differences.

Interestingly, 3,5-dihalogenation (R = -F, -Cl, -Br and -I; Fig. 15) resulted in worse *in vitro* cyclooxygenase inhibition as well as *in vivo* analgesic activity than PAR, which was most likely due to their higher oxidation potentials than PAR (Bessems *et al.*, 1995). For the dihalogenated compounds that were studied as such (R = -F, -Cl, and -Br), cytotoxicity was comparable to that of PAR. Moreover, structural modulation by these halogens (R = -F, -Cl, and -Br) did not block the phase I bioactivation in an *in vitro* peroxidase system as revealed by ESR-detection of the respective phenoxy free radicals (Bessems *et al.*, 1998). As for the series of 3,5-disubstituted analogues with electron-donating substituents mentioned before, this

TABLE 7 Main *in vitro* and *in vivo* biotransformation products of paracetamol analogues

PAR Analogues	P450 Metabolites		GSH Conjugates			Effect	Reference
 2'-HAA	 2',3'-diHAA	 2',5'-diHAA (minor)					Hamilton and Kissinger, 1986
 3'-HAA	 2',3'-diHAA						
	 2',5'-diHAA (minor)	 2-APBQ				Protein binding without toxicity 2',5'-HAA also formed as a very minor metabolite	Streeter *et al*, 1984a; Hamilton and Kissinger, 1986; Rashed and Nelson 1989a, b; Rashed *et al.*, 1990
	 3',4'-diHAA	 4-AOBQ				Ditto	
 PAR (4'-HAA)	 NAPQI	 3',4'-diHAA (minor)	 PAR-SG	 *ipso*-adduct		Protein binding and toxicity. *Ipso*-adduct proposed by Ketterer *et al.*, 1988	Fernando *et al.*, 1980; Streeter *et al.*, 1984; Hamilton and Kissinger, 1986; Harvison *et al.*, 1986b; Rashed and Nelson, 1989a, b; Rashed *et al.*, 1990

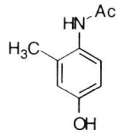


***N*-CH_3-PAR**

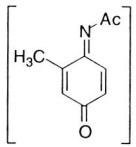
No P450 substrate due to out of plane torsion of the acetyl moiety

No toxicity

Harvison *et al.*, 1986b

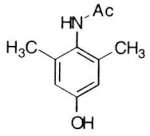


2-CH_3-PAR



Less hepatotoxic than PAR, probably due to less P450 dependent bioactivation (slight torsion of the *N*-acetyl group out of the plane of the phenyl ring)

Harvison *et al.*, 1986b

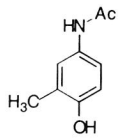


2,6-diCH_3-PAR

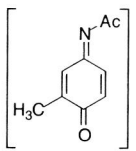
Less favoured P450 oxidation probably due to out of plane torsion of the acetyl moiety

Significantly less hepatotoxic than PAR

Fernando *et al.*, 1980

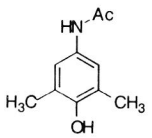


3-CH_3-PAR

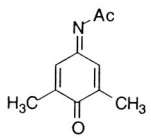


As hepatotoxic as PAR

Harvison *et al.*, 1986b; Van de Straat *et al.*, 1986



3,5-diCH_3-PAR



3,5-diCH_3-NAPQI

Significant less cytotoxicity in rat liver hepatocytes

Fernando *et al.*, 1980; Van de Straat *et al.*, 1986; Bessems *et al.*, 1996

substantiates the general assumption that cyclooxygenase inhibition is dependent on one-electron oxidation of phenolic compounds. Again, the formation of the respective phenoxy free radicals was in line with *ab intio* calculations that showed that hydrogen abstraction from the phenolic hydroxyl group of the PAR-analogues used in the calculations (R = -F and R = -Cl) is energetically more favourable than hydrogen abstraction from the acetylamino nitrogen (Bessems *et al.*, 1998).

Moreover, in hepatocytes from ßNF-induced rats, the toxicity of a whole series of 3,5-dihalogenated PAR analogues (3,5-diF-, 3,5-diCl-, and 3,5-diBr-PAR; Fig. 15) was in the same order of magnitude as the cytotoxicity of PAR. It should be noted, however, that an increase in the size of the substituent seemed to be related to a decrease in cytotoxicity (Bessems *et al.*, 1997). This trend might be caused by structure-related detoxification by glucuronidation of the 3,5-dihalogenated PAR-analogues. Glucuronidation increases with the size of the substituents adjacent to the phenolic hydroxyl group which seems feasible as the reactivity towards the cofactor uridine 5'-diphosphoglucuronic acid (UDPGA) is known to be dependent on the nucleophilicity of the structure, both due to electronic and steric factors, but also on lipid solubility (Mulder *et al.*, 1990). As no redox-reaction is involved (glucuronidation is an SN_2 reaction), the ease of the *O*-glucuronidation is not directly related to the oxidation potential. The latter varies only between 0.032 and 0.034 Volt for 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR (Bessems *et al.*, 1995).

Analogous to PAR, the observed cytotoxicity was due to bioactivation of substantial amounts of the 3,5-dihalogenated PAR analogues, predominantly by CYP1A, as observed also in microsomal incubations. As expected to be formed via a nucleophilic (SN_2) addition-elimination mechanism, in microsomal incubations GSH-conjugates and in hepatocyte incubations covalent protein were observed for most halogenated analogues (Bessems *et al.*, 1996; Bessems *et al.*, 1997). For 3,5-diCl-PAR and 3,5-diBr-PAR but not 3,5-diF-PAR, protein adducts at regio-specific aromatic positions were formed (Bessems *et al.*, 1997). As far as GSH adduct formation of 3,5-diBr-PAR is concerned, these *in vitro* results are supported by a study in which analogous *S*-conjugate formation was studied in male rats with the analgesic 'amino analogue' of 3,5-diBr-PAR, 4-amino-3,5-dibromoacetanilide. The mercapturic acid degradation product of 3-bromo-4-amino-5-glutathionyl-acetanilide was found as the main urinary metabolite (Prox *et al.*, 1987).

VII.8 Nuclear effects

A specific activity of PAR that was described is inhibition of replicative DNA synthesis. As this might eventually cause apoptosis or necrosis it is regarded highly significant. A structure-activity study of PAR analogues on this inhibitory activity as measured in V79 Chines hamster cells revealed that PAR, just like *p*-cresol, *m*-aminophenol and *p*-hydroxyphenol has moderate replicative DNA synthesis inhibiting potencies, whereas 2,4-diaminophenol, *o*-aminophenol, *p*-aminophenol and *p*-methylaminophenol exhibited high inhibiting potencies and a variety of other analogues exhibited low to no inhibiting potencies. Based on these results it was hypothesized that the observed inhibitory activity variation of the PAR analogues was

based on the relative abilities of these compounds to undergo hydrogen atom loss at the phenolic oxygen and on the relative stabilities of the resulting free-radical species (Richard *et al.*, 1991).

VII.9 Summary

A large number of reports describe investigations into structural modification of PAR in order to improve its analgesic and safety properties. Valuable knowledge on the analgesic and toxic activities of PAR and their underlying mechanism was obtained. Analgesic activity appears to be confined to acetanilides with an -OH or an $-NH_2$-group at *para*-position and the acetanilide moiety 'in plane'. The phenolic structure could also be created by *in situ* oxidation of an acetanilide although absence of the -OH would change lipophilicity and thus disposition significantly. Substitution *ortho* to the -OH groups with electron-donating substituents could improve the analgesic properties as the cyclooxygenase inhibition probably involves oxidation of this -OH group.

No radicals were detected in rat liver cytochrome P450-containing microsomal or reconstituted systems in which rat liver CYP1A1 or CYP2E1 (for which PAR is known to be a substrate) were present. The failure to experimentally detect phenoxy radicals in cytochrome P450-catalysed oxidation of any of the eight 3,5-disubstituted PAR analogues, even of those analogues that provide very stable phenoxy radicals, indicates that the quantity of these radicals remains below the detection level of the ESR-analysis. This could indicate that phenoxy radicals do not leave the active site of the P450 involved at all or in very low quantities. In addition, the reducing effects that agents like NADPH and protein thiol groups have on phenoxy radicals rather than the physical instability of the respective radicals might prevent detection by ESR. Thus, the findings substantiate the fact that formation of PAR-dependent free phenoxy radicals is relevant in tissues rich in peroxidase-activity such as the kidneys whereas it is probably irrelevant in P450-dependent hepatotoxicity.

Hepatotoxic activity appears to be confined to compounds that are capable of forming quinoid structures which are, susceptible to both irreversible and, importantly, reversible attack by soluble and nonsoluble thiols. If only irreversibly bound *S*-conjugates are formed, such as with the metabolites of 3'-HAA (a regioisomer of PAR), this covalent binding seems to be confined mainly to microsomal proteins in hepatocytes. If also reversible adduct formation is possible, such as with NAPQI (a reactive PAR-metabolite), the number of proteins and the possible sites where adduct formation becomes feasible, is importantly increased. Evidence is even growing that by GSH-conjugation or protein-SH adduct formation, the reactive metabolite is not unerringly detoxified. Maybe these *S*-conjugates and -adducts exist in two forms, as a stable Michael adduct and as an *ipso*-conjugate, with the latter being prone to release of NAPQI distinct from the endoplasmatic reticulum or even at extracellular molecular targets.

It should be mentioned that although hepatotoxic activities can be diminished by blocking the proximate electrophilic sites *ortho* to the -OH group of PAR (or $-NH_2$ group of an amino analogue) by e.g. alkyl substituents, toxicity to other organs may become manifest, e.g. renal toxicity. This may be due to renal *N*-deacetylase activity

by *N*-acetyltransferase, giving rise to toxic aminophenols. The *N*-acetyl group could be modified as to prevent *N*-deacetylation. However, variations might influence the lipophilicity of the parent compound which could direct it to other compartments of the body. Also, substitution *ortho* to the -OH (or $-NH_2$) by substituents that can be removed too easily by nucleophilic thiols in an addition-elimination reaction, after formation of corresponding NAPQI-analogues, is unwarranted. In addition, the substituents themselves should not be inert to biotransformation since this could direct biotransformation to *N*-deacetylation of the parent compound to nephrotoxic aminophenols. Examples of these compounds might be PAR derivatives that are disubstituted *ortho* to the -OH group with $-OCH_3$, $-SCH_3$, -OCOOH or $-OC_2H_5$ groups, leaving some possibilities of phase I biotransformation at this position (e.g. *O*- and *S*-dealkylation). Interestingly, *in vitro* experiments already provided strong indications for analgesic activity as well as decreased hepatotoxicity for 3,5-diOCH_3-PAR and 3,5-diSCH_3,-PAR (Bessems *et al.*, 1995). The *in vivo* relevance of these findings as well as the potential toxicity for other organs remains to be established, however.

A new lead for structural modification of PAR in order to obtain a safer analgesic substance should combine the two traces, one that is aimed at retainment of improval of analgesic properties, the other one that is aimed at decrease of the hazardous properties.

PART VIII GENERAL CONCLUSIONS

As established in numerous investigations, the acute and fulminant liver toxicity due to a large dose of paracetamol (PAR) is mainly dependent on P450-catalysed oxidative biotransformation to *N*-acetyl-*p*-benzoquinone imine (NAPQI). The reactive NAPQI subsequently reacts with soluble (glutathione; GSH) and nonsoluble (protein) thiols. The analgesic and anti-pyretic properties are due to the anti-cyclooxygenase properties of PAR. Inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase (PGES) results in decreased formation of prostaglandins.

Significant species differences exist with respect to susceptibility to PAR-dependent hepatic and renal toxicity, the latter possibly occurring together with acute liver failure. Mice and hamsters are relatively sensitive whereas rat, rabbit and guinea pig are rather resistant. The sensitivity positively and negatively correlates with urinary excretion of toxication pathway-related metabolites, i.e. breakdown products of the glutathione conjugate of PAR (PAR-SG) and secondary metabolites thereof, and inactivation pathway-related metabolites such as the sulphate and the glucuronide conjugate of PAR and 3-hydroxyparacetamol (3-OH-PAR) or secondary metabolites thereof, respectively. Except differences in the glutathione-conjugation pathway, also species differences in *N*-acetyl transferase activity may render species vulnerable to renal toxicity of PAR, as illustrated by the renal *N*-acetyltransferase-dependent formation of *p*-aminophenol, a well known nephrotoxicant, in rabbits.

In combination with species differences in phase II biotransformation enzymes (expression level as well as localisation), diversity in hepatic phase I oxidative enzymes is importantly correlated with sensitivity of PAR-toxicity. In mice and hamsters, phase I biotransformation seems to be mostly limited to the P450 enzymes CYP2E1 and CYP1A2, both activating P450s. In contrast, rats seem to exhibit a variety of P450s that are active in oxidation of PAR, including P450s, such as CYP1A1 and CYP2C11 that catalyse the oxidative activation as well as inactivation of PAR to NAPQI and 3-OH-PAR, respectively. In addition, some rat P450s such as CYP2B1 exhibit significant regioselective catalytic oxidation in favour of 3-OH-PAR. Moreover, it should be noted that acute renal toxicity is largely dependent on species and even gender specific activation of PAR by renal CYP2E1 with male mice being sensitive in contrast to female mice. Although the sensitivity of the human species has not been compared in detail with that in other species, in man, probably CYP3A4 is mainly involved in oxidative biotransformation of PAR at therapeutic intake whereas CYP2E1 and CYP1A2 become increasingly involved at high intake levels. A combination of species differences in phase II biotransformation with activity of the second phase I biotransformation group of enzymes, i.e. the peroxidases (such as PGES), may cause species selective formation of radical metabolites from PAR via *p*-aminophenol. With respect to the mechanism of oxidation of PAR to NAPQI by P450s, a direct two-electron oxidation mechanism is most likely. A single hydrogen abstraction mechanism, resulting in a phenoxy radical intermediate (NAPSQI), and followed by a second hydrogen abstraction or hydroxyl radical recombination, resulting in NAPQI and 3-OH-PAR, respectively, has been suggested but lacks as yet experimental proof.

Uncertainty still exists as to the mechanism of targetting of NAPQI to sites distinct from the site of formation, presumably and largely the liver, although some formation may occur in e.g. the kidneys. Possibly two structures exist for covalent binding of NAPQI to sulfhydryl groups, i.e. the relatively inert one formed upon 1,4-Michael addition and a relatively unstable *ipso*-adduct at the C1-carbon (to which the *N*-acetyl group is linked). NAPQI could be released from the *ipso*-adduct, either with GSH or protein, at sites distinct from the endoplasmatic reticulum or even at extracellular molecular targets. Thus, cytoplasmatic, mitochondrial, plasma membrane as well as nuclear proteins could be covalently modified as such in the early hours after administration of a toxic dose of PAR, a stage which has been designated as Stage I of PAR-toxicity.

Formation 3-(cystein-*S*-yl)paracetamol protein adducts, as even found in plasma of PAR-intoxicated patients, probably exibits a dominant mechanistic role in acute hepatic necrosis. These PAR-arylated proteins appear time- and dose-dependently in liver fractions mice upon administration of PAR. In addition, the adduct levels found in serum correlates with serum ALT levels and peak several hours later than the adduct levels in liver fractions, suggesting a threshold level of adduct formation in liver for the development of hepatotoxicity. Plasma membrane and mitochondrial fractions appear to contain most covalently modified proteins whereas a 55 to 58 kDa cytosolic protein appears to be the most intense arylated individual protein. Many microsomal, mitochondrial, cytosolic and even nuclear proteins that have been shown the past

decade to become arylated, catalyse important biochemical events and may exhibit cellular signalling functions. The major alkylated proteins, of which the actual functions remain to be elucidated, have been studied thoroughly in the nineties and designated as the 55 to 58 kDa 'acetaminophen-binding proteins'. One of these proteins appeared in several tissues such as liver and kidneys in mice after addition of a toxic dose of PAR. These proteins may act as scavengers of reactive metabolites of xenobiotics with oxidant properties. Moreover, it has been suggested that the 58 kDa 'acetaminophen-binding protein' may have a nuclear signalling function.

An emerging number of *in vitro* results as well as epidemiological reports point to all kinds of nuclear effects. The most prominent findings are impaired DNA repair and even DNA-adduct formation at low target concentrations. In addition to necrosis, also apoptosis may be involved in some stages of the highly integrated process of PAR-induced toxicity. It remains to be established, however, what the relevance as well as the possible consequences of the *in vitro* findings as well epidemiological findings are for PAR-intoxicated patients as well as humans taking PAR at therapeutic levels.

It was envisaged in the past decade also that many extracellular events emerged upon administration of a toxic dose of PAR to mice after the first hours and spread in liver tissue. These events, designated as Stage II phenomena, include excretion of growth factors, inflammatory mediators and reactive oxygen species by hepatocytes as well as non-parenchymal cells such as the Kupffer cells. Some of these mediators stimulate tissue repair whereas others provoke tissue damage. Up till now it is not clear whether a relation exists between the nuclear effects and these Stage II phenomena. However, these findings may provide interesting leads for clinical treatment of acute hepatic failure.

Many agents that have been investigated for modulation of liver toxicity of PAR are of little value in the clinic as they are to be administered before (chemoprevention) or concomitantly with PAR (chemoprotection). They are aimed at inhibition of oxidative bioactivation of PAR of increased phase II detoxification. However, most primary damage (Stage I) often has taken place before admittance to a hospital. Therefore, a new guide might be to protect susceptible molecular targets by stimulation or improvement of the functioning of the 'acetaminophen-binding proteins', e.g. by selenium containing compounds.

Lastly, structural modification was a lead for numerous investigations into improvement of analgesic and safety properties of PAR. Valuable knowledge on the analgesic and toxic activities of PAR and their underlying mechanisms was obtained. E.g., 3,5-disubstitution of PAR with electron-donating substituents facilitates one-electron oxidation, leading to phenoxy free radicals in phase I biotransformation (including peroxidase reactions), which has implications for analgesic activity as well as the hazardous properties compared to PAR. The opposite holds for electron-withdrawing substituents. Phenoxy free radical formation is relevant in the toxicity mechanism in tissues rich in peroxidase, such as the kidneys, whereas it is probably irrelevant in the P450-dependent hepatotoxicity. A new lead for structural modification of PAR in order to obtain a safer analgesic substance should combine two traces. The analgesic trace prescribes an acetanilide with an -OH (or eventually an

-NH_2) at *para*-position and the *N*-acetyl group 'coplanar'. The *N*-acetyl group could be modified as to prevent *N*-deacetylation. However, variations might influence the lipophilicity of the parent compound which could direct it to other compartments of the body with potential toxicity. The 'toxicologically safe' trace prescribes substituents *ortho* to the -OH (or maybe -NH_2) group that are not liable to addition-elimination reactions by sulfhydryls after formation of a corresponding NAPQI-analogue. In addition, the substituents themselves should not be inert to biotransformation since this could direct biotransformation to *N*-deacetylation of the parent compound to aminophenols. Substitution by alkyl groups via an ether or thioether bond appear to fulfil both analgesic and safety requirements. *In vitro* experiments already provided strong indications for some of these compounds exhibiting analgesic properties combined with decreased hepatotoxicity although the *in vivo* relevance of these findings as well as the potential toxicity for other organs remains to be established.

In general, it is concluded that notwithstanding the huge amount of investigations on chemoprevention, on chemoprotection and on various analogues of PAR, which were all aimed at modulation mainly of PAR-dependent hepatotoxicity, very few clinically useful results have been obtained. However, it is also accomplished that perception of the moleculair mechanisms of the PAR-dependent toxicity, mainly in liver, but also in other organs, is extremely valuable. Mechanism-based development of chemoprotective agents and progress in the development of structural analogues with an improved therapeutic index may be expected. In addition, this understanding of the molecular toxicological aspects of the model-toxicant PAR, with dose- and time-dependent covalent modification of critical and non-critical proteins are important in the comprehension of toxicity-mechanisms of many clinically relevant and clinically non-relevant chemical substances. Perception of these molecular and biochemical mechanisms may help in the development of improved methods for early treament of intoxications as well as refined methods in toxicological risk assessment of chemicals (e.g. the delineation of safe levels of covalent modification of specific proteins). Lastly, insight in the physiological processes that follow primary damage close to the site of formation of reactive metabolites is valuable for the elaboration of chemoprotective agents that can be used clinically in situations when fulminant liver failure has progressed significantly.

Note

While this manuscript had reached its final stage, a paper appeared in which for the first time, unequivocal evidence was presented for the formation of a labile *ipso* adduct between NAPQI and both protein and nonprotein cysteinyl thiols (W. Chen *et al.*, 1999, *Biochemistry*, **38**, 8159-8166).

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SECTION II SYNTHESIS OF ANALOGUES, SCREENING OF BIOLOGICAL ACTIVITIES AND PURIFICATION OF P450 ENZYMES

Chapter 3 3,5-Disubstituted analogues of paracetamol - Synthesis, analgesic activity and cytotoxicity

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3,5-Disubstituted analogues of paracetamol. Synthesis, analgesic activity and cytotoxicity

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Abstract

Seven 3,5-disubstituted analogues of paracetamol were synthesised in order to compare their physicochemical, pharmacological and toxicological properties with those of paracetamol (4′-hydroxyacetanilide, acetaminophen). Oxidation of the phenolic structure is likely involved in the analgesic action of paracetamol as well as in its toxification by cytochrome P450. The effect of disubstitution adjacent to the phenolic hydroxyl group was studied in order to establish possible structure-activity relationships. 3,5-Substituents with electron-donating capacities (R = $-CH_3$, $-OCH_3$, $-SCH_3$) decreased the half-wave oxidation potential substantially by 0.07 V to 0.16 V, whereas electron-withdrawing substituents (R = −F, −Cl, −Br, or −I) increased the oxidation potential by 0.04 V to 0.06 V when compared to paracetamol. Electron-donating substituents (R = $-CH_3$, $-OCH_3$, $-SCH_3$) increased the mouse brain

Abbreviations: DiBr-PAR, 3,5-dibromo-paracetamol; $DiCH_3$-PAR, 3,5-dimethyl-paracetamol; DiCl-PAR, 3,5-dichloro-paracetamol; DiF-PAR, 3,5-difluoro-paracetamol; DiI-PAR, 3,5-diiodo-paracetamol; $DiOCH_3$-PAR, 3,5-dimethoxy-paracetamol; $DiSCH_3$-PAR, 3,5-dithiomethyl-paracetamol; EI-MS, electron impact mass spectrometry; IC_{50}, 50% inhibiting concentration; LDH, lactate dehydrogenase; PAR, paracetamol.

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cyclooxygenase inhibiting capacity of paracetamol. Electron-withdrawing halogen substituents (R = −F, −Cl, −Br or −I) decreased this inhibiting capacity. In agreement with this, the in vivo analgesic activity of the 3,5-dihalogenated analogues was lower when compared to paracetamol. Electron-donating substituents (R = $-CH_3$, $-OCH_3$, $-SCH_3$) decreased the cytotoxicity of paracetamol, when measured as leakage of lactate dehydrogenase from freshly isolated rat hepatocytes, almost completely. Most 3,5-dihalogen substituents (R = −F, −Cl or −Br) diminished it slightly. The fourth electron-withdrawing substituent (R = −I) strongly lowered the cytotoxicity of paracetamol in this test system. In conclusion, a higher cyclooxygenase inhibitory potency of 3,5-disubstituted analogues of paracetamol seemed to correlate with a lower cytotoxicity. 3,5-Disubstituted analogues with electron-donating substituents might therefore be safer analgesics than paracetamol itself. The opposite probably applies to analogues of paracetamol with electron-withdrawing substituents at the 3- and 5- positions of the aromatic nucleus.

Keywords: Paracetamol; Paracetamol analogues; Disubstituted analogues; Synthesis; Analgesic activity; Cytotoxicity

1. Introduction

Paracetamol (acetaminophen, 4′-hydroxyacetanilide, PAR; Table 1) is a widely used non-prescription analgesic and antipyretic drug of rather low efficacy. Although little is known of the mechanism of its analgesic properties, the pharmacological effects of PAR are generally considered to be based on inhibition of prostaglandin synthesis [1,2]. Prostaglandin synthase exhibits two inseparable activities: a cyclooxygenase activity which catalyses the bis-dioxygenation of arachidonic acid to its hydroperoxy endoperoxide (i.e. the prostaglandin PGG_2) and a hydroperoxidase activity which reduces PGG_2 to the corresponding hydroxy-endoperoxide PGH_2 [3]. In vitro, at high concentrations (>10 mM) PAR inhibits the cyclooxygenation of arachidonic acid. At low concentrations (<0.2 mM) however, PAR is acting as a hydrogen donor in the hydroperoxidase reaction [3–5]. Cyclooxygenase inhibition in the central nervous system is likely involved in the analgesic activity of paracetamol, analogously to other prostaglandin synthase inhibiting analgesics.

PAR is also known to be hepatotoxic in man and various experimental animals upon overdose [6–8]. In addition to being a substrate for prostaglandin synthase at low concentrations [5], PAR is also oxidised by cytochrome P450 into the reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI) [9–11].

Taking the presumed molecular mechanisms of analgesic activity as well as of the hepatotoxicity of PAR into consideration, there have been several efforts to improve its analgesic activity while preventing its toxicity by modifying its structure [12–14]. In an attempt to improve the analgesic activity of PAR by mono-substitution *ortho* to the hydroxyl group, Harvison et al. [13] showed that 3-methylparacetamol was equipotent to PAR with respect to analgesic activity in mice. Unfortunately however, hepatotoxicity was also equal. The hepatotoxicity of PAR was decreased by 2-methyl substitution (meta to the hydroxyl group), however, the analgesic activity

was also decreased. *N*-Methylparacetamol was found to be completely devoid of hepatotoxicity but also of analgesic activity. Overall, analgesic activities of the forementioned three monomethylated analogues paralleled their hepatoxicity [13]. In addition to mono-substitution, it has been shown that dialkyl-substitution at the 3- and 5- positions of the aromatic nucleus of PAR did not reduce the analgesic activity [15]. A toxicological study showed that the in vivo hepatotoxicity of the 3,5-dialkylated analogues was reduced almost completely [16].

Recently, it was reported that aromatic ring-substitution by one or two fluorines decreased the analgesic activity of paracetamol in mice [17]. This lead to the suggestion that ease of oxidation might be an important factor for analgesic activity: due to strong electron-withdrawing properties of fluorine, the oxidation potentials of the fluorinated analogues were markedly increased when compared to PAR. It was also shown that these modifications decreased the in vivo toxicity [18].

To establish the consequence of effects of *ortho*-substitution adjacent to the hydroxyl group in PAR on pharmacological and toxicological properties in more detail, we set out to synthesise two new series of 3,5-substituted analogues of PAR (Table 1). The electron-donating substituents R = $-CH_3$, $-OCH_3$ and $-SCH_3$, and the electron-withdrawing substitutuents R = –F, 1Cl, –Br and –I were chosen for evaluation. The in vitro inhibition of mouse brain cyclooxygenase was explored and compared with regard to its predictive value for the mouse writhing test, an accepted in vivo test for analgesic activity [19]. As it is considered to be a reliable in vitro test system for in vivo hepatotoxicity [20], LDH leakage was measured in incubations of the analogues with freshly isolated hepatocytes from rats pretreated with β-naphthoflavone.

2. Materials and methods

2.1. Analytical methods

^{1}H-NMR spectra were recorded using a Bruker 250 MHz NMR. Electron impact (EI-MS) mass spectra were recorded on a Finnigan MAT90 (Finnigan MAT, San José, CA, USA) using 70 eV electron impact and direct introduction of the samples. ^{125}I-radioactivity was counted in an Auto-Gamma scintillation spectrometer (Packard, USA).

2.2. Chemicals

Paracetamol was obtained from Brocacef (Delft, The Netherlands), collagenase (type B) from Boehringer (Mannheim, Germany). HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] was from Merck (Darmstadt, Germany). NADH was obtained from Sigma Chemical Co. (St. Louis, USA). General laboratory chemicals and reagents were obtained in the highest grade possible from local suppliers.

3,5-Dimethyl-paracetamol. 3,5-Dimethyl-paracetamol ($DiCH_3$-PAR; 3′,5′-dimethyl-4′-hydroxyacetanilide [22 900-79-4]) was synthesised as described by Dearden and O'Hara [21].

3,5-Dimethoxy-paracetamol. 3,5-Dimethoxy-paracetamol ($DiOCH_3$-PAR; 3′,5′-

dimethoxy-4′-hydroxyacetanilide) was prepared from 2,6-dimethoxyphenol by diazotation. After reduction with sodium dithionite the crude product was filtered off and the resulting 2,6-dimethoxy-4-aminophenol was finally *N*-acetylated with acetic anhydride.

3,5-Dithiomethyl-paracetamol. 3,5-Dithiomethyl-paracetamol ($DiSCH_3$-PAR; 3′,5′-dithiomethyl-4′-hydroxyacetanilide) was synthesised via 2,6-dithiomethylphenol. 2,6-Dithiomethylphenol was prepared from cyclohexanone and dimethyldisulfide analogously to the method described by Trost et al. [22]. After diazotation of 2,6-dithiomethylphenol and reduction by sodium dithionite, the crude product was filtered off. The resulting 2,6-dithiomethyl-4-aminophenol was dissolved in glacial acetic acid and finally *N*-acetylated with acetic anhydride.

3,5-Difluoro-paracetamol. 3,5-Difluoro-paracetamol (DiF-PAR; 3′,5′-difluoro-4′-hydroxyacetanilide [151 414-41-4]) was prepared from 2,6-difluorophenol by nitration [23]. The resulting 2,6-difluoro-4-nitrophenol was reduced to 2,6-difluoro-4-aminophenol with the sodium borohydride-nickelous chloride system [24] and subsequently *N*-acetylated with acetic anhydride [25].

3,5-Dichloro-paracetamol. 3,5-Dichloro-paracetamol (DiCl-PAR; 3′,5′-dichloro-4′-hydroxyacetanilide [79 694-26-1]) was synthesised from 2,6-dichloro-4-aminophenol by *N*-acetylation with acetic anhydride [25].

3,5-Dibromo-paracetamol. 3,5-Dibromo-paracetamol (DiBr-PAR; 3′,5′-dibromo-4′-hydroxyacetanilide [63 558-07-6]) was prepared from 2,6-dibromo-4-nitrophenol, which was reduced to 2,6-dibromo-4-aminophenol with sodium borohydride-nickelous chloride and subsequently *N*-acetylated with acetic anhydride as described for 3′,5′-difluoro-4′-hydroxyacetanilide.

3,5-Diiodo-paracetamol. 3,5-Diiodo-paracetamol (DiI-PAR; 3′,5′-diiodo-4′-hydroxyacetanilide [27 721-00-2]) was synthesised from 2,6-diiodo-4-aminophenol analogously to the procedure described above for 3′,5′-difluoro-4′-hydroxyacetanilide.

2.3. Oxidation potentials

Half-wave redox potentials ($E_{1/2}$) were determined at a sweep rate of 2 mV/s from the first sweep of the oxidation wave of cyclic voltammograms. Solutions (50% ethanol in 100 mM sodium phosphate buffer; pH 7.4) contained 0.1 mM of PAR or of its 3,5-disubstituted analogues. Voltammograms were recorded with a gold wire working electrode, a Ag/AgCl/3 M KCl reference electrode and a platinum auxiliary electrode. Each voltammogram was generated using a freshly prepared electrode surface. An Autolab type potentiostat was used controlled by a GPES 3.1© software package developed by Eco Chemie (Utrecht, The Netherlands).

2.4. Animals

For antinociception testing and the isolation of brain microsomes male Swiss Webster mice were used. Male Wistar rats were used for the isolation of hepatocytes. All animals had free access to food and tap water. For induction of cytochrome P450, the rats were pretreated twice with β-naphthoflavone (i.p.; 100 mg/kg, in arachidis oil) 48 and 24 h before they were anaesthetized for hepatocyte isolation.

2.5. Antinociceptive activity

Mice were weighed and placed in 9 × 9 cm clear plexiglas® cubicles. The compounds were dissolved in 50% (v/v) PEG 200/water and administered to the animals intravenously in a final volume of 10 ml/kg. Five min after the intravenous injection, acetylcholine bromide (3.2 mg/kg) was administered by the intraperitoneal route and animals were observed, by an observer, being unaware of the dosing regime, for abdominal constrictions (writhing) for a period of 3 min. The percentage of mice in each group protected against acetylcholine-induced writhing was determined and the ED_{50} was calculated by the method of Litchfield and Wilcoxin [26].

2.6. Isolation of mouse brain microsomes

Mice were decapitated and the cerebral cortex, hypothalamus and brainstem rapidly dissected over ice. The tissue was homogenized in 3 volumes of ice cold 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM EDTA and 0.1% BSA (w/v). The homogenate was centrifuged at 10 000 × *g* for 15 min. The resulting supernatant was decanted and subsequently centrifuged at 100 000 × *g* for 30 min yielding a crude microsomal pellet. The pellet was washed 3 times with 3 volumes of 0.1 M potassium phosphate buffer (pH 7.4).

2.7. Inhibition of brain cyclooxygenase

Cyclooxygenase activity was determined by the formation of prostaglandin PGE_2 from arachidonic acid in brain microsomes [27,28]. PGE_2 formation was quantitated using a RIA-kit with ^{125}I-PGE_2 (New England Nuclear Research Products) according to instructions of NEN Research Products.

2.8. Hepatocytes

Liver parenchymal cells were isolated from β-naphthoflavone-pretreated rats by collagenase perfusion of the liver as described [29]. Hepatocytes were incubated in Krebs-Henseleit buffer (pH 7.4) containing 2.5 mM HEPES and 10 mM glucose. The cells were equilibrated ~5 min at 37°C with carbogen (95% O_2/5% CO_2) prior to the addition of 0.5 mM of paracetamol or the synthesised disubstituted analogues of paracetamol (all dissolved in DMSO).

2.9. Lactate dehydrogenase (LDH) leakage

After 3 h of incubation, 0.5 ml aliquots were removed. In these samples, medium was separated from the cells by centrifugation (50 × *g* for 5 min). LDH leakage in the supernatant fraction was then assayed as described by Moldéus et al. [30], using a Philips PU-8720 UV/vis spectrophotometer. Values were expressed as percentage of 100% leakage which was determined in a Triton X-100 treated incubation.

3. Results

3.1. Synthesis

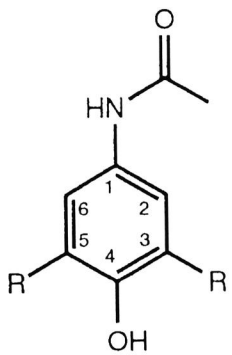
The synthesis of two series of 3,5-disubstituted analogues of PAR was successful. The yields of the crude synthetic reaction products were in the range of 70–90%.

Purity of the compounds was established by TLC, ^{1}H-NMR and determination of melting points. The range of the melting points was < 2°C and with NMR and TLC only one product was found, meaning that purity is >97%. Chemical shifts upon ^{1}H-NMR analysis and EI-MS data are summarized in Table 2, and the melting points in Table 1. All NMR signals could be assigned. For each compound, the difference between experimentally determined exact masses of the respective molecular ions and the calculated masses was no more than 0.0040 amu.

3.2. Oxidation potentials

The half-wave oxidation potentials ($E_{1/2}$) as determined by cyclic voltammetry are shown in Table 1. Oxidation potentials ($E_{1/2}$) of $DiCH_3$-PAR, $DiOCH_3$-PAR and $DiSCH_3$-PAR were substantially lower than $E_{1/2}$ of PAR. $E_{1/2}$ of $DiCH_3$-PAR was decreased to 0.21 V, which is 0.07 V lower than $E_{1/2}$ of PAR. 3,5-Disubstitution with $-OCH_3$ and $-SCH_3$ lowered the half-wave oxidation potentials even further

Table 1
Substituents, abbreviations, names, halfway oxidation potentials ($E_{1/2}$) and melting points (uncorrected) of paracetamol and 7 synthetical 3,5-disubstituted analogues



Substituted –R	Code	Compound	$E_{1/2}$[a]	m.p.[c]
–H	PAR	paracetamol	0.28 V	—
Electron-donating				
$-CH_3$	$DiCH_3$-PAR	3,5-dimethylparacetamol	0.21 V	162°C
$-OCH_3$	$DiOCH_3$-PAR	3,5-dimethoxyparacetamol	0.12 V	141°C
$-SCH_3$	$DiSCH_3$-PAR	3,5-dithiomethylparacetamol	0.12 V	135°C
Electron-withdrawing				
–F	DiF-PAR	3,5-difluoroparacetamol	0.32 V	180°C
–Cl	DiCl-PAR	3,5-dichloroparacetamol	0.34 V	157°C
–Br	DiBr-PAR	3,5-dibromoparacetamol	0.32 V	171°C
–I	DiI-PAR	3,5-diiodoparacetamol	—[b]	188°C

[a]Half-wave oxidation potentials ($E_{1/2}$ vs. Ag/AgCl), determined as described in Materials and methods. Values given are mean (n = 3).
[b]No oxidation potential could be determined.
[c]Melting points (uncorrected)

Table 2
[1]H-NMR and electron impact (EI) mass spectrometric data of the ions and the most abundant fragments of the synthesised 3,5-disubstituted paracetamol analogues

Compound	[1]H-NMR: δ in ppm[a]	HRMS and partial mass spectra: *m/z* of ion or fragment (intensity)
$DiCH_3$-PAR	2.1 (s, 3H, acetyl), 2.2 (s, 6H, methyl), 7.05 (s, 2H, aromatic) ($CDCl_3$)	
$DiOCH_3$-PAR	2.0 (s, 3H, acetyl), 3.75 (s, 6H, methoxy), 6.9 (s, 2H, aromatic), 8.05 (s, 1H, OH or NH), 9.7 (s, 1H, OH or NH) (DMSO-d_6)	HRMS: Observed 211.0840, calcd for $C_{10}H_{13}NO_4$, 211.0845 Partial mass spectrum: 211 (100%, $M^{+\bullet}$), 169 (75%, $[M\text{-}CH_2CO]^{+\bullet}$)
$DiSCH_3$-PAR	2.0 (s, 3H, acetyl), 2.3 (2s, 6H, thiomethyl), 7.3 (s, 2H, aromatic), 9.75 (1H, br., OH or NH), 9.8 (s, 1H, OH or NH) (DMSO-d_6)	HRMS: Observed 243.0392, calcd for $C_{10}H_{13}NO_2S_2$, 243.0388 Partial mass spectrum: 243 (100%, $M^{+\bullet}$), 201 (42%, $[M\text{-}CH_2CO]^{+\bullet}$), 186 (57%, $[M\text{-}NHCOCH_3]^+$)
DiF-PAR	2.0 (s, 3H, acetyl), 7.25 (d, 2H, aromatic, J_{FH} = 10 Hz), 9.8 (s, 1H, OH or NH), 10.0 (s, 1H, OH or NH) (DMSO-d_6)[b]	HRMS: Observed 187.0448, calcd for $C_8H_7NO_2F_2$, 187.0445 Partial mass spectrum: 187 (38%, $M^{+\bullet}$), 145 (100%, $[M\text{-}CH_2CO]^{+\bullet}$)
DiCl-PAR	2.0 (s, 3H, acetyl), 7.55 (s, 2H, aromatic), 9.8 (1H, br., OH or NH), 10.0 (s, 1H, OH or NH) (DMSO-d_6)	HRMS: Observed 220.9795, calcd for $C_8H_7NO_2{}^{37}Cl{}^{35}Cl$, 220.9824 Partial mass spectrum: 219 (34%, $M^{+\bullet}$ $^{35}Cl_2$), 177 (100%, $[M\text{-}CH_2CO]^{+\bullet}$)
DiBr-PAR	2.0 (s, 3H, acetyl), 7.75 (s, 2H, aromatic), 9.65 (s, 1H, OH or NH), 9.95 (s, 1H, OH or NH) (DMSO-d_6)	HRMS: Observed 306.8879, calcd for $C_8H_7NO_2{}^{79}Br_2$, 306.8844 Partial mass spectrum: 309 (41%, $M^{+\bullet}$ $^{79}Br^{81}Br$), 267 (100%, $[M\text{-}CH_2CO]^{+\bullet}$)
DiI-PAR	2.0 (s, 3H, acetyl), 7.95 (s, 2H, aromatic), 9.3 (s, 1H, OH or NH), 9.85 (s, 1H, OH or NH) (DMSO-d_6)	HRMS: Observed 402.8586, calcd for $C_8H_7NO_2I_2$, 402.8564. Partial mass spectrum: 403 (100%, $M^{+\bullet}$), 361 (95%, $[M\text{-}CH_2CO]^{+\bullet}$)

[a]Internal standard was TMS.
[b]Due to low resolution fluorine coupling could not be resolved.
[c]HRMS, high resolution mass spectrometry.

down to 0.12 V. 3,5-Dihalogenation of PAR increased the half-wave oxidation potential by 0.04–0.06 V. 3,5-Difluorination and -bromination increased $E_{1/2}$ to 0.32 V and 3,5-dichlorination increased it to 0.34 V.

3.3. Antinociceptive activities

The experiments conducted to determine and to compare the antinociceptive activities of 4 analogues to that of PAR are shown in Table 3. PAR was used as a control and inhibited acetylcholine-induced writhing (ED_{50} of 77 mg/kg with 95% confidence limits of 52–115 mg/kg). No side effects were observed at the doses tested. At 100 mg/kg, the 4 dihalogenated analogues were less potent than PAR. Each of the 4 analogues protected for 25% against acetylcholine-induced writhing, whereas PAR protected 70% of the animals. At 300 mg/kg, between 85% and 100%

Table 3
Inhibition of acetylcholine-induced writhing and side-effects in male Swiss Webster mice by paracetamol and 4 synthetical 3,5-dihalogenated analogues

Compound	Dose (mg/kg)	% Animals protected	% Antinoci-ception[a]	% Side effects
PAR	30	10 (n = 10)	0	0
	55	20 (n = 10)	11	0
	100	70 (n = 10)	67	0
	173	90 (n = 10)	89	0
	300	90 (n = 10)	89	0
DiF-PAR	100	25 (n = 4)	25	0
	300	85 (n = 8)	87	25
DiCl-PAR	100	25 (n = 4)	25	0
	300	100 (n = 8)	100	37
DiBr-PAR	100	25 (n = 4)	25	0
	300	100 (n = 8)	100	75
DiI-PAR	100	25 (n = 4)	25	0
	300	85 (n = 8)	87	25

[a]Antinociception = [(% protected − % control protected)/(100% − % control protected)] × 100.

were protected against the acetylcholine-induced writhing, with 25–75% of the mice having observable side effects ranging from sedation to depression.

3.4. Inhibition of cyclooxygenase

As can be seen in Table 4, all compounds inhibited cyclooxygenase. 3,5-Disubstitution with the $-CH_3$, $-OCH_3$ or $-SCH_3$ substituents resulted in a stronger inhibition of cyclooxygenase than PAR itself (i.e. IC_{50} ranged from 0.35 mM for PAR to values of 0.20 mM and less for $DiCH_3$-PAR, $DiOCH_3$-PAR or $DiSCH_3$-

Table 4
IC_{50} values and LDH leakage found for paracetamol and 7 synthetical 3,5-disubstituted analogues

Compound	IC_{50} (mM) ± S.E.M.[a]	LDH (%) ± S.E.M.[b]
BLANK	—	8.2 ± 1.0
PAR	0.35 ± 0.08	50.6 ± 2.8
$DiCH_3$-PAR	0.16 ± 0.00	15.0 ± 0.9
$DiOCH_3$-PAR	0.20[c]	12.4 ± 0.5
$DiSCH_3$-PAR	0.14 ± 0.03	14.5 ± 1.2
DiF-PAR	1.14 ± 0.07	42.3 ± 0.9
DiCl-PAR	0.56 ± 0.13	36.0 ± 3.5
DiBr-PAR	0.48 ± 0.07	26.1 ± 3.2
DiI-PAR	1.11 ± 0.16	14.7 ± 1.4

[a]IC_{50} values, representing mean ± S.E.M. (n = 6).
[b]LDH leakage in hepatocytes after 3 h of incubation. Values represent mean ± S.E.M. (n = 3).
[c]Single experiment.

PAR). In contrast, 3,5-disubstitution with –Cl and –Br decreased the cyclooxygenase inhibiting capacity only slightly when compared to PAR (IC_{50} of 0.56 mM and 0.48 mM for DiCl-PAR and DiBr-PAR, respectively). 3,5-Disubstitution by –F or –I, however, caused a substantial decrease in the cyclooxygenase inhibiting capacity (IC_{50} 1.11 mM for both DiF-PAR and DiI-PAR).

3.5. LDH leakage in hepatocytes

The obtained results on LDH leakage showed a wide range of cytotoxicity. In comparison to PAR, two groups of analogues could be distinguished with regard to their property to induce cytotoxicity in freshly isolated hepatocytes (Table 4). 3,5-Disubstitution with R = $-CH_3$, $-OCH_3$ and $-SCH_3$ decreased LDH leakage after 3 h of incubation to about 15%, 12% and 15%, respectively, compared to about 51% for PAR itself. Thus, in comparison to blank incubations (8%), LDH leakage was only slightly elevated in this group of PAR analogues with electron-donating substituents. The second group of analogues, with electron-withdrawing substituents at the 3,5-positions (R = –F, –Cl, –Br and –I), was moderately to strongly cytotoxic. LDH leakage was about 42%, 36% and 26%, respectively, for incubations with DiF-PAR, DiCl-PAR and DiBr-PAR. Only DiI-PAR was an exception in the group of analogues with electron-withdrawing substituents with only about 15% of LDH leakage after 3 h of incubation.

4. Discussion

The aim of the present study was to synthesise two series of 3,5-disubstituted analogues of paracetamol and to explore possible structure-activity relationships with respect to their cyclooxygenase inhibiting properties in mouse brain fractions and their cytotoxicity in freshly isolated rat hepatocytes. The first series of analogues that was synthesised successfully contained compounds with two electron-donating substituents at the 3- and 5- postition in the aromatic ring ($-CH_3$, $-OCH_3$ and $-SCH_3$). The second series was composed of PAR analogues with two electron-withdrawing halogen substituents, varying from –F, –Cl, and –Br to –I. To our knowledge, $DiOCH_3$-PAR and $DiSCH_3$-PAR were synthesised for the first time. Syntheses of DiBr-PAR and DiI-PAR have only been described briefly in patents. Since with NMR, IR and TLC only one product was found in the purified products of all syntheses it was concluded that their purity is over 97%.

Considering the half-wave oxidation potentials ($E_{1/2}$), as expected, electron-donating substituents (R = $-CH_3$, $-OCH_3$ and $-SCH_3$) decreased the oxidation potential of PAR substantially. These results are in agreement with previous data with 3,5-dimethyl substitution of paracetamol [3,5]. In contrast, 3,5-disubstitution with electron-withdrawing halogens (R = –F, –Cl and –Br) increased the oxidation potential. This is also in conformity with a recent report in which an increase in oxidation potential was found when paracetamol was ring-substituted by 1, 2 or 4 electron-withdrawing fluorine atoms [17].

Previously, it has been shown that variation in lipophilicity causes variation in analgesic potency in a series of 3- and 3,5-, mono- and dialkyl substituted paraceta-

mol derivatives [15]. Also, the analgesic potency has been suggested to be related to oxidisability [5,18]. Lastly, coplanarity of the acetamide group with the aromatic nucleus has been argued to be important for cyclooxygenase inhibition [17,21]. In the present study, compounds were investigated that were deliberately varied in the substituents at the 3- and 5-positions, in which the coplanarity of the acetamide is not disturbed according to computational conformation analysis (data not shown). The present results of the acetylcholine-induced writhing test in mice, as presented in Table 3, showed that DiF-PAR, DiCl-PAR, DiBr-PAR and DiI-PAR were slightly less potent than PAR. Whether this difference is due to differences in potency to inhibit prostaglandin synthesis or to differences in the pharmacokinetics or disposition of the compounds is still uncertain.

Inhibition of cyclooxygenase, being a specific activity of prostaglandin synthase, is considered to be involved in analgesic activity of compounds like PAR [1,2]. Therefore, the inhibition of cyclooxygenase by the newly synthesised analogues of PAR was also studied by measuring the formation of prostaglandin PGE_2 from arachidonic acid in mice brain microsomes. Comparison between the in vivo writhing test in mice and the in vitro inhibition of cyclooxygenase suggests a relation between IC_{50} values, as determined in vitro (Table 4), and the analgesic activity of PAR and its 3,5-dihalogenated analogues, as determined in vivo (Table 3). The acetylcholine-induced writhing test showed that at 100 mg/kg dosage, the four 3,5-dihalogenated analogues (R = –F, –Cl, –Br and –I) were less potent than PAR itself. In line with this, the 3,5-dihalogenation of PAR decreased its cyclooxygenase inhibitory properties.

Electron-withdrawing substituents at the 3,5-position of PAR tend to decrease the inhibitory potency towards cyclooxygenase and electron-donating substituents tend to increase this pharmacological property. When comparing IC_{50} values and $E_{1/2}$ values (Fig. 1), it seems that a higher oxidation potential is indicative for a weaker cyclooxygenase inhibition. The fact that a higher half-wave redox potential is inversely related with the relative potencies of the analogues of PAR is in agreement with previous in vitro and in vivo results with two analogues of PAR, i.e. 2-methyl-paracetamol and 2,6-dimethyl-paracetamol [5,13]. Also, recent data from Barnard et al. support the hypothesis that an increase in oxidation potentials as a result of aromatic fluorine substitution at 3-, 3,5-, 2,6-, and 2,3,5,6-positions, results in decreased analgesic activity [17].

Because of the limited number of analogues and biological data no solid and appropriate correlation analysis could be performed with respect to physico-chemical parameters such as Hammett σ_m and Hammett $\sigma_{o=p}$, Charton's inductive parameter σ_I, Taft's E_s steric parameter and $f_{revised}$ [31]. Nevertheless, preliminary calculations indicated that the parameter Hammett σ_m together with Charton's inductive parameter σ_I had some predictive value (data not shown).

As shown in Table 3, at 300 mg/kg dose, 3,5-dihalogenated PAR analogues exhibited side effects as well. With respect to toxicity, previous investigations [20] have shown a good correlation between in vivo hepatotoxicity and in vitro LDH leakage from freshly isolated rat hepatocytes for PAR and eight 3-mono- and 3,5-dialkyl substituted derivatives. In the present study, DiF-PAR, DiCl-PAR and DiBr-PAR

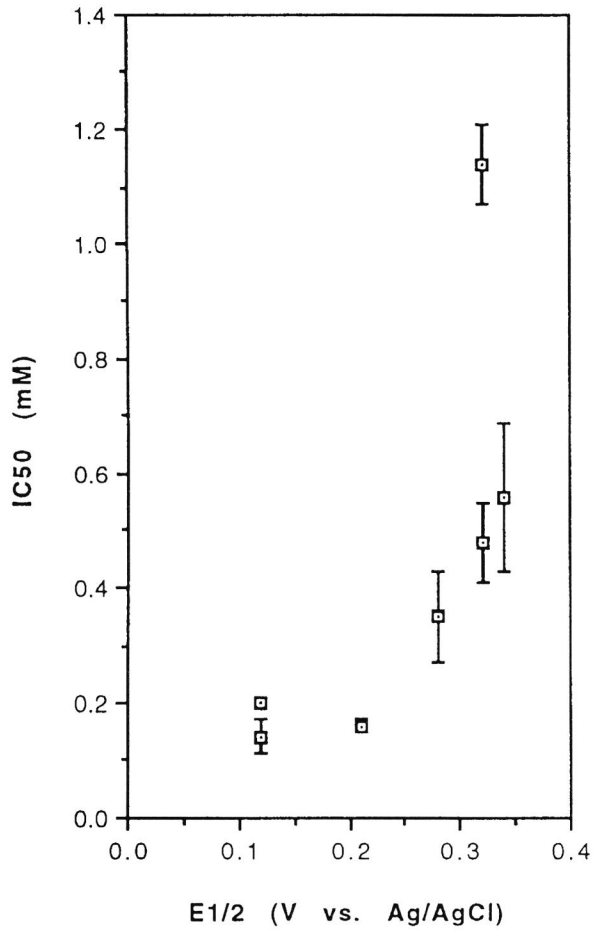


Fig. 1. Visualisation of the relation between cyclooxygenase inhibiting capacity (IC_{50}) and half-wave oxidation potential ($E_{1/2}$) of PAR and 7 synthetical 3,5-disubstituted analogues.

did present significant cytotoxicity in freshly isolated hepatocytes. In contrast, $DiCH_3$-PAR, $DiOCH_3$-PAR and $DiSCH_3$-PAR did cause only very minor LDH leakage after 3 h of incubation in freshly isolated hepatocytes from β-naphthoflavone-pretreated rats (Table 4). The LDH leakage observed upon incubation of PAR and $DiCH_3$-PAR was comparable to that reported earlier [20].

Graphical presentation of LDH leakage vs. $E_{1/2}$ of the 3,5-disubstituted PAR analogues investigated in the present study, suggests the cytotoxicity to be related to their oxidisability (Fig. 2). Analogously to the inhibition of cyclooxygenase, with regard to the cytotoxicity, a certain role may be assigned as well to the electronic properties of the PAR analogues. The toxicity of PAR is generally accepted to be mediated by *N*-acetyl-*p*-quinone imine (NAPQI) and its subsequent reaction with

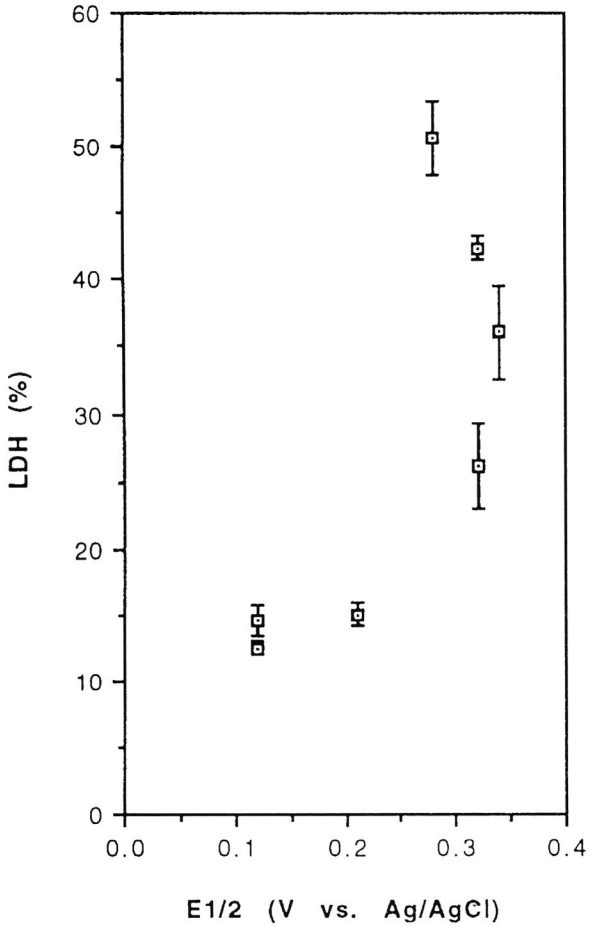


Fig. 2. Visualisation of the relation between LDH leakage caused by PAR and 7 synthetical 3,5-disubstituted analogues and their half-wave oxidation potential ($E_{1/2}$).

protein and non-protein thiol groups. Therefore, dependency of toxicity on only the oxidisability of the PAR analogues is not to be expected unless the primary oxidation step would be rate-limiting in the overall process [8].

In summary, varying cyclooxygenase inhibiting properties were found upon 3,5-disubstitution of PAR. Analogues with two electron-donating substituents ($DiCH_3$-PAR, $DiOCH_3$-PAR and $DiSCH_3$-PAR) seem to be promising candidates for further research with respect to their analgesic and cyclooxygenase inhibiting properties. For these PAR analogues, lower IC_{50} values as well as a lower cytotoxicity were found. The suggested link between oxidisability and cyclooxygenase inhibition suggests that for inhibition of prostaglandin synthase, one- or two-electron oxidation is a prerequisite [5]. With respect to cytotoxicity, electron-donating substituents (R = $-CH_3$, $-OCH_3$ and $-SCH_3$) tended to decrease LDH leakage, whereas most

electron-withdrawing substituents (R = −F, −Cl, −Br and −I) did not. The series of analogues used in this study may be interesting as a lead for further exploration of the mechanisms of both prostaglandin synthase inhibition and the oxidative biotransformation by cytochrome P450.

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Chapter 4 High-performance ion-exchange chromatography preparative scale purification of rat hepatic microsomal cytochromes P450

High-performance ion-exchange chromatography preparative scale purification of rat hepatic microsomal cytochromes P450

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Abstract

A preliminary investigation of a rapid preparative scale purification of rat liver microsomal cytochrome P450 1A1 (CYP1A1; pilot scale)*, P450 2E1 (CYP2E1) and NADPH-dependent cytochrome P450 reductase (P450-RED), based on high-performance ion-exchange chromatography (ion-exchange fast protein liquid chromatography; ion-exchange FPLC) is presented.

A pilot scale CYP1A1 purification procedure was performed using liver microsomes of rats, pretreated with ß-naphthoflavone (ßNF) and 0.3% Emulgen 911 for solubilisation. For further removal of non-CYP1A1 proteins, only High Performance (HP) Q-Sepharose ion-exchange chromatography was performed. After removal of Emulgen 911 by hydroxyapatite chromatography CYP1A1 was apparently pure as judged by SDS-PAGE and Western blotting using anti-CYP1A1.

In the large scale CYP2E1 purification procedure, proteins were solubilised from microsomes of pyrazole-treated rats with 0.4% Emulgen 911. Anionic proteins were removed by retention on High Performance (HP) Q-Sepharose. CYP2E1 emerged in the pass-through fraction and was subsequently applied to a HP S-Sepharose cation-exchange column. Hydroxyapatite chromatography with unconventional gradient elution removed further impurities and detergent. Starting from 310 nmol total cytochrome P450, the overall recovery (yield) was 1.1% as the CYP2E1-containing fraction emerging from hydroxyapatite contained 3.5 nmol of cytochrome P450; only one impurity (45 kDa) was still visible with silver staining. The ultimate specific content of the CYP2E1 was 3.5 nmol mg^{-1} protein with an overall purification factor of 6.6. The ultimate specific activity in a reconstituted system was 0.37 nmol min^{-1} $nmol^{-1}$ P450 with *p*-nitrophenol hydroxylation.

NADPH-dependent cytochrome P450 reductase (P450-RED) was isolated parallel on HP Q-Sepharose and emerged with an NaCl gradient around 0.5 M NaCl. Subsequent affinity chromatography on a 2',5'-ADP-Sepharose column ultimately resulted in apparently pure P450-RED. Starting with about 62 nmol of P450-RED, the ultimate purified fraction contained 2.9 nmol (yield 4.7%). The specific content was increased from 0.11 to 3.1 nmol mg^{-1} protein (purification factor 28).

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* *Abbreviations used:* ßNF, ß-naphthoflavone; CM, carboxymethyl; CYP1A1, cytochrome P450 1A1; CYP2E1, cytochrome P450 2E1; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylene diamine tetra acetic acid; FPLC, Fast Protein Liquid Chromatography; HP, High Performance; P450, cytochrome P450; P450-RED, NADPH dependent cytochrome P450 reductase; PMSF, phenylmethanesulfonylfluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

INTRODUCTION

In order to study the biochemical and toxicologically relevant properties of a specific cytochrome P450 enzyme, large scale isolation of this microsomal protein and NADPH-dependent cytochrome P450 reductase (P450-RED) from other cytochromes P450 (P450's) as well as other non-cytochrome P450 proteins is often a prerequisite (Thomas *et al.*, 1981). Next to *in vivo* induction of one or a few specific P450's and inhibition of enzymatic activity by specific monoclonal anti-P450 antibodies, the availability of purified P450's is a powerful tool in mechanistic toxicological research (Van Bladeren *et al.*, 1987; Guengerich *et al.*, 1991; Boyd *et al.*, 1993). As P450's are microsomal enzymes, the separation of one specific P450 from other P450's has always been somewhat more complicated than e.g. the purification of most glutathione *S*-transferase isoenzymes as most glutathione *S*-transferases are cytosolic enzymes, precluding protein solubilisation.

Some P450 enzymes, such as P450 1A1 (CYP1A1) and P450 2B1 (CYP2B1), have been purified on a preparative scale already from the seventies on (Ryan *et al.*, 1979; West *et al.*, 1979). For some others, such as P450 2E1 (CYP2E1), this has awaited until the eighties (Ryan *et al.*, 1985; Patten *et al.*, 1986). However, due to several reasons, such as protein instability in the absence of (pseudo)substrates and the pI of CYP2E1 which requires modified purification methods, its large-scale isolation is still laborious and time-consuming. As CYP1A1 as well as CYP2E1 are known to be important enzymes in the bioactivation of paracetamol and other toxicants, at least in rat (Ryan *et al.*, 1985; Patten *et al.*, 1986; Harvison *et al.*, 1988), it was felt necessary to investigate potential and significant improvements in the purification procedures of CYP1A1 and even more strongly for CYP2E1 as published so far.

INTERMEZZO

Together with hydrophobic interaction chromatography (octylamino Sepharose 4B), ion-exchange chromatography still is a very common technique for isolation and characterization of different isoforms of cytochrome P450. For anion and cation-exchange chromatography, respectively diethylaminoethyl (DEAE) and carboxymethyl (CM) are frequently used as functional groups exhibiting weak ion-exchange characteristics. Initially DEAE-cellulose was used for the preparative scale isolation of cytochrome P450 (Guengerich and Martin, 1980)*, while later DEAE-Sepharose resolved one apparently homogeneous isoform as eluted from DEAE-cellulose, into four different P450 isoforms with very similar molecular masses* (Bansal *et al.*, 1985)*. The combination of octylamino Sepharose 4B and DEAE-Sepharose CL-6B chromatography provided large amounts of homogeneous enzymes to be used for example in toxicological research* (Goeptar *et al.*, 1993)*. Unfortunately however, the physical limitations (pressure, sensitivity to alkaline or acidic conditions) of the classical methods for the isolation of cytochrome P450 isoforms from microsomal fractions from various species hampered the development of rapid separation procedures. In the late seventies, advances in high-performance liquid*

chromatography (HPLC) enabled the rapid resolution of complex mixtures of soluble peptides and proteins (references 13-16 in (Kotake and Funae, 1980)*). Its speed and resolution capacity makes HPLC methods superior to conventional, low pressure chromatographic techniques.*

Kotake and Funae were among the first to use HPLC with ion-exchange media for analytical (small scale) separation of membrane-bound hepatic proteins such as cytochrome P450 (Kotake and Funae, 1980)*. Later, they pioneered in the use of strong cation-exchange HPLC colums (sulfopropyl functional groups) for P450 profiling* (Imaoka and Funae, 1986)*. One major drawback, however, was the lack of functional activity of the enzymes as a result of possible loss of the heme group during HPLC* (Bansal *et al.*, 1985) *First in 1986, catalytic activity of microsomal cytochrome P450 was maintained in a preparative scale purification procedure in which HPLC was used for monitoring (analytical scale)* (Bornheim and Correia, 1986)*. The reason of retainment of enzyme activity was not discussed but might be due to the use of safer colums resins. Subsequently, Funae et al. were the first to purify active P450 enzymes from solubilized microsomes solely by HPLC in two steps (anion-exchange and hydroxyapatite)* (Funae *et al.*, 1986)*.*

HPLC was used mostly for profiling of microsomal P450's from rat, rabbit, and man (Holm and Kupfer, 1985; Bornheim and Correia, 1986; Imaoka *et al.*, 1990; Roos, 1990)*. A few studies were reported so far were preparative HPLC with a DEAE anion-exchange column was used for large scale purification of microsomal cytochrome P450 from rats* (Imaoka *et al.*, 1987; Funae *et al.*, 1988; Suzuki *et al.*, 1992)*. One started with over 2000 nmol of P450 and about 600 nmol was applied to a DEAE anion-exchange column* (Funae *et al.*, 1988)*. For preparative scale purification usually large bed volumes were needed. To speed up purification, the availability of media that could stand high flow rates without concomitant increase in back-pressure was needed. Furthermore, the resolution of P450 isoforms appeared also to be affected by the buffer, the concentration of glycerol, the kind of detergent, and thepore size of the column* (Kastner and Schulz, 1987)*. In order to perform multiple purifications with one and the same column, inert support material was needed. Unfortunately, weak ion-exchangers (such as DEAE and CM) based on monobeads are not available, and the existing weak ion-exchange media are not resistant to the acidic or basic conditions needed for in situ regeneration.*

In 1987, a specific HPLC method that was known for protein purification, called Fast Protein Liquid Chromatography (FPLC) and based on the strong ion-exchange media Q- and S-Sepharose (quaternair ammonium and sulfopropyl) in Mono-Q and Mono-S columns, was used for the first time for P450 profiling (Kastner and Schulz, 1987). Later on, scaling up for cytochrome P450 fractionation was performed by adapting the optimized FPLC procedures for Mono-Q and Mono-S to Q- and S-Sepharose Fast Flow media (Roos, 1990). In addition, FPLC introduced the stepwise gradient elution, which appeared to exhibit significant better separation of different forms of cytochrome P450 (Roos, 1990; Kastner and Neubert, 1991), The

usefulness, applicability and optimization of a wide range of commercially available, prepacked FPLC columns and labororatory-packed columns were reviewed in these papers (Roos, 1990; Kastner and Neubert, 1991).

In this paper, investigations are described for improvements of P450 and P450-RED purification procedures with respect to reproducibility and number of purification steps (total time period needed). In rat, CYP1A1, CYP2E1, CYP3A3, and to a lesser extent, CYP1A2, are important P450 enzymes involved in the bioactivation of paracetamol (acetaminophen) (Harvison *et al.*, 1988; Patten *et al.*, 1993). As conventional purification of rat liver microsomal CYP2E1 was published to request multiple chromatography steps (Ryan *et al.*, 1985), it was aimed at improvement of the isolation in sense of speed and reproducibility. Thus, our primary aim was to set-up a rapid preparative scale isolation method for CYP2E1 (and P450-RED). For reasons of convenience (CYP1A1 is more stable and classical purification is more straightforward than isolation of CYP2E1), first a pilot purification was performed using CYP1A1. For this purpose, it was decided to investigate the performance characteristics of the HiLOAD Sepharose High Performance media for anion- and cation-exchange chromatography with regard to P450 purification without the commonly used first chromatography step on octylamino Sepharose. These HiLOAD media were specially designed for FPLC, including the possibility of simple variation of the gradient properties such as introducing a stepwise gradient.

Materials and methods

Chemicals and apparatus

3-Amino-1,2,4-triazole (3-AT), 2'-adeninemonophosphate (AMP), 5-bromo-4-chloro-3-indolylphosphate (BCIP), cytochrome *c*, dithiotreitol (DTT), ethoxyresorufin and resorufin, glucose-6-phosphate and glucose-6-phosphate-dehydrogenase, L-α-phosphatidylcholinedilauroyl, 4-methylpyrazole, $NADP^+$, NADPH, Tris-HCl and Tween 20 were purchased from Sigma (St. Louis, MO, USA). Acrylamide and bisacrylamide were from Serva (Heidelberg, Germany). Ammonium persulfate (AMPS), ethylenediaminotetraacetic acid (EDTA), glycerol (Baker analyzed grade) and *p*-nitrophenol were obtained from Baker (Deventer, The Netherlands).

Bromophenol blue, sodiumdeoxycholate, glycine, 2-mercaptoethanol, nitrobluetetrazolium (NBT), phenylmethanesulfonylfluoride (PMSF) and pyrazole were purchased from Merck (Darmstadt, Germany). α-Naphthoflavone (αNF) was from Aldrich (Brussels, Belgium), ß-naphthoflavone (ßNF), *p*-nitrocatechol, diethylenetriaminepentaacetic acid (DETAPAC) and N,N,N',N'-tetramethylethylene-diamine (TEMED) from Janssen Chimica (Beerse Belgium) and sodiumdodecylsulfate (SDS) from Brocades acf (Maarssen, The Netherlands).

Emulgen 911 was from Kao Atlas Chemicals (Tokyo, Japan). Bio-Gel HTP (hydroxyapatite; $(Ca_5(PO_4)_3OH)_2$) and molecular-weight markers for the SDS-PAGE (silver stain SDS-PAGE standards, low range) were obtained from Bio-Rad Laboratories (Richmond, CA). 2',5'-ADP-Sepharose was purchased from Pharmacia

(Uppsala, Sweden). All other chemicals were analytical grade. Monoclonal antibodies were free gifts from P. Kremers (K06: mouse anti-rat CYP1A1) and F. Gonzalez (rabbit anti-rat CYP2E1). Ultraconcentrators were from Amicon Division (W.R. Grace & Co.-Conn, Beverly, CA)

Animals and microsomes

Male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 180-220 grams, were housed in plastic cages in temperature (22°C) and humidity (50%)-controlled rooms with a 12-h lighting cycle. Water and commercial laboratory chow (HOPE Farms, Woerden, The Netherlands) were available *ad libitum*.

For CYP1A1 induction, five rats were injected i.p. with 40 mg/kg ßNF, dissolved in arachides oil (8 mg/ml) for two consecutive days. For CYP2E1 induction, five rats were injected i.p. with 200 mg/kg pyrazole (40 mg/ml), dissolved in a physiological saline solution for two consecutive days (Winters and Cederbaum, 1992). Fed rats were sacrificed the third day by decapitation under light diethyl ether narcosis.

Livers from five rats belonging to either the ßNF- or the pyrazole-group were quickly excised, weighed and homogenised at 4°C with a Waring blender and Elvjehem teflon-glass potter in two volumes (one volume equals the total liver weight) buffer **A** (50 mM potassium phosphate, 0.9% NaCl, pH 7.4 at 4°C). The supernatant after centrifugation (20 min x 12,000g at 4°C) was ultracentrifugated (75 min x 100,000g at 4°C). The microsomal pellet was resuspended in two volumes of buffer **B** (100 mM potassium phosphate, 25% glycerol (*v/v*), 0.1% EDTA (*m/v*), pH 7.4 at 4°C), with 0.4 mM PMSF (from 100 mM stock in isopropanol) that was added just before use. The ultracentrifuge step was repeated once after which the resuspended microsomes were stored at -80°C. PMSF, that was also used in the purification buffers, was always added just shortly before use.

Purification of CYP1A1

Step 1: Solubilisation After quick thawing of the microsomal fraction from ßNF-induced rats under running tap water, solubilisation of the microsomal proteins was performed at 4°C. A 10% (*m/v*) solution of Emulgen 911 in buffer **C** (100 mM potassium phosphate, 25% glycerol (*v/v*), EDTA (1 g/liter) at pH 7.4) was added dropwise to the microsomal solution (4 mg protein ml^{-1}) up to a concentration of 0.3%. After stirring slowly for 20 min, the solubilised microsomal protein was centrifuged for 80 min at 100,000 x g at 4°C in order to remove membranes and non-solubilised material. The supernatant containing solubilised protein was kept on ice whereas the pellet was discarded.

Step 2: Q-Sepharose A HiLOAD 26/10 Q-Sepharose High Performance column from Pharmacia/LKB (#17-1066-01, bed volume 53 ml) was equilibrated at room temperature against freshly made degassed buffer **D** (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.2% (*m/v*) Emulgen 911, 20% (*v/v*) glycerol, 0.4 mM PMSF (from 100 mM stock solution in isopropanol added just before use), pH 7.7). The solubilised protein (about 10 ml) from step 1 was filtrated (0.45 µm) and applied immediately to the Q-Sepharose column via an external pump at a flow rate of 2 ml/min.

Preconcentration of the solubilised protein sample with an Amicon ultraconcentrator is not advisable since it increased the viscosity of the sample leading to increased pressure at loading. By switchting to the internal pump, the resin was washed with two column bed volumes (100 ml) of degassed buffer **D**. After washing with two column volumes buffer **D** (100 ml), elution of proteins was started with a stepwise gradient (see Figures) upto 40% of buffer **E** (buffer **D** including 1M NaCl) and then two column bed volumes of 100% **E**. Elution of the proteins was detected at 254 nm (protein) and 405 nm (heme). Detection at 280 nm for protein was not possible due to absorption by Emulgen (Roos, 1990). Fractions of 10 ml were collected using a Pharmacia RediFrac fraction collector which was cooled at 0°C. After 2-3 runs, the HiLOAD column were regenerated by standard procedures according to instructions provided by the manufacturer.

Step 3: Hydroxyapatite Fractions from a sharp peak eluting from Q-Sepharose, containg most P450 according to the absorbance ratio A_{405}/A_{254} were subjected to hydroxyapatite chromatography (column 1.1 cm diameter containing 4 ml Bio-Gel HTP from Bio-Rad) at 4°C with a flow of 1.3 ml/min as follows: (a) equilibration with 20 ml buffer **F** containing potassium phosphate (30 mM), 0.2% (*m/v*) Emulgen 911 and 20% glycerol, pH 7.25, (b) loading of the semi pure P450 sample, (c) washing with 20 ml buffer **G** (like **F** except Emulgen), (d) elution by 60 ml buffer **H** (300 mM potassium phosphate with 20% glycerol, pH 7.25). The P450-containing fractions (A_{405}/A_{254}) were pooled and frozen in small aliquots at -80°C.

Purification of CYP2E1

Step 1: Solubilisation Solubilisation was identical except that Emulgen was added to a concentration of 0.4%.

Step 2: Q-Sepharose The solubilised sample was applied to a Q-Sepharose column in an identical manner as for CYP1A1 purification. Thereafter, CYP2E1 was eluted directly using two column bed volumes (100 ml) of degassed buffer D and collected at 0°C.

Step 3: S-Sepharose The flow-through fractions from Q-Sepharose that contained most P450 according to A_{405}/A_{254} (approx. 50 ml) were pooled and applied directly to a pre-equilibrated (buffer **D**; room temperature) cation-exchange column (HiLOAD 26/10 S-Sepharose HP, #17-1067-01). At room temperature, elution was performed with a stepwise gradient of degassed buffer **E** (buffer **D** including 1M NaCl) at a flow rate of 5 ml/min upto 40% of buffer **E** and then two column volumes of 100% **E** (see Figures). Fractions of 10 ml were collected on ice. Pooled fractions with high 405 nm absorbance were concentrated by ultrafiltration (Amicon PM30 filter, 400 ml device) in a cold room to about 10 ml. After 2-3 runs, the HiLOAD colums were regenerated according to manufacturer's instructions.

Step 4: Hydroxyapatite The pooled fractions from step 3 with high A_{405}/A_{254} were subjected to hydroxyapatite chromatography. The procedure was similar to the one used in the CYP1A1 purification although some important modifications were introduced: (a) identical, (b) loading of the pooled S-Sepharose sample, (c) identical, (d) gradient elution of in total 60 ml buffer (potassium phosphate concentration in

buffer **G** was increased to 300 mM). Subsequently, the P450-containing fractions (A_{405}/A_{254}) were pooled and frozen in small aliquots at -80°C.

Purification of P450-RED

Step 1: Solubilisation Solubilisation was identical to the method used for CYP2E1 purification.

Step 2: Q-Sepharose The solubilised protein sample was applied to a preequilibrated Q-Sepharose column (2 ml/min), followed by washing of the column (2 ml/min), both identical to the procedure for CYP2E1 purification. P450-RED was eluted at room temperature with a stepwise gradient of buffer **E** (buffer **D** including 1M NaCl) at a flow rate of 5 ml/min upto 40% of buffer **E** (see Figures), analogous to P450 purification. Fractions of 10 ml were collected on ice and those 6 fractions with high 280 nm absorbance eluting upon increase of the gradient to 30% of buffer **E** were concentrated by ultrafiltration (Amicon PM30 filter, 400 ml device) in a cold room to about 10 ml. P450-RED activity was determined by its ability to reduce cytochrome *c* using $\varepsilon = 21\ mM^{-1}cm^{-1}$ (Vermilion and Coon, 1978).

Step 3: 2',5'-ADP-Sepharose The concentrated Q-Sepharose eluate (Amicon) containing P450-RED was further purified using 2',5'-ADP-Sepharose affinity chromatography essentially as described for 2',5'-agarose and using 5 mM 2'-adenosinemonophosphate added to buffer in order to elute P450-RED (Guengerich and Martin, 1980).

Step 4: Dialysis The pooled P450-RED containing fractions were dialysed two times 16 hours against 30 mM potassium phosphate buffer including 20% glycerol, 0.1 mM DETAPAC, and 0.4 mM PMSF at pH 7.7 in order to remove 2'-adenosinemonophosphate.

Determination of protein and P450

Protein concentrations were estimated using the Pierce bincinchonic acid procedure, according to the manufacturer's directions with bovine serum albumine as the standard (Pierce Chemical Co., Rockford, IL, USA). The cytochrome P450 content was estimated spectrally from Fe^{2+}-CO versus Fe^{2+} difference spectra ($\varepsilon = 91\ mM^{-1}\ cm^{-1}$) essentially as described and modified (Omura and Sato, 1964; Schoene *et al.*, 1972).

Enzyme assays

Microsomal incubations consisted of substrate in 100 mM potassium phosphate buffer (pH 7.8 for CYP1A1 and pH 6.8 for CYP2E1 activity determinations) and 1 mg/ml induced microsomal protein or a reconstituted system. After 2 min preincubation at 37°C, reactions were started by addition of 1 mM NADPH (final conc.) or a regenerating system consisting of 1 mM $NADP^+$ and 5 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase (1 U/ml).

Reconstitution with purified enzymes was performed as described by Rietjens *et al.* (Rietjens *et al.*, 1989). Briefly, 10 pmol CYP1A1/CYP2E1, 10 pmol P450-RED

(about 0.027 units) and 2 µg L-α-phosphatidylcholinedilauroyl were preincubated for 5 min before addition to the substrate containing incubation mixture.

Ethoxyresorufin *O*-dealkylase activity (EROD; CYP1A1) was assayed as described previously (Burke and Mayer, 1975) and presented with Table I.

p-Nitrophenol hydroxylation was assayed as described by Koop (Koop, 1986). Briefly, reactions were stopped with perchloric acid on ice. After centrifugation, sodium hydroxide was added and absorption was measured at 511 nm to determine the concentration of *p*-nitrocatechol by comparison with a calibration line.

Table I: Enzyme assays#

Enzyme system	Ethoxyresorufin *O*-dealkykase (pmol min^{-1} $nmol^{-1}$ P450)	*p*-Nitrophenol hydroxylase (nmol min^{-1} $nmol^{-1}$ P450)
ßNF-microsomes	1.7	
Reconstituted CYP1A1	0.41	
Pyrazole-microsomes		3.0
Reconstituted CYP2E1		0.37

Reaction mixtures contained 100 mM potassium phosphate buffer at pH 6.8 with 100 µM *p*-nitrophenol (*p*-nitrophenol hydroxylase assay) or pH 7.8 with 0.25 µM ethoxyresorufin (ethoxyresorufin *O*-dealkykase assay), induced microsomes (1 mg/ml), or a reconstituted system (Rietjens, 1989)consisting of CYP2E1 or CYP1A1 (10 pmol), P450-RED (10 pmol), and LPCD (2 µg). Reactions were initiated with the addition of 1 mM NADPH (or a regenerating system). Product formations were measured as described (Burke, 1975; Koop, 1986)

Electrophoresis

Discontinuous (3% and 10%, respectively) polyacrylamide gel electrophoresis was carried out in the presence of 0.1% SDS (Laemmli, 1970). The proteins were reduced with about 2.5% 2-mercaptoethanol and separated by electrophoresis (0.3 µg - 5 µg protein per lane) at 50 mA/100 V and 100 mA/200 V for electrophoresis in the stacking and separating gel, respectively. SDS-PAGE was always done in duplicate: one gel for silver staining, the other gel (with prestained markers, Bio-Rad) for Western blotting. Silver staining was performed with 12.5 mM $AgNO_3$ as described (Merril *et al.*, 1981). After staining gels were dried in a Rapidry-mini AE-3711 (Atto).

Western blotting

Immunoblotting (300 mA, 96 Volt) was performed as described by Towbin *et al.* (Towbin *et al.*, 1979). For CYP1A1 and CYP2E1 blotting, monoclonal antibodies mouse anti-rat CYP1A1 (K06) and rabbit anti-rat CYP2E1, respectively, were used. Alkaline phosphatase labeled anti-mouse IgG and anti-rabbit IgG (Promega) respectively were used for staining.

RESULTS

Purification of CYP1A1

Microsomal fractions from ßNF-induced rats were used to start the purification procedure for cytochrome P450 1A1 (CYP1A1). A final concentration of 0.3% (*w/v*) of Emulgen 911 was found to be optimal for solubilisation of cytochromes P450 out of

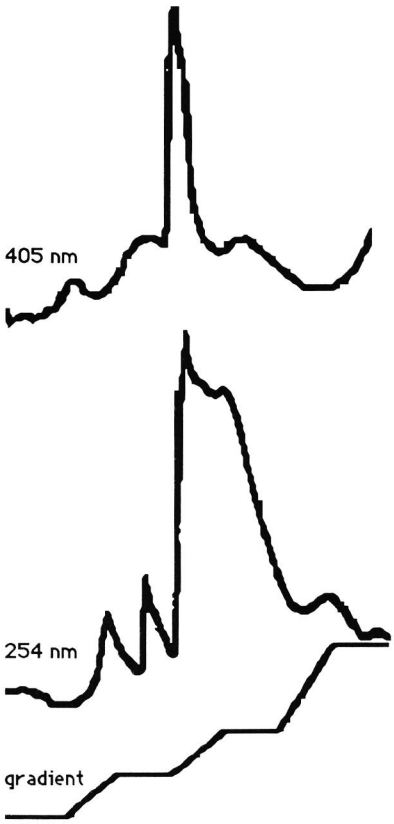

Fig. 1: Q-Sepharose (HP) chromatography of solubilised ßNF-induced rat liver microsomes. Detection at 254 nm and 405 nm, stepwise salt gradient is indicated (0-40% buffer E)

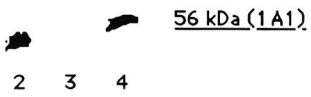

Fig. 2: Westen blot of SDS-PAGE analysis of isolation of CYP1A1

ßNF-microsomes. At lower concentrations, the solubilisation was not complete. At higher concentrations of Emulgen 911, more cytochrome P450 denaturated to cytochrome P420 (data not shown). Subsequently and as expected, CYP1A1 was found

to bind to the anion-exchanger Q-Sepharose. In Fig. 1 it is shown that after loading of solubilised ßNF-induced microsomes onto the Q-Sepharose column, washing with equilibration buffer and elution with an NaCl gradient, a sharp P450 (heme-containing) peak eluted between 0.15 and 0.2 M NaCl, as judged by absorption at 405 nm.

Although the peak eluting from Q-Sepharose still contained some proteins other than CYP1A1, as judged from SDS-PAGE (data not shown), fractions with the highest A_{405}/A_{254} ratio were pooled and used for hydroxyapatite chromatography in order to remove Emulgen 911. A stepwise elution was used with succes in attempting to remove these contaminating proteins. At 300 mM potassium phosphate, a sharp peak eluted from the hydroxyapatite column. This appeared to be one single protein of about 56 kD as judged by SDS-PAGE (data not shown). A Western blot demonstrated the protein eluting at 300 mM potassium phosphate to be CYP1A1 (Fig. 2).

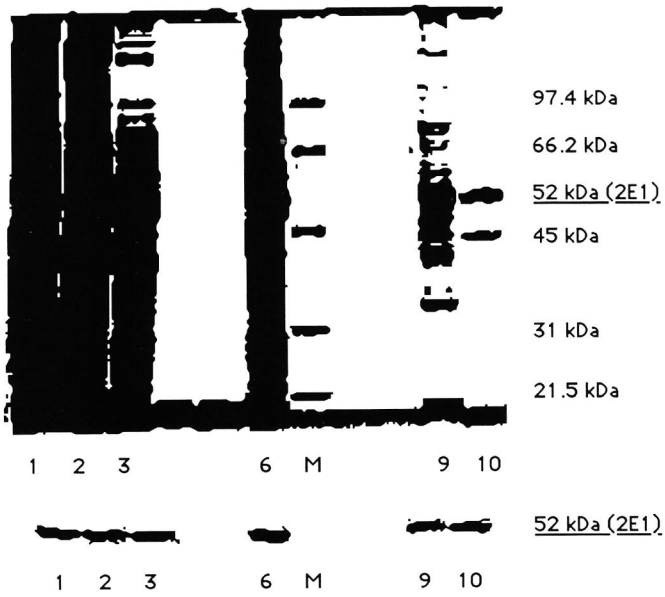

Fig. 3: Top SDS-PAGE analysis (10% gel) of the purification procedure for CYP2E1 from pyrazole-induced rat liver microsomes. Molecular weights of markers are given on the right side. Molecular weight of purified CYP2E1 was determined to be about 52 kD.
Bottom Western blot of this SDS-PAGE. Lanes: [1] pyrazole-induced microsomes; [2] solubilised microsomes; [3] pass-through Q-Sepharose; [6] pass-through S-Sepharose; [M] Marker lane; [9] CYP2E1 containing peak of S-Sepharose (2 µg of protein); [10] CYP2E1 containing peak of hydroxyapatite (0.4 µg of protein)

Ethoxyresoryfine *O*-dealkylase activities of ßNF-induced microsomes and a reconstituted system containing purified CYP1A1 are shown in Table I. The ßNF-microsomes and the reconstituted CYP1A1 exhibited an ethoxyresorufin *O*-dealkylase

activity of 1.7 and 0.41 pmol resorufine formed min^{-1} $nmol^{-1}$ P450, respectively. In Table II, the purification parameters of this pilot purification with a limited amount of microsomal protein are shown. The Soret peak of the CO-dithionite difference spectrum was found at 448.3 nm.

Purification of CYP2E1

The purification of cytochrome P450 2E1 (CYP2E1) started with the solubilisation of the pyrazole-induced P450's. In contrast to ßNF-induction, for solubilisation of the pyrazole-induced P450's, an Emulgen 911 concentration of 0.4% (*w/v*) was found to be the optimal (data not shown). Upon loading of the solubilised proteins onto the Q-Sepharose column, no retention of CYP2E1 was observed as can be seen from the Western blot of the SDS-PAGE. CYP2E1 was clearly present in lane 3, the eluate of Q-Sepharose (Fig. 3).

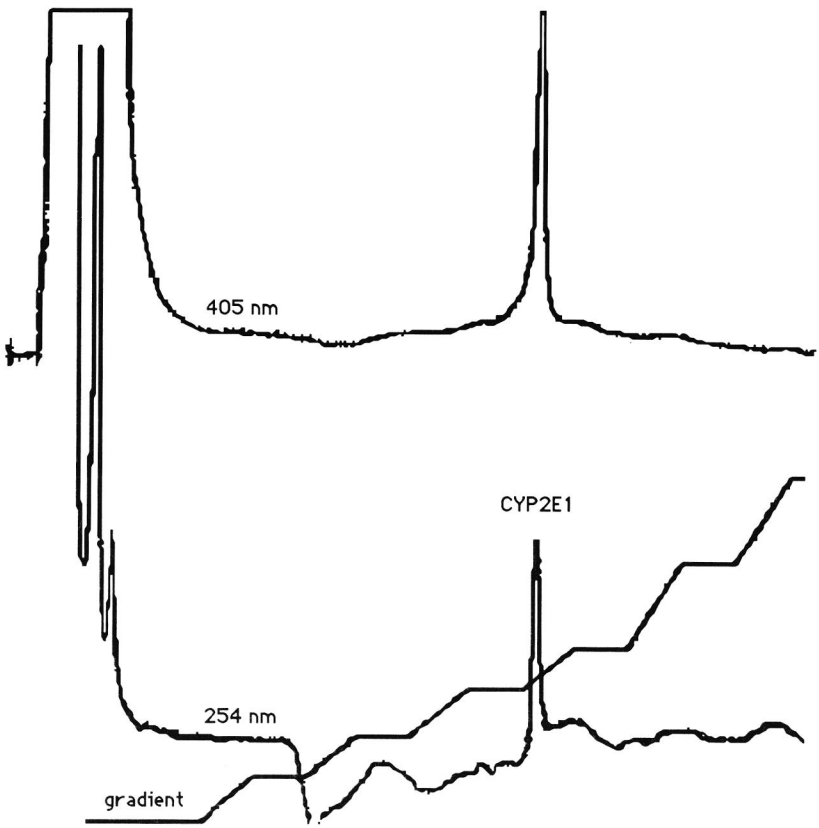

Fig. 4: S-Sepharose chromatography of pass-through from Q-Sepharose to which solubilised pyrazole induced rat liver microsomes were applied: Detection at 254 nm and 405 nm; stepwise gradient elution is indicated (0-40% of buffer E)

Subsequently and as expected, this pass-through eluate of Q-Sepharose was retained upon loading onto S-Sepharose as is obvious from a thorough evaluation of Fig. 3. Salt gradient elution of the cation exchanger resulted in a sharp haem-containing peak after about 13 min, corresponding to 0.16 M NaCl (Fig. 4). This peak appeared to be CYP2E1 (the line in the chromatogram indicates the percentage of high salt buffer on top of the column). From the Western blot (Fig. 3), it might seem that CYP2E1 is not retained as again, the pass-through eluate, but now of S-Sepharose (lane 6), appears to contain CYP2E1. However, thorough evaluation presents the immunoreactive spot at a slightly higher molecular weight (lower in the blot) than observed for the other lanes, indicating cross-reactivity with anti-CYP2E1, possibly from denaturated CYP2E1.

As obvious from lane 9 (Fig. 3), the CYP2E1 containing peak from S-Sepharose still contained many impurities. Therefore, the hydroxyapatite chromatography, which is mostly used only for removal of detergent, was investigated for further purification of the CYP2E1-containing pool from S-Sepharose. After loading, washing, and application of gradient elution, hydroxyapatite chromatography finally resulted in a protein fraction with only one contaminating protein as is shown in Fig. 3. Western blot analysis (Fig. 3) showed that only one of these represents CYP2E1.

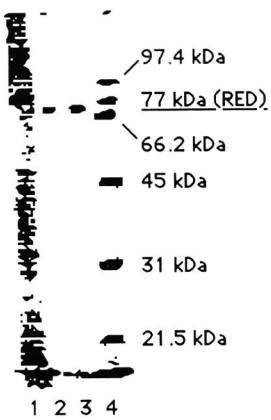

Fig. 5: SDS-PAGE (10%) analysis of purification of P450-RED. Lanes: [1] cytochrome c reduction activity containing peak from Q-Sepharose; [2] fraction from ADP-chromatography; [3] same fraction after dialysis.

Purification factors and other parameters are presented in the purification table (Table III). The Soret peak of the CO-dithionite difference spectrum was found at 450 nm. Some P420 was present. Activity measurements of CYP2E1 are presented in Table I. The pyrazole microsomes and the reconstituted CYP2E1 exhibited a *p*-nitrophenol hydroxylase activity of 3.0 and 0.37 nmol min^{-1} $nmol^{-1}$ P450, respectively.

Purification of P450-RED

The results of purification of NADPH-dependent cytochrome P450 reductase (P450-RED) are presented in Table IV. It was found that P450-RED binds firmly to Q-Sepharose and could be eluted as a broad peak with a salt gradient around 0.5 M NaCl (data not shown). Although in comparison with solubilised microsomes, the specific content was increased almost eight-fold by Q-Sepharose chromatography, the pooled fractions containing cytochrome *c* reductase activity still contained many proteins as observed by SDS-PAGE and presented in Fig. 5 (lane 1). After affinity chromatography (2',5'-ADP-Sepharose), a 77 kD protein was apparently homogeneous as can be seen from the lanes 2 and 3 (Fig. 5). The cytochrome *c* reductase activity of this purified P450-RED was found to be 3.1 μmol min^{-1} mg^{-1} protein (Table IV).

DISCUSSION

Preliminary but promising investigations are described to develop a rapid method for the purification of CYP1A1 as well as CYP2E1 from liver microsomal fractions from rats, pretreated with ßNF and pyrazole, respectively. Isolated CYP1A1 and CYP2E1 from rat and other species are used widely for investigation of the bioactivation of paracetamol and other chemical substances (Harvison *et al.*, 1988; Raucy *et al.*, 1989; Guengerich *et al.*, 1991; Lee *et al.*, 1991; Chen *et al.*, 1998). The major improvement was the use of ion-exchange resins with better performance characteristics with respect to speed and capacity of loading, compared to media for classical chromatography. The use of stepwise in stead of a linear gradients resulted in very sharp elution of the individual P450 enzymes from the HiLOAD S-Sepharose High Performance column.

At start of the investigations described here, the widely used Lubrol PX (Sigma) was utilised as detergent for solubilisation of the P450's from the microsomes (Guengerich and Martin, 1980). However, as the study continued, Lubrol PX had to be replaced because it was not commercially available anymore in the purity needed with respect to contaminating peroxides. Emulgen 911 was used previously for the purification of cytochrome P450 using preparative DEAE-5PW chromatography (Imaoka and Funae, 1987) or analytical Mono Q and Mono S chromatography (Kastner and Schulz, 1987) and turned out to be a good substitute. As protein monitoring at 280 nm was difficult because of absorption of the phenyl group in Emulgen, it was changed to 254 nm where absorption of Emulgen 911 is low (local absorption minimum at 244 nm) (Imaoka *et al.*, 1987). Solubilisation of the microsomal protein was found to be optimal at 0.3% and 0.4% Emulgen 911 for microsomes from ßNF- and pyrazole-induced rat liver, respectively.

Favourable results were obtained in the pilot experiment for the purification of **CYP1A1** using Q-Sepharose anion exchange chromatography only. It is known that ßNF-, but also phenobarbital-inducible P450's (CYP1A1, CYP1A2, respectively CYP2B1) bind to anion-exchange resins (Guengerich and Martin, 1980).

Furthermore, ßNF-inducible P450 isoforms were shown to be resolved by Mono Q (Kastner and Schulz, 1987), having the same functional group as the HiLOAD Q-Sepharose HP as used in the present study. Also it was shown that a stepwise gradient resulted in better separation of different P450 enzymes (Roos, 1990). Therefore, analogously to Ryan *et al.* (Ryan *et al.*, 1982), for further purification, stepwise elution on hydroxyapatite was performed which resulted in one single protein band of about 56 kD on SDS-PAGE (Fig. 3). However, the specific content was rather low (3.9 nmol mg^{-1} protein) compared to what can be reached theoretically (about 13-17 nmol mg^{-1} protein) (Guengerich and Martin, 1980).

Table II: Purification of CYP1A1 from ßNF-induced rat liver

Step	Treatment	Total protein (mg)	Total P450 (nmol)	Yield (%)	Spec. content (nmol mg^{-1} prot.)	Purif. factor
1.	0.3 % Emulgen	40	41	100	1.0	1
2.	Q-Sepharose	0.34	0.77	1.9	2.3	2.3
3.	Hydroxyapatite	0.08	0.31	0.76	3.9	3.9

Except by immunoblotting, the presence of CYP1A1 in the isolated fractions was indicated by the finding that in the reconstituted system, enzyme activity was measured with ethoxyresorufin as substrate, which could be inhibited by 80% when the incubation mixture was bubbled with carbonmonoxide. Also 50 µM α-NF, a potent inhibitor of, amongst others, the P450's induced by ßNF (CYP1A1 and CYP1A2) (Chang *et al.*, 1994), completely blocked the ethoxyresorufin *O*-dealkylase activity.

Briefly, the use of HiLOAD Q-Sepharose HP chromatography in an FPLC system seems to offer new possibilities and could be beneficial in the purification of CYP1A1 and other P450's from various species as long as they have anion characteristics. For this purpose, the octylamino Sepharose chromatography is mostly used and followed by single gradient chromatography using a DEAE-coupled resin and finished with hydroxyapatite chromatography for removal of detergent, all with open columns (Narimatsu *et al.*, 1990; Shimada *et al.*, 1992). However, an HPLC-system was used in an analytical setting, e.g. for making P450 'handprints' to be used for preliminary screening of e.g. induction (Tarr and Crabb, 1983; Iversen and Franklin, 1985). Preparative purification in an HPLC-system was also used already although still only op to quantities of 200-300 nmol at once (Shimeno *et al.*, 1991). It should be mentionend that in addition, immunoaffinity chromatography, using a highly specific antibody raised against a certain P450 isoform, offers interesting possibilities (Maurice *et al.*, 1991). It remains to be established how our preparative FPLC method compares to immunoaffinity chromatography in various aspects. Also, hydroxyapatite chromatography could be improved if necessary as hydroxyapatite can be used also in an HPLC-system (Kawasaki *et al.*, 1985).

Preliminary but encouraging investigations are also described to develop a rapid method for the purification of **CYP2E1** (after pyrazole-induction) on a preparative scale almost to homogeneity (as demonstrated by SDS-PAGE), based on High Performance Q- and S-Sepharose ion-exchange FPLC. Only one contaminating non-CYP2E1 protein remained. The major improvement was the use of ion-exchange resins with better performance characteristics with respect to speed and capacity of loading, compared to media for classical chromatography. The use of stepwise in stead of a linear gradient resulted in very sharp elution of CYP2E1 from the HiLOAD S-Sepharose High Performance column.

Considering the adherence to either anion- or cation-exchange resins in the purification procedure of CYP2E1, no retention on anion-exchanger Q-Sepharose was observed. Ryan *et al.* already mentioned that rat liver microsomal CYP2E1 was not retained at anion-exchange resins under various conditions (Ryan *et al.*, 1985). In most cases, including the isolation of liver microsomal CYP2E1 of other species, pH 7.7 is used for anion-exchange resins like Q-Sepharose (Ryan *et al.*, 1982; Wrighton *et al.*, 1987; Larson *et al.*, 1991b), whereas a pH of about 6.6 is used for cation-exchange chromatography (e.g. S-Sepharose) (Ryan *et al.*, 1985; Larson *et al.*, 1991b; Larson *et al.*, 1991a). Hamster liver CYP2E1 for example adheres to Q-Sepharose at pH 7.7 (Puccini *et al.*, 1992). For purification of an ethanol-induced rabbit liver P450, CM-cellulose was used at pH 6.0 (Koop *et al.*, 1982). Therefore it may be concluded that CYP2E1 has a pI somewhere around 7.5 and that it is a mainly a cation at a pH below 6.5. The present study confirms that CYP2E1 is a cationic protein at pH 7.7 which does not bind to anion-exchange media. The pass-through fraction of Q-Sepharose therefore was applied to S-Sepharose.

Table III: Purification of CYP2E1 from pyrazole-induced rat liver

Step	Treatment	Total protein (mg)	Total P450 (nmol)	Yield (%)	Spec. content (nmol mg^{-1} prot.)	Purif. factor
1.	0.4 % Emulgen	550	310	100	0.56	1
2.	Q-Sepharose*	250	120	39	0.48	0.86
3.	S-Sepharose	4.7	5.8	1.9	1.2	2.1
4.	Hydroxyapatite	0.98	3.5	1.1	3.6	6.4

As a major goal of the preliminary investigations described in this chapter was to find a rapid purification method for CYP2E1, a buffer change to a pH of about 6.5 of the protein sample as well as equilibration of the S-Sepharose column to pH 6.5 was omitted. Although the CYP2E1 was not expected to bind strongly to the cation-exchange S-Sepharose at pH 7.7, significant binding was observed. A pale red band representing P450(s) appeared upon application of the pooled pass-through fractions from Q-Sepharose. It has to be noted, however, that this pale red band moved downward slowly

even during the washing procedure, although it was still on the column when gradient elution was started. As a result of this gradient elution, for this cation-exchange chromatography step at pH 7.7, a purification of a factor 2.1 was achieved (Table III: specific contents ratio 1.2/0.48).

In this study hydroxyapatite chromatography was found to be suitable for further purification next to the removal of detergent, for which purpose it was used for originally (Ryan *et al.*, 1985; Wrighton *et al.*, 1987; Larson *et al.*, 1991b; Puccini *et al.*, 1992). One prerequisite however, was the use of a continuous gradient elution after the detergent had been removed with low salt buffer. This resulted in an almost homogeneous protein fraction exhibiting only one contaminating protein of about 45 kD next to a 52 kD protein on SDS-PAGE, the latter one which is in agreement with published data for the size of CYP2E1 (Fig. 3) (Larson *et al.*, 1991b).

One of the special aspects that makes CYP2E1 a rather special form of P450 is the stability of CYP2E1 which is very dependent on the presence of (pseudo)substrates like imidazole (Eliasson *et al.*, 1992). In the presence of (pseudo)substrates, degradation of the substrate-bound CYP2E1 follows only the slow lysosomal degradation process since substrate availability prevents phosphorylation of this enzyme which keeps it less liable to degradation by protein kinases. The stability of CYP2E1 appeared to be increased four times in the presence of 50 μM 4-methylpyrazole in all purification buffers (Larson *et al.*, 1991a). The likely positive effects of addition of a (pseudo)substrate like 4-methylpyrazole in the purification method described here need further investigation.

Some discrepancy seems to exist between the final purity, as expressed in nmol P450 mg^{-1} protein (Table III), and the electrophoretic homogeneity. Theoretically, for homogeneous rat or hamster liver CYP2E1, with a MW of 51.5-55 kDa, the specific content amounts to 18-19 nmol mg^{-1} protein, although only contents of 9.2-13 nmol mg^{-1} protein have been published previously (Ryan *et al.*, 1985; Puccini *et al.*, 1992). In the investigations described in the underlying report, a specific content of only 3.5 nmol mg^{-1} protein was found (Table III). This would mean that the specific content could still be increased by a factor 3 to 4, whereas the SDS-PAGE indicates the impurity at 45 kD (Fig. 3) to be less than 50%. However, quantification of specific protein levels using SDS-PAGE is unreliable as staining intensity per mg protein can be different for various proteins. The yield of the complete purification, related to the P450 content of the solubilised microsomes (1.1%), was in the same range as found by others (1.1% - 2%) (Ryan *et al.*, 1985; Puccini *et al.*, 1992)).

In recent years, molecular biological techniques introduced the possibility of expressing (slightly modified) cDNA of a specific cytochrome P450 of a mammalian species, including man, in high level expression systems such as yeasts, bacteria and cell lines (Mapoles *et al.*, 1993). Slight modifications are introduced into the cDNA such as addition of a histidine-tag, a codon change or a deletion of several codons in order to facilitate introduction into DNA of the host or to increase the expression. For example, cDNA of a human liver CYP2E1 (minus the first 21 codons of the native sequence and a codon change (mutation) resulting in a tryptophane to alanine transition) has been prepared with reverse transcriptase and polymerase. Via an

expression vector, this cDNA was introduced into *Escherichia coli* DNA, followed by large scale production of the modified human CYP2E1. Subsequently, this modified human CYP2E1 was purified to apparent homogeneity using 'classical' solubilisation, ion-exchange chromatography(anion- and cation-) and hydroxyapatite chromatography (Gillam *et al.*, 1994). These techniques provide an enormous increase of the ratio of the specific P450 of interest to the total amount of P450 present, thereby facilitating purification procedures and providing large amounts of 'human' P450's (Chen *et al.*, 1998). Using such expression systems, even single-step purification was described using only S-Sepharose cation-exchange chromatograpy using a pH gradient for elution (Larson *et al.*, 1991a; Larson *et al.*, 1991b). However, the isolation of specific P450s from natural matrices such as liver and kidneys will remain necessary in order to investigate the retainment of the natural characteristics of the modified P450 with respect to spectral characteristics, enzymatic activity, substrate specificity and so on.

Table IV: Purification of P450-RED from pyrazole-induced rat liver

Step	Treatment	Total protein (mg)	Total activity (μmol min^{-1})	Yield (%)	Spec. content (μmol min^{-1} mg^{-1} prot.)	Purif. factor
1.	0.4 % Emulgen	550	62	100	0.11	1
2.	Q-Sepharose	15	13	21	0.87	7.9
3.	ADP-Sepharose	3.0	4.1	6.6	1.4	13
4.	Dialysis	0.94	2.9	4.7	3.1	28

Shortly, a promising alternative is presented for the laborious and time-consuming conventional isolation procedure of CYP2E1 which needs up to six purification steps after solubilisation and which usually lasts much more than a working week. The purification procedure, as described in this report, lasts only about one or two working days from solubilisation of the microsomes on. Furthermore, it is to be expected that working with an automated system will increase the reproducibility of the purification compared to 'classical' systems. In comparison with the time-consuming conventional chromatography methods as known in literature, containing hydrophobic interaction chromatography (octylamino Sepharose), anion-exchange chromatography, cation-exchange chromatography, phosphocellulose chromatography and hydroxyapatite chromatography (Ryan *et al.*, 1985), at least the hydrophobic interaction chromatography and the phosphocellulose chromatography are omitted. Of course, some further investigations are needed with respect to pH and the presence of (pseudo)substrates in order to further increase the specific content and yield (Wu *et al.*, 1990; Larson *et al.*, 1991a). However, the presence of the contaminating 45 kD protein does not necessarily pose a problem for specific P450 activity determinations. In the case that it is not a P450 (which is very likely as it is outside the molecular

weight range of the P450's), it might not be necessary to remove it, depending on the sort of experiments are planned with the purified CYP2E1. Lastly, addition of Zn^{2+} (Zhukov *et al.*, 1993), inhibition of serine proteases (Eliasson *et al.*, 1992), should be considered as possible modifications of the purification procedure with potentially positive effects.

P450-RED purification was easily started together with purification of CYP2E1 on Q-Sepharose and finalised by performing 2',5'-ADP-Sepharose chromatography only. However, the cytochrome *c* reductase activity and yield (3.1 μmol min^{-1} mg^{-1} protein and 4.7%, respectively) were rather low compared to values published previously (65 μmol min^{-1} mg^{-1} and 63%, respectively) (Guengerich and Martin, 1980). Although investigations are needed in orther to rationalise these differences it is concluded that even without specific induction of P450-RED, which is optimal with phenobarbital, some apparently pure P450-RED can be isolated concomitantly with CYP2E1 after induction with pyrazole.

Summarising, rapid methods, based on Sepharose ion-exchange in addition with hydroxyapatite chromatography, were developed to isolate cytochromes P450 from rat liver microsomal fractions. HiLOAD ion-exchange media for FPLC seem to be well suited for preparative scale purification of microsomal CYP1A1 and CYP2E1. Quantities of upto 1000 nmol of cytochrome P450 can be loaded at once which is important especially for rat liver microsomal CYP2E1 because recovery in general is very low compared to isolation of other P450 enzymes (Ryan *et al.*, 1985). In a pilot experiment, liver CYP1A1 was purified to electrophoretic homogeneity from βNF-induced rats, by using mainly Q-Sepharose HiLOAD chromatography. CYP2E1 was purified from liver microsomal fractions of pyrazole-treated rats, almost to electrophoretic homogeneity, by using Q-Sepharose and S-Sepharose chromatography and hydroxylapatite chromatography with gradient elution. How far the presented method is applicable to microsomal P450's from different organs and species that were isolated recently in multi-step procedures (Ohgiya *et al.*, 1996; Longo *et al.*, 1997; Shimuzu *et al.*, 1997), remains to be established.

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SECTION III MECHANISTIC INVESTIGATIONS - CYTOCHROME P450-DEPENDENT OXIDATIVE BIOTRANSFORMATION

Chapter 5 Hydrogen atom abstraction of 3,5-disubstituted analogues of paracetamol by horseradish peroxidase and cytochrome P450

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Hydrogen atom abstraction of 3,5-disubstituted analogues of paracetamol by horseradish peroxidase and cytochrome P450

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1. The formation of free radicals during enzyme catalysed oxidation of eight 3,5-disubstituted analogues of paracetamol (PAR) has been studied. A simple peroxidase system as well as cytochrome P450-containing systems were used. Radicals were detected by electron spin resonance (ESR) on incubation of PAR and 3,5-diCH_3-, 3,5-diC_2H_5-, 3,5-di*t*C_4H_9-, 3,5-diOCH_3-, 3,5-diSCH_3-, 3,5-diF-, 3,5-diCl- and 3,5-diBr-substituted analogues of PAR with horseradish peroxidase in the presence of hydrogen peroxide (H_2O_2). Initial analysis of the observed ESR spectra revealed all radical species to be phenoxy radicals, based on the absence of dominant nitrogen hyperfine splittings. No radicals were detected in rat liver cytochrome P450-containing microsomal or reconstituted systems.

2. To rationalize the observed ESR spectra, hydrogen atom abstraction of PAR and four of the 3,5-disubstituted analogues (3,5-diCH_3-, 3,5-diOCH_3-, 3,5-diF- and 3,5-diCl-PAR) was calculated using *ab initio* calculations, and a singlet oxygen atom was used as the oxidizing species. The calculations indicated that for all compounds studied an initial hydrogen atom abstraction from the phenolic hydroxyl group is favoured by approximately 125 kJ/mol over an initial hydrogen atom abstraction from the acetylamino nitrogen atom, and that after hydrogen abstraction from the phenolic hydroxyl group, the unpaired electron remains predominantly localised at the phenoxy oxygen atom ($\pm 85\,\%$).

3. The experimental finding of phenoxy radicals in horseradish peroxidase/H_2O_2 incubations paralleled these theoretical findings. The failure to detect experimentally phenoxy radicals in cytochrome P450-catalysed oxidation of any of the eight 3,5-disubstituted PAR analogues is more likely due to the reducing effects that agents like NADPH and protein thiol groups have on phenoxy radicals rather than on the physical instability of the respective substrate radicals.

Introduction

Paracetamol (4′-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol, PAR) is a widely used analgesic and antipyretic drug. Besides detoxification by glucuronidation and sulphation, PAR is bioactivated by the microsomal cytochrome P450-containing mixed-function oxidase system (P450) (Potter *et al.* 1973, Jollow *et al.* 1974, Prescott 1980, Hinson *et al.* 1981). In addition, enzymes with peroxidase activity, like prostaglandin synthase, can catalyse the metabolism of PAR to a reactive metabolite (Moldéus *et al.* 1982). Evidence has been presented that *N*-acetyl-*p*-benzoquinone imine (NAPQI) is the reactive electrophilic intermediate responsible for the observed toxicity (Albano *et al.* 1985, Van de Straat *et al.* 1988), although *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) has also been proposed (De Vries 1981). It

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has been shown that an acute overdose of PAR and its long-term use can lead to acute renal failure and an increased risk of chronic renal disease respectively (Cobden *et al.* 1982, Björck *et al.* 1988, Sandler *et al.* 1989). The kidney is typically rich in prostaglandin synthase.

It is known that oxidation of PAR by peroxidase-like enzymes *in vitro* occurs via a hydrogen atom abstraction to form a *N*-acetyl-*p*-benzo-semiquinone imine radical (NAPSQI). Depending on the relative concentrations of the enzyme and reactants, oxidation by peroxidases proceeds with a second hydrogen atom abstraction (Fischer and Mason 1984, Kobayashi *et al.* 1990, Sayo and Saito 1990). In case of PAR, this subsequent reaction is the oxidation of NAPSQI to NAPQI. Whether the mechanism of oxidation of PAR to NAPQI by cytochrome P450 involves a direct two-electron oxidation by abstraction of two hydrogen atoms or two one-electron oxidations by two subsequent abstractions of one hydrogen atom remains unclear. A radical mechanism has previously been suggested by several authors because a persistent melanin polymer signal was detected in a hamster hepatic microsomal incubation system under oxidative (aerobic) conditions (Rosen *et al.* 1983). Under reductive (anaerobic) conditions in rat liver microsomal and reconstituted P450-catalysed incubations of NAPQI, a persistent, single-line ESR (electron spin resonance) spectrum was also detected (Van de Straat *et al.* 1987). However, ESR detection of an elusive short-lived PAR phenoxy radical has never been reported in aerobic microsomal systems, despite the fact that observation of such a transient species would be the ultimate evidence for a free-radical mechanism for the bioactivation of PAR (West *et al.* 1984). In a peroxidase-catalysed reaction, however, a phenoxy radical (NAPSQI) has been detected using fast-flow ESR analysis (West *et al.* 1984). Moreover, 3,5-dimethylated PAR has been shown to be liable to peroxidase-mediated oxidation to a free and stable phenoxy radical (Fischer and Mason 1984, Fischer *et al.* 1985).

Using *ab initio* molecular orbital calculations, Koymans *et al.* (1989) provided a rationale for spin delocalization of NAPSQI. The observation of a phenoxy radical in contrast with a nitrogen-based radical in the case of PAR (West *et al.* 1984) is in agreement with the *ab initio* calculations, i.e. hydrogen atom abstraction from the phenolic hydroxyl group being 30 kcal/mol more favourable than hydrogen atom abstraction from the acetylamino nitrogen and 86% of the unpaired spin being retained at the phenoxy oxygen (Koymans *et al.* 1989).

Recently, 3,5-dialkylated and 3,5-dihalogenated PAR analogues have also been observed to be metabolized by cytochrome P450-dependent oxidative biotransformation in microsomal as well as hepatocyte systems (Van de Straat *et al.* 1986, Bessems *et al.* 1996, Bessems *et al.* 1997). In agreement with the cytochrome P450-dependent formation of NAPQI from PAR, 3,5-diCH_3-PAR, 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR have been reported to be oxidized to their respective NAPQI analogues by microsomal cytochrome P450 (Bessems *et al.* 1996). Few attempts have been reported to date to determine whether the mechanism of P450-catalysed oxidation of 3,5-disubstituted analogues of PAR involves detectable free radicals. 3,5-DiCH_3-PAR has been reported to be converted to a fairly stable phenoxy radical upon oxidation by horseradish peroxidase/H_2O_2, i.e. the 3,5-dimethylated *N*-acetyl-*p*-benzosemiquinone imine (3,5-diCH_3-NAPSQI), but not by P450 (Fischer and Mason 1984). However, 3,5-diCH_3-NAPSQI was detected by ESR analysis on microsomal and reconstituted P450-catalysed reduction of 3,5-diCH_3-NAPQI (Van de Straat *et al.* 1987).

In the current study, representatives of the series of 3,5-disubstituted PAR analogues, including three 3,5-dialkylated PAR analogues, 3,5-diOCH_3-PAR, 3,5-diSCH_3-PAR and three 3,5-dihalogenated PAR analogues have been investigated with respect to initial hydrogen atom abstraction and formation of free radicals with varying stabilities in the presence of horseradish peroxidase/H_2O_2 and microsomal and reconstituted P450. Horseradish peroxidase/H_2O_2 was used because it is an efficient system for the *in vitro* modelling of abstraction of one and two hydrogen atoms of various exogenous molecules such as *p*-aminophenol (Josephy *et al.* 1983), phenetidine (Ross *et al.* 1985), PAR (Nelson *et al.* 1981, West *et al.* 1984) and other phenolic species (Thompson *et al.* 1989, Valoti *et al.* 1989). In addition, the free radical formation from 3,5-disubstituted PAR analogues by horseradish peroxidase/H_2O_2 *in vitro* might provide preliminary indications for the existence of similar free radical formation *in vivo* in organs that contain enzymes of the peroxidase family, such as myeloperoxidase and prostaglandin synthase. Moreover, *ab initio* calculations were performed to investigate whether the formation of a free radical phenoxyl or anilinyl radical and a hydroxyl radical from a 3,5-disubstituted PAR analogue and a singlet oxygen (as model for cytochrome P450) would be energetically favourable in theory (*in vacuo*) and to rationalize the structure of the radical species as observed by ESR spectra.

Materials and methods

Materials

Glucose 6-phosphate and glucose 6-phosphate-dehydrogenase, L-α-phosphatidylcholinedilauroyl, $NADP^+$, and NADPH were purchased from Sigma Chemical Co. (St Louis, MO, USA). β-Naphthoflavon (βNF) was from Janssen Chimica (Beerse, Belgium). Paracetamol was from Brocacef (Delft, The Netherlands). 3,5-diCH_3-PAR and 3,5-diC_2H_5-PAR were synthesized as described by Dearden and O'Hara (1978). 3,5-di*t*C_4H_9-PAR were a generous gift from Dr J. C. Dearden. Other 3,5-disubstituted PAR analogues were synthesized as described by Bessems *et al.* (1995). All other chemicals were of analytical grade.

Animals and treatments

Male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 180–220 g, were housed in plastic cages in temperature- (22 °C) and humidity- (50 %) controlled rooms with a 12-h lighting cycle. Water and commercial laboratory chow (HOPE Farms, Woerden, The Netherlands) were available *ad libitum*. For CYP1A1 induction, five animals were injected i.p. with 40 mg/kg βNF, dissolved in arachides oil (8 mg/ml) for 2 consecutive days, and killed on the third day.

Microsomes

Unfasted rats were sacrificed on the third day by decapitation after light ether narcosis. Livers were quickly excised, weighed and homogenized at 4 °C with a Waring blender and Elvjehem Teflon–glass potter in 2 vols (1 vol. equals the total liver weight) buffer A (50 mM potassium phosphate, 0.9 % NaCl, pH 7.4, at 4 °C). The supernatant after centrifugation (20 min × 12000*g* at 4 °C) was ultracentrifuged (75 min × 100000*g* at 4 °C). The microsomal pellet was resuspended in 2 vols buffer B (100 mM potassium phosphate, 25 % glycerol (v/v), 0.1 % EDTA (m/v), pH 7.4 at 4 °C). The ultracentrifuge step was repeated once after which the resuspended microsomes were stored at −80 °C.

Liver microsomes from phenobarbital-induced rats and the purified P450 isoform CYP2B1 were gifts from Dr Goeptar in our department (Goeptar *et al.* 1993). Purification of cytochrome P450 isoforms CYP1A1 and CYP2E1 and NADPH cytochrome P450-dependent reductase are described elsewhere (Bessems *et al.*, in preparation). Reconstituted P450 isoforms CYP1A1 and CYP2E1 were active in ethoxyresorufin *O*-dealkylase (EROD) and *p*-nitrophenol hydroxylase assays respectively, as described previously (Burke and Mayer 1975, Koop 1986).

ESP spectroscopy

ESR spectra were recorded on a Bruker ESP300 ESR spectrometer equipped with a 4102 ST/8627 cavity at room temperature. After incubation, samples (about 0.8 ml) were transferred to a flat cell and mounted into the ESR cavity immediately. A typical instrumental condition was 100 kHz modulation

frequency, 20 mW microwave power, 9.8 GHz microwave frequency, 0.5 G modulation amplitude, 5×10^5 receiver gain and midfield set at 3480 G. Spectra were detected usually 5–10 min after starting the incubation (needed for positioning and fine tuning), using the Bruker ESP300/ESP 1600 data processor program. For detection of the free-radical product of PAR in horseradish peroxidase/H_2O_2 incubations, fast-flow ESR was used as previously described (Fischer *et al.* 1986). Shortly, PAR/H_2O_2 in phosphate buffer (50 mM, pH 7.4) was mixed with an equal volume of horseradish peroxidase in phosphate buffer, milliseconds prior to entering a flat cell at a total flow rate of about 100 ml/min.

Except for minor deviations, the conditions of horseradish peroxidase/H_2O_2 incubations were 1 μg/ml horseradish peroxidase, 2.5 mM substrate (from 100 mM stock solution in acetonitrile) in 50 mM potassium phosphate buffer pH 7.4 at room temperature. Reactions were started by adding H_2O_2 (2.5 mM final concentration).

Microsomal incubations consisted of 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.1 mg/ml microsomes and 2.5 mM substrate. After 2-min preincubation at 37 °C, reactions were started by the addition of 1 mM NADPH or a regenerating system consisting of 1 mM $NADP^+$ and 5 mM glucose 6-phosphate and glucose 6-phosphate dehydrogenase (1 U/ml) in a total incubation volume of 1 ml.

Reconstitution experiments with purified CYP1A1, CYP2B1 or CYP2E1 and NADPH-cytochrome P450 reductase were performed as described (Rietjens *et al.* 1989).

Ab initio *calculations*

To reduce computational efforts, a simplified model system was used for the peroxidase and cytochrome P450 catalytic cycles. Singlet oxygen was suggested as a possible reactive species involved in horseradish peroxidase-catalysed oxidation reactions (Kohda *et al.* 1990, Metodiewa *et al.* 1992) and was also used successfully in studies concerning P450 catalysis (Koymans *et al.* 1989, De Groot *et al.* 1995). The initial conformation of PAR was generated using the molecular modelling package ChemX (Chemical Design Ltd 1990). Following a preoptimization using molecular mechanics and the semiempirical package MOPAC 6.00 (Stewart and Seiler 1990), the quantum chemical program package GAMESS-UK (Dupuis *et al.* 1980, Guest *et al.* 1993) was used for the *ab initio* calculations. The geometry of PAR was *ab initio* optimized using RHF (Restricted Hartree Fock) whereas UHF (Unrestricted Hartree Fock) was used for the open shell (radical) species. A STO-3G (Slater Type Orbital comprised of 3 Gaussians) (Hehre *et al.* 1969, 1970) minimal basis set was used for these optimizations. The optimized geometry of PAR was used to construct the 3,5-disubstituted PAR analogues and their respective radicals in a similar way. On the resulting STO-3G geometry of each of the analogues and radicals, a single point energy calculation, a DMA (Distributed Multiple Analysis) calculation, and, in the case of radicals, a calculation of spin distributions (Stone 1981), was performed using the RHF method in a SV (Split Valence) 6–31 G (Binkley *et al.* 1980, Gordon *et al.* 1982, Frisch *et al.* 1984) basis set.

Simulation of ESR spectra

ESR spectra were simulated using the EPRcalc program running on the Bruker ESP300 spectrometer ($g = 2.0043$ was used). Hyperfine couplings were determined for the relevant atoms using the spin distribution of the *N*-acetyl-*p*-amino-phenoxy radical (NAPSQI). The atoms with expected hyperfine coupling are the hydrogen-, fluorine-, chlorine- (all spin quantum number $\frac{1}{2}$; causing a doublet), and nitrogen-atom (spin quantum number 1; causing a triplet). As the observed net spin on the different hydrogen atoms was zero, the values of the spin distribution in the PAR skeleton were used (this is the atomic spin population), based on the assumption of π/p-interaction of a carbon or nitrogen atom with the 1*s*-orbital of an adjacent hydrogen (Wertz and Bolton 1972). To calculate the hyperfine splitting constants due to fluorine or chlorine nuclei, *ab initio* calculated net spins on the fluorine and chlorine were used respectively.

Results

ESR analysis of horseradish peroxidase/H_2O_2 incubations

Paracetamol. The free radical formation of PAR in horseradish peroxidase/H_2O_2 incubations was measured first in a fast-flow ESR experiment (figure 1). The observed three-line pattern for *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) is characteristic of a *para*-substituted phenoxy-radical exhibiting a dominant large coupling with the two equivalent *ortho* hydrogen atoms in the aromatic nucleus ($a^{H}_{ortho} = 5.11$ G (2H), $a^{H}_{N} = 1.35$ G (1H), $a^{H}_{C(O)CH_3} = 1.01$ G (3H), $a^{N} = 0.81$ G (1N),

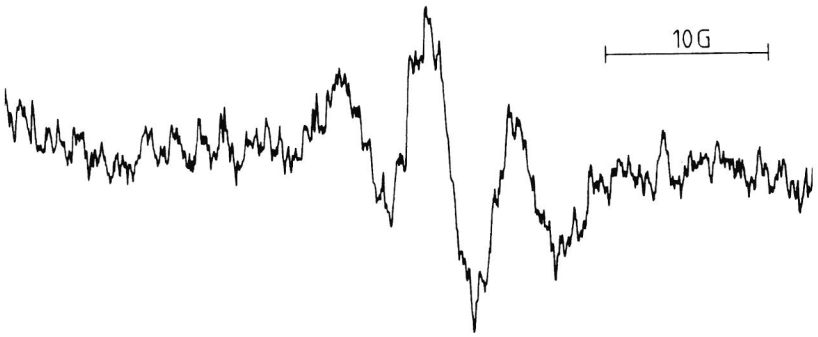

Figure 1. ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of PAR in a fast flow experiment. Receiver gain was 10^5, modulation amplitude 0.99 G, center field 3480 G, sweep width 50 G, conversion time 20.48 ms and time constant 81.92 ms.

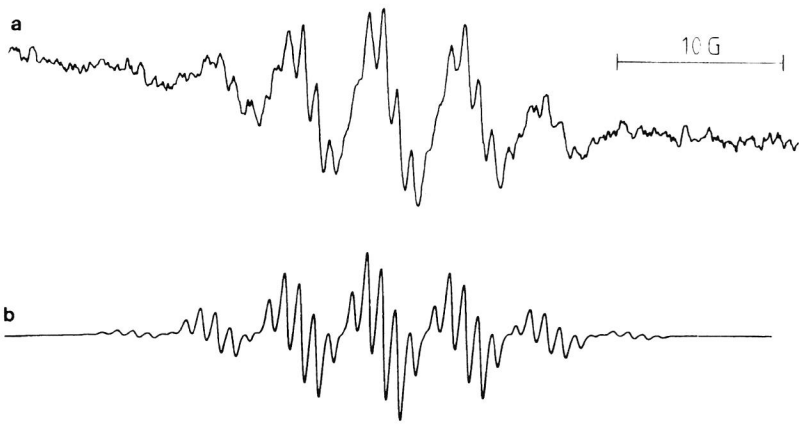

Figure 2. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diCH_3-PAR. Receiver gain was 2×10^4, modulation amplitude 1.00 G, centre field 3480 G, sweep width 50 G, conversion time 5.12 ms and time constant 1.28 ms. (b) Simulation of an ESR spectrum of the phenoxy radical of 3,5-diCH_3-PAR with hyperfine splitting constants as calculated in this paper (table 4).

$a^{H}_{meta} = 0.64$ G (2H)) and is similar to a previously reported ESR spectrum (Fischer *et al.* 1986).

3,5-Dialkylated PAR analogues. On incubation of 3,5-diCH_3-PAR in horseradish peroxidase/H_2O_2 incubations (figure 2a), a relatively strong ESR signal was observed for *N*-acetyl-3,5-dimethyl-*p*-benzosemiquinone imine (3,5-diCH_3-NAPSQI), characteristic of *para*-substituted phenoxy-radicals and exhibiting dominant coupling with the six equivalent hydrogen atoms of the *ortho* methyl groups ($a^{H}_{3',5'\text{-}CH_3} = 5.05$ G (6H)). Further coupling is observed with the *N*-acetyl and the aromatic hydrogen atoms at the *meta* positions of the atomatic nucleus ($a^{H}_{C(O)CH_3} = 1.0$ G (3H); $a^{H}_{2',6'} = a^{H}_{N} = 0.82$ G (3H)). Because a modulation amplitude of 0.5 G was used here, no coupling with the acetyl-nitrogen ($a^{N} = 0.404$ G (1N)) was observed (Fischer and Mason 1984).

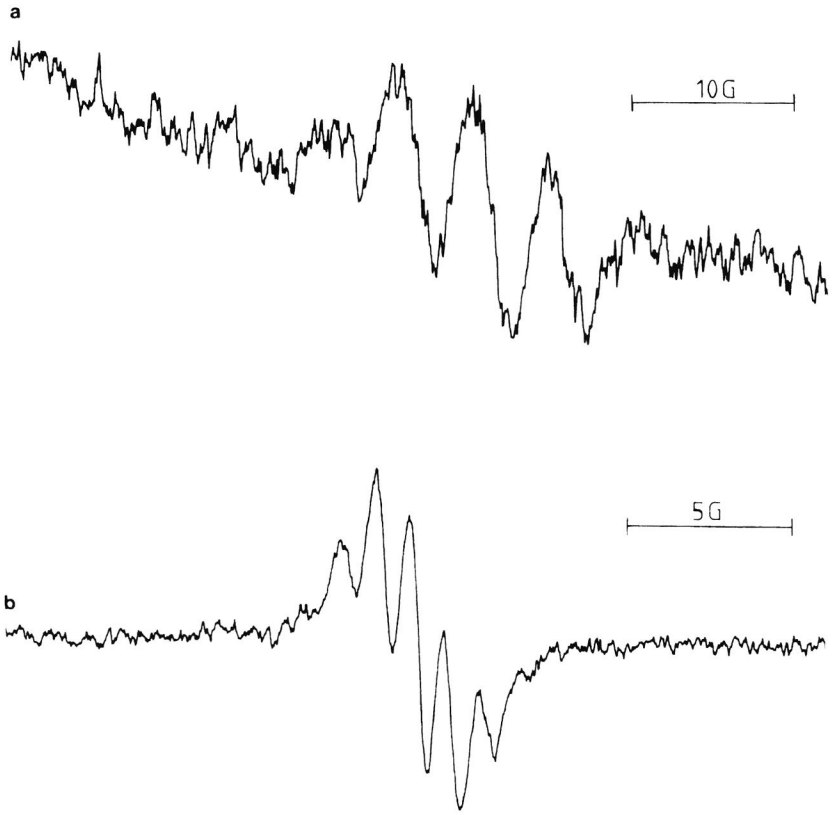

Figure 3. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diC_2H_5-PAR. Receiver gain was 10^5, modulation amplitude 1.975 G, centre field 3480 G, sweep width 50 G, conversion time 20.48 ms and time constant 81.92 ms. (b) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-di*t*C_4H_9-PAR, as described in the text. Receiver gain was 2×10^6, modulation amplitude 0.25 G, centre field 3483 G, sweep width 25 G, conversion time 163.84 ms and time constant 655.36 ms.

As can be seen in figure 3, clear ESR signals were also detected in the horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diC_2H_5-PAR and 3,5-di*t*C_4H_9-PAR. The observed ESR spectra appeared to be derived from oxygen-phenoxy radicals, since, for nitrogen-based radicals, a large triplet splitting ($a^N \approx$ 14 G) would be expected. The latter type of splitting has been reported for phenothiazine cation radicals observed during horseradish peroxicase/H_2O_2-mediated oxidation of phenothiazine (Stolze and Mason 1991). However, none of the ESR spectra in the present study exceeded 20 Gauss.

The ESR spectrum observed on oxidation of 3,5-diC_2H_5-PAR in horseradish peroxidase/H_2O_2 incubations (figure 3a) exhibited a five-line pattern characteristic for four equivalent γ-hydrogen atoms of two methylene groups *ortho* to the phenoxy group, although the fifth peak was not very clear due to the small signal-to-noise ratio. Hyperfine splitting constants $a^H_{CH_2}$ were $\approx$ 4.1 G (2H) for 3,5-diC_2H_5-PAR. Oxidation of 3,5-di*t*C_4H_9-PAR (figure 3b), resulted in a similar five-line spectrum, with smaller hyperfine splitting of $\approx$ 1 G. This could be due to the methyl hydrogen atoms in the *N*-acetyl group with coupling in the same order of magnitude as that of the -NH hydrogen, analogous to corresponding coupling effects (1.0 and 0.82 G

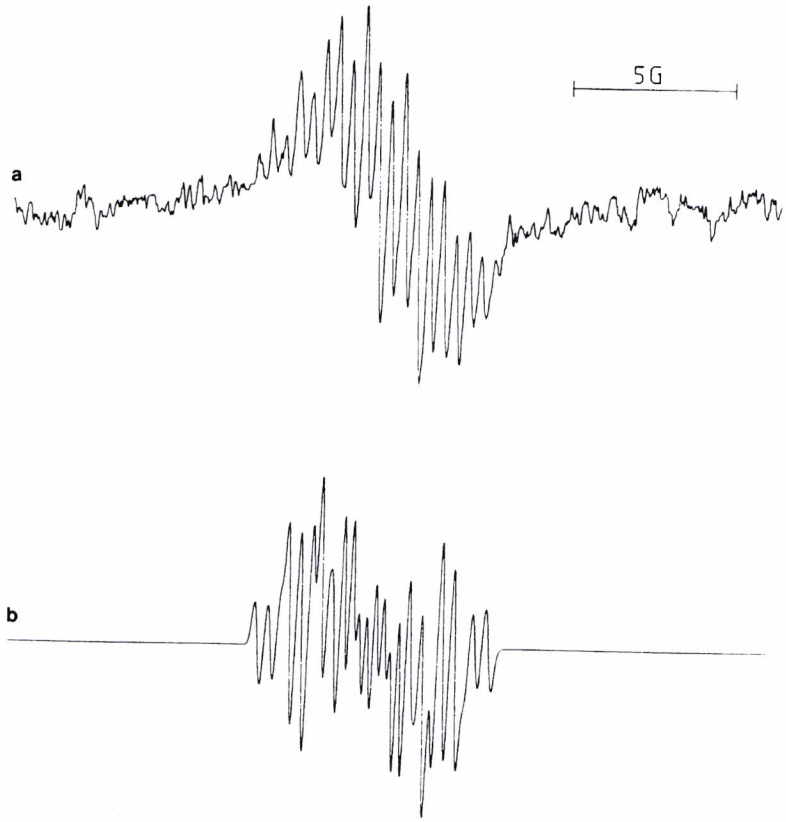

Figure 4. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diOCH_3-PAR. Receiver gain was 2×10^6, modulation amplitude 0.25 G, sweep width 25 G, centre field 3483 G, conversion time 163.84 ms and time constant 655.36 ms. (b) Simulation of an ESR spectrum of the phenoxy radical of 3,5-diOCH_3-PAR with hyperfine splitting constants, as calculated in this paper (table 4).

respectively) reported for 3,5-diCH_3-PAR (Fischer and Mason 1984). The spectrum could not be resolved completely but an extra splitting due to coupling with the aromatic hydrogen atoms is expected to be responsible for the weak hyperfine splitting. Hyperfine splitting in the ESR spectrum due to the 3,5-di*t*C_4H_9 substituents, which is expected to be about 0.36 G (18 H) (Valoti *et al.* 1989), could not be observed because a modulation amplitude of only 0.5 G was used.

The observed resonances were always dependent on the substrates and substrate concentrations. In the absence of a 3,5-dialkylated PAR analogue, horseradish peroxidase or H_2O_2, no ESR signals were observed (data not shown).

3,5-DiOCH_3-PAR and 3,5-diSCH_3-PAR. A very strong and clear ESR spectrum was obtained on oxidation of 3,5-diOCH_3-PAR in horseradish peroxidase/H_2O_2 incubations (figure 4a). Clearly, hyperfine splittings were observed, although we have not been able to assign these to couplings with specific atoms. Since the complete spectrum exhibits > 20 peaks and covers only about 7.5 G, several minor couplings will be involved.

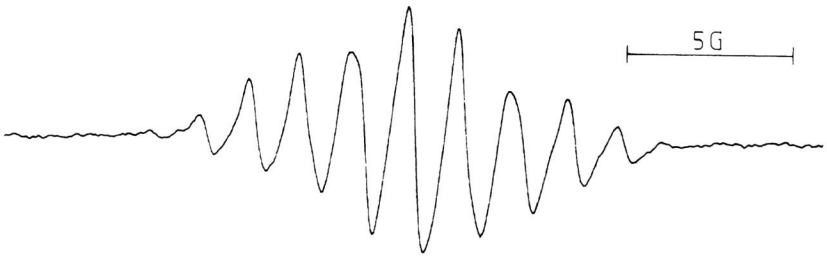

Figure 5. ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diSCH_3-PAR. Receiver gain was 2×10^4, modulation amplitude 1.00 G, centre field 3480 G, sweep width 50 G, conversion time 5.12 ms and time constant 1.28 ms.

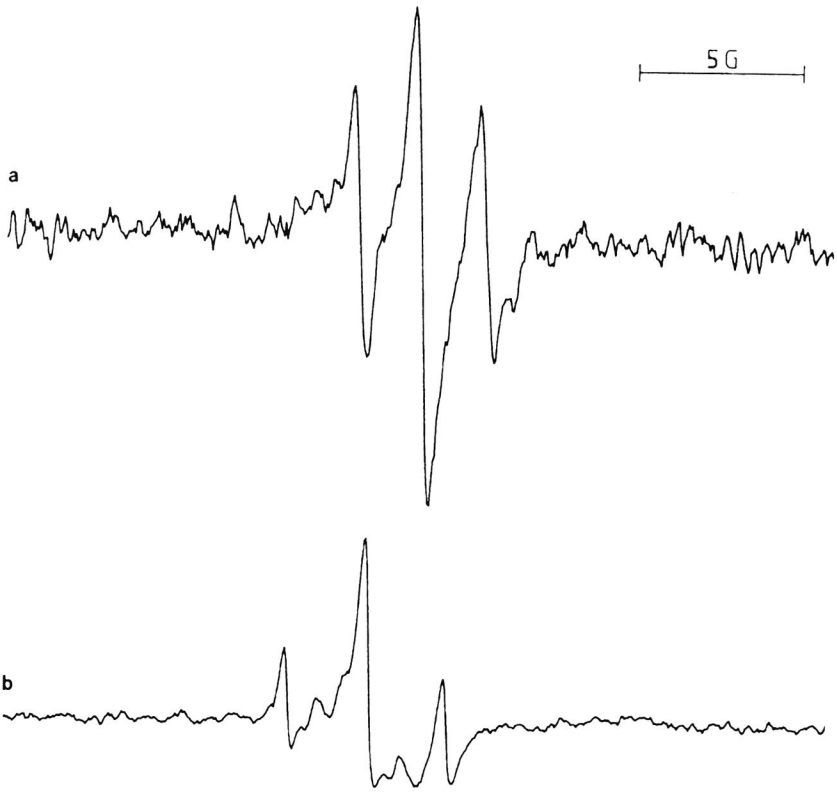

Figure 6. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diF-PAR. Receiver gain was 5×10^5, modulation amplitude 0.5 G, centre field 3480 G, sweep width 50 G, conversion time 40.96 ms and time constant 81.92 ms. (b) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diCl-PAR. Receiver gain was 10^5, modulation amplitude 0.099 G, centre field 3485 G, sweep width 50 G, conversion time 20.48 ms and time constant 81.92 ms.

For the oxidation of 3,5-diSCH_3-PAR (figure 5), a clear ESR spectrum was also observed. Importantly, as soon as H_2O_2 was added to the incubation mixtures the sample permanently turned into a bright orange solution. In the ESR signal, at least nine peaks were observed within a width of 15 G. At least four peaks seemed a

summation of several smaller peaks with almost identical hyperfine splittings. Specifically, the fourth and seventh large positive peaks contained shoulders. Also the first and second negative peak were not as sharp as several others. Furthermore, the spectrum shown in figure 5 seems not completely symmetrical, indicating that more than one radical may be involved.

The observed resonances were always dependent on the substrates and substrate concentrations. For all experiments, blank incubations containing the complete incubation mixture but not the substrates, did not show any ESR-signal (data not shown).

3,5-Dihalogenated PAR analogues. On oxidation of 3,5-diF-PAR, and 3,5-diCl-PAR in horseradish peroxidase/H_2O_2 incubations, clear ESR-signals were also observed. The observed hyperfine interactions were relatively small (< 5 G), indicating oxygen-derived phenoxy radicals (figure 6). However, in contrast with PAR and the 3,5-dialkylated PAR analogues, ESR signals were not directly observed, but increased steadily 10–20 min after starting the incubation. Clear hyperfine splittings can be observed in the ESR-signals; however, we have not yet been able to assign specific coupling constants to specific atoms.

On one-electron oxidation of 3,5-diCl-PAR, ESR spectra were obtained at modulation amplitudes of 1, 0.5 and 0.1 G with increasing hyperfine splittings (figure 7a–c). The signal appeared a superposition of two independent signals; a more intense three-line signal with an intensity ratio of 1:2:1 (hyperfine splitting $a^H = 2.34$ G), and a less intense signal of which the hyperfine splitting and intensity ratios could not be determined exactly (figure 7a–c).

The ESR spectrum observed on oxidation of 3,5-diF-PAR exhibited a three-line pattern (figure 6a). However, the hyperfine interaction of about 1.8 G was smaller than that observed for NAPSQI (5.11 G, figure 1). Oxidation of 3,5-diBr-PAR in a horseradish peroxidase-mediated incubation resulted in a weak though clear signal without significant hyperfine splitting (figure 8).

Omission of either of the reactants or horseradish peroxidase failed to show any ESR signals in these incubations (shown only for 3,5-diCl-PAR in figure 7e–g).

ESR analysis of microsomal and reconstituted P450 incubations

As presented above, the horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diCH_3-PAR, 3,5-ditC_4H_9-PAR, 3,5-diOCH_3-PAR and 3,5-diSCH_3-PAR resulted in the rapid formation of stable ESR signals, in contrast with incubations using the 3,5-dihalogenated PAR analogues. As the ESR parameters, such as receiver gain, modulation amplitude and power, were already optimized for each of the 3,5-disubstituted PAR analogues, these were not significantly modified. We focused mainly on incubation conditions and concentrations of the various constituents in the cytochrome P450-containing incubations.

First, liver microsomal fractions from βNF-induced rats were used at protein concentrations varying from 0.05 to 1 mg protein/ml. In addition, liver microsomes from phenobarbital-induced rats were used in several incubations. Concentrations of the 3,5-disubstituted PAR analogues were varied from 0.1 to 5 mM and concentrations of NADPH from 0.1 to 1 mM. Furthermore, a NADPH-regenerating

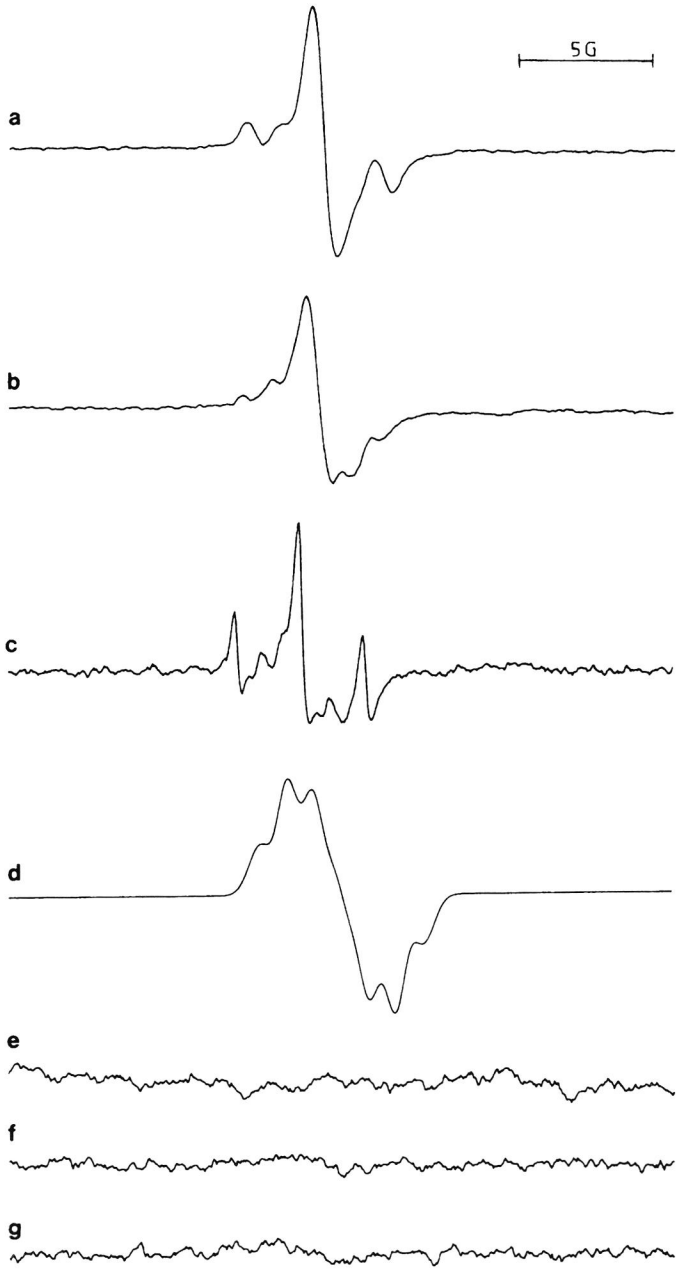

Figure 7. ESR spectra of radicals observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diCl-PAR using various modifications. Instrumental settings were as described for figure 6b (figure 7c is identical to 6b). The modifications included modulation amplitude 0.99 G (a), 0.496 G (b), without H_2O_2 (e), without horseradish peroxidase (f), and without 3,5-diCl-PAR, horseradish peroxidase and H_2O_2 (only phosphate buffer) (g). Simulation of an ESR spectrum of the phenoxy radical of 3,5-diCH_3-PAR with hyperfine splitting constants, as calculated in this paper (table 4) (d).

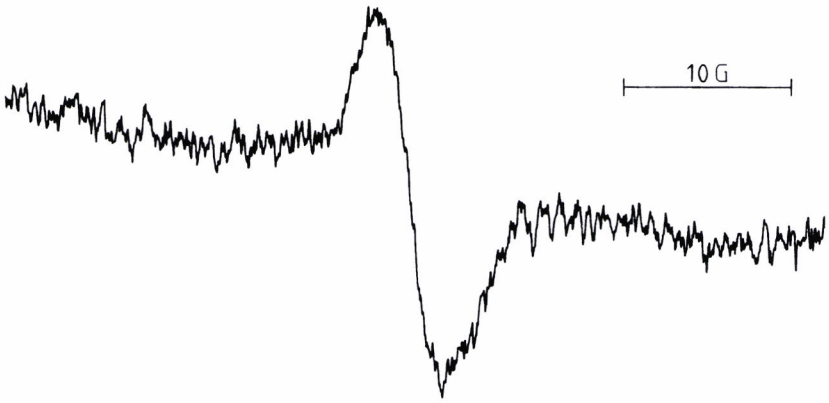

Figure 8. ESR spectrum of the radical observed during horseradish/H_2O_2-mediated oxidation of 3,5-diBr-PAR. Receiver gain was 2×10^4, modulation amplitude 1.0 G, centre field 3480 G, sweet width 50 G, conversion time 5.12 ms and time constant 1.28 ms.

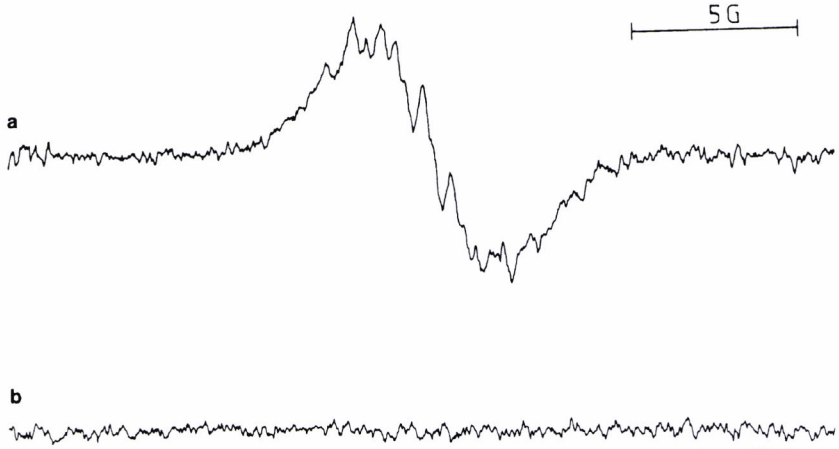

Figure 9. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-diOCH_3 (incubation and instrumental conditions were as in figure 4a). (b) ESR analysis of a microsomal incubation as described in the Materials and methods using 3,5-diOCH_3 as substrate (instrumental conditions were as in figure 4a).

system was used to provide a sustainable source of NADPH. Unfortunately, none of these experiments resulted in significant and reproducible ESR signals (only shown for one representative, 3,5-diOCH_3-PAR, in figure 9b versus a).

Second, incubations using reconstituted cytochrome P450-containing monooxygenase systems were performed. Purified P450 isoforms CYP1A1, CYP2B1 as well as CYP2E1, were used with the various 3,5-dialkylated PAR analogues. However, in none of these systems significant or reproducible signals were observed in ESR analysis (data not shown). Modifications of the ratio of purified P450, NADPH-cytochrome P450 reductase, and NADPH, nor the incorporation of an NADPH-regenerating system resulted in detection of ESR signals upon analysis (data not shown).

Some additional experiments were performed to determine the influence that constituents like microsomal lipids and protein, and NADPH, would have on ESR signals once generated upon horseradish peroxidase/H_2O_2 catalysed incubation

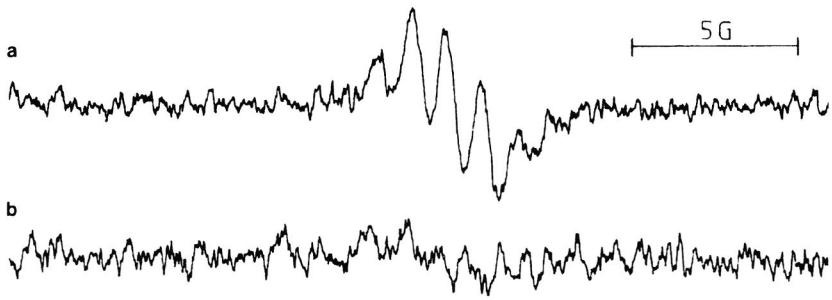

Figure 10. (a) ESR spectrum of the radical observed during horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-ditC_4H_9-PAR (incubation and instrumental conditions comparable with those in figure 3b). (b) ESR analysis of the same horseradish peroxidase/H_2O_2-mediated oxidation of 3,5-ditC_4H_9-PAR but with 0.5 mM NADPH added (without change of instrumental conditions).

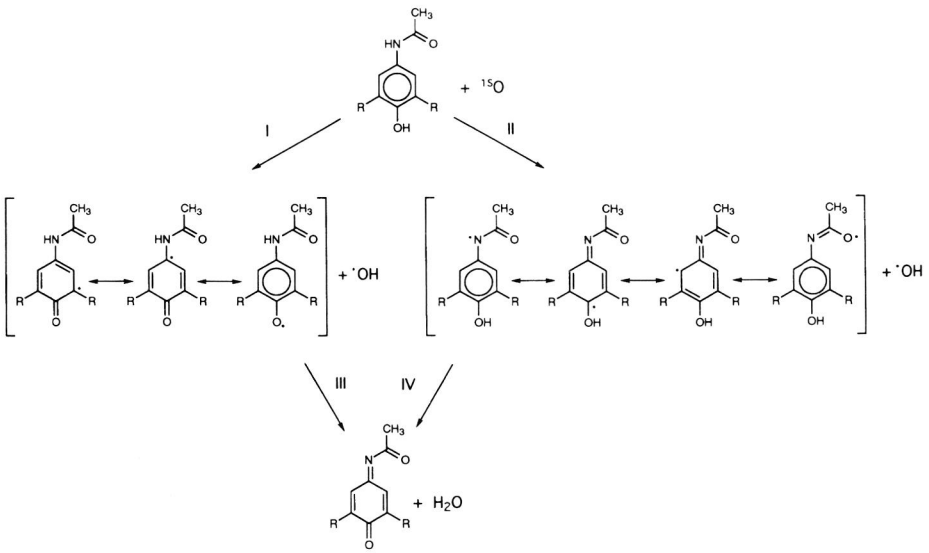

Figure 11. Hypothetical radical pathways for the oxidation of PAR (R = H) and 3,5-disubstituted PAR analogues. An initial hydrogen atom abstraction is assumed to take place either at the phenolic hydroxyl group or at the nitrogen atom in the acetylamino side chain of these PAR analogues. Routes III and IV lead to NAPQI or the respective 3,5-disubstituted NAPQI-analogues and water.

with several 3,5-disubstituted PAR analogues. Addition of microsomal protein (0.1 mg/ml) to a horseradish peroxidase/H_2O_2 incubation of 3,5-diCH_3-PAR reduced the ESR signal by a factor 2–3 (data not shown). Addition of microsomal protein to an analogous incubation of 3,5-ditC_4H_9-PAR resulted in a comparable reduction of the corresponding ESR signal (data not shown). Moreover, when NADPH (0.5 mM) was added instead of microsomal protein, the ESR signal was lost completely (figure 10b versus a). The same phenomenon was observed when NADPH (0.5 mM) was added to a horseradish peroxidase/H_2O_2 incubation of 3,5-diOCH_3-PAR in which a very stable ESR signal had been detected just 5 min previously (data not shown).

Table 1. Calculated energy differences (RHF/SV 6–31 G energies), ΔE (kJ/mol) for hydrogen atom abstractions from PAR and four 3,5-disubstituted PAR analogues[a].

Route[b]	PAR	3,5-diF	3,5-diCl	3,5-diCH_3	3,5-diOCH_3
I	−455	−438	−430	−473	−431
II	−332	−308	−314	−329	−319
III	−7	6	−7	−11	−12
IV	−130	−124	−123	−155	−124

[a] Energy differences between reactants, intermediates and products (i.e. 3,5-disubstituted PAR analogues, singlet oxygen, disubstituted phenoxyl radical intermediates, hydroxyl radical, 3,5-disubstituted NAPQI analogues and water).

[b] Routes indicated are as depicted in figure 11.

Finally, horseradish peroxidase/H_2O_2-mediated oxidation samples were split before starting the reaction with H_2O_2. Subsequently, microsomal protein or NADPH, or an equal amount of buffer (positive control samples), were added to either reaction tube. Then, on addition of H_2O_2 and ESR analysis, signals were observed in the positive control samples, whereas in the split samples where microsomes or NADPH were added, no ESR signals were observed at all (data not shown).

Energy differences

Figure 11 represents hypothesized radical pathways for the oxidation of PAR and the 3,5-disubstituted analogues as initiated by singlet oxygen, in analogy to what has been reported for PAR and cytochrome P450 (Koymans *et al.* 1989). Single hydrogen atom abstractions from the phenolic hydroxyl group of PAR and 3,5-disubstituted analogues (I) would yield each a phenoxy and a hydroxyl radical. Single hydrogen atom abstractions from the acetylamino nitrogen (II) for each compound, would yield a *p*-hydroxy-*N*-acetyl-anilinyl and a hydroxyl radical. An initial hydrogen atom abstraction from the phenolic hydroxyl group (route I) was calculated to be energetically favoured over an initial hydrogen atom abstraction from the acetylamino nitrogen (route II) by more than 100 kJ/mol for PAR as well as the 3,5-disubstituted analogues (table 1).

Reactions III and IV are secondary hydrogen atom abstractions from the phenoxy radical (route III) and the anilinyl radical (route IV) respectively, both resulting in the formation of NAPQI and 3,5-disubstituted NAPQI analogues and water. These reactions were also found to be exothermic except the secondary hydrogen atom abstraction from the 3,5-difluoro-*N*-acetyl-*p*-amino-phenoxy radical (table 1).

Atomic spin population

As can be seen from table 2a, for phenoxy radicals more than 83 % of the atomic spin population was calculated to be located at the phenoxy oxygen atom. About 4.5 % was located at each of the *ortho* positions (C_3 and C_5) and about 3 % at *para* position (C_1) relative to the phenolic hydroxyl substituent.

Assuming the initial hydrogen atom abstraction occured at the nitrogen atom in the acetylamino side chain, resulting in *p*-hydroxy-*N*-acetyl-anilinyl radicals,

Table 2a. Calculated atomic spin populations of the *N*-acetyl-*p*-amino-phenoxy radical of PAR and four 3,5-disubstituted PAR analogues[a].

Atom	PAR	3,5-diF	3,5-diCl	3,5-diCH_3	3,5-diOCH_3
C_3	0.045	0.039	0.043	0.048	0.046
C_3-substituent		0.003 (F)	0.003 (Cl)	0.000 (C)	0.002 (O)[b]
C_5	0.046	0.040	0.043	0.049	0.040
C_5-substituent		0.003 (F)	0.003 (Cl)	0.000 (C)	0.005 (O)[b]
C_2	0.002	0.002	0.003	0.002	0.003
C_6	0.001	0.001	0.002	0.001	0.001
C_1	0.027	0.024	0.025	0.028	0.025
C_4	0.014	0.015	0.011	0.025	0.022
O_7	0.860	0.868	0.860	0.838	0.848
N_8	0.003	0.003	0.004	0.003	0.003
C_9	0.001	0.001	0.001	0.001	0.001
O_{11}	0.001	0.001	0.001	0.001	0.001
C_{10}	0.000	0.000	0.000	0.000	0.001

[a] Atomic spin populations on all hydrogen atoms were insignificant (< 0.003) and were therefore omitted.

[b] Atomic spin populations on methyl carbon atoms were insignificant (< 0.002) and were therefore omitted.

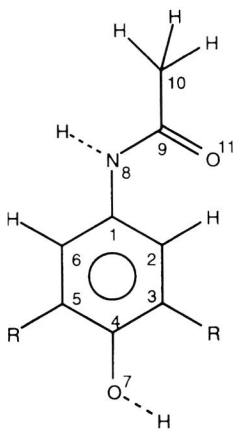

Table 2b. Calculated atomic spin populations of the *p*-hydroxy-*N*-acetyl-anilinyl radical of PAR and four 3,5-disubstituted PAR analogues[a].

Atom	PAR	3,5-diF	3,5-diCl	3,5-diCH_3	3,5-diOCH_3
C_3	0.002	0.001	0.001	0.001	0.001
C_3-substituent		0.000 (F)	0.000 (Cl)	0.000 (C)	0.000 (O)[b]
C_5	0.005	0.003	0.003	0.004	0.006
C_5-substituent		0.000 (F)	0.000 (Cl)	0.000 (C)	0.001 (O)[b]
C_2	0.052	0.050	0.049	0.053	0.065
C_6	0.061	0.052	0.056	0.062	0.048
C_1	0.029	0.014	0.017	0.027	0.025
C_4	0.039	0.035	0.034	0.041	0.044
O_7	0.006	0.006	0.006	0.006	0.007
N_8	0.693	0.733	0.728	0.690	0.690
C_9	0.028	0.022	0.023	0.028	0.028
O_{11}	0.083	0.081	0.081	0.083	0.082
C_{10}	0.001	0.001	0.001	0.001	0.001

[a] Atomic spin populations on all hydrogen atoms were insignificant (< 0.003) and were therefore omitted.

[b] Atomic spin populations on methyl carbon atoms were insignificant (< 0.001) and were therefore omitted.

Table 3. *N*-acetyl-*p*-amino-phenoxy radical: hyperfine splitting constants (a), *ab initio* calculated atomic spin populations (ρ) and calculated Q[a].

Position	a (G)[b]	ρ	Q
C_3-proton	5.11	0.04499	114
C_5-proton	5.11	0.04559	112
C_2-proton	0.64	0.00177	362
C_6-proton	0.64	0.00107	598
N_8	0.81	0.00327	248
N_8-proton	1.35	0.00327	413
$C(O)CH_3$-protons	1.01	0.00001	[c]

[a] Q for the atoms showing atomic spin populations $\geqslant 0.001$ (table 2a) was calculated using equation 1 (Heller and McConnell 1960).

[b] Hyperfine splitting constants (a) taken from Fischer *et al.* (1986).

[c] Due to very small atomic spin population, the calculation of Q is not accurate.

calculated atomic spin populations are as presented in table 2b. Spin delocalizations were significantly higher in this case. On average, about 70% of the spin was calculated to be localized at the anilinyl nitrogen atom. Approximately 8% was found at the acetyl oxygen atom and 5–6% at the *ortho* position. The spin delocalization in phenoxy and anilinyl radicals is visualised by several mesomeric structures as presented in figure 11.

ESR simulations

To simulate the ESR spectra from phenoxy radicals of the 3,5-disubstituted PAR analogues for which energy and atomic spin population calculations were performed, the reported hyperfine splitting constants of the phenoxy radical of PAR were used (Fischer and Mason 1984). According to Heller and McConnell (1960) and Wertz and Bolton (1972), hyperfine splitting as caused by a σ proton ($a_{\alpha H}$), is directly proportional to the π-electron density (ρ_C) on the neighbouring carbon atom (I), based on the assumption of π/p-interaction of a carbon atom with the 1s-orbital of a neighbouring H atom.

$$a_{\alpha H} = Q.\rho_C \qquad (1)$$

Using equation 1, Q relevant for hyperfine splitting of the ESR signal was calculated for each atom in the phenoxy radical of PAR (table 3). Equation 1 was used to calculate Q for nitrogen and the -NH proton, although it was not completely clear whether equation 1 does apply to other groups than C-H bonds. The atomic spin population on nitrogen was used for nitrogen itself as well as for the hydrogen atom attached. The hyperfine splitting constants derived (equation 1) using the calculated Q for the atoms in the *N*-acetyl-*p*-amino-phenoxy radical (table 3), are presented in table 4. Using the calculated hyperfine splitting constants, several ESR spectra were computer simulated. Results of simulations of the ESR spectra are presented for 3,5-diCH_3-PAR (figure 2b), 3,5-diOCH_3-PAR (figure 4b) and 3,5-diCl-PAR (figure 7d).

As can be seen, the simulated ESR spectrum of the phenoxy free radical of 3,5-diCH_3-PAR is similar to the experimental spectrum (figure 2a and b). Furthermore, the hyperfine splitting constants used (especially those for the aromatic

Table 4. Calculated hyperfine coupling constants a (G) for the phenoxy radicals of PAR and 3,5-disubstituted PAR analogues[a].

Position	PAR[b]	3,5-diF	3,5-diCl	3,5-diCH_3[c]	3,5-diOCH_3
C_3-substituent	5.11	[d]	[d]	5.50 (5.05)	[d]
C_5-substituent	5.11	[d]	[d]	5.51 (5.05)	[d]
C_2-proton	0.64	0.76	0.94	0.79 (0.82)	0.96
C_6-proton	0.64	0.83	1.03	0.81 (0.82)	0.41
N_8[f]	0.81	0.82	0.92	0.82 (0.404)	0.77
N_8-proton[f]	1.35	1.37	1.54	1.37 (0.82)	1.28
C(O)CH_3-protons	1.01	[e]	[e]	[e] (1.0)	[e]

[a] Hyperfine splitting constants were derived (equation 1) using the atomic spin populations (ρ) and Q, as presented in table 3.

[b] Hyperfine splitting for PAR is given for comparison and taken from Fischer *et al.* (1986).

[c] Parentheses indicate the hyperfine splitting constants as observed experimentally by Fischer and Mason (1984).

[d] McConnell equation probably does not apply to these nuclei.

[e] Due to the unavailability of a reliable Q, no hyperfine splitting constants could be calculated.

[f] Due to the small spin on nitrogen as well as the nitrogen hydrogen atom, calculation of hyperfine splitting constants with the McConnell equation is unreliable.

hydrogen atoms), as calculated using equation 1 (table 4), are similar to the ones previously published (Fischer and Mason 1984). For other 3,5-disubstituted PAR analogues, however, none of the simulated spectra corresponds well to the experimental ESR spectra (figures 4b and 7d).

Discussion

Paracetamol (PAR) and 3,5-disubstituted analogues are susceptible to horseradish peroxidase/H_2O_2 oxidation to NAPSQI and 3,5-disubstituted analogues of NAPSQI (Fischer and Mason 1984, Fischer *et al.* 1985) and liver microsomal cytochrome P450 catalysed oxidation to NAPQI and 3,5-disubstituted analogues of NAPQI (Dahlin *et al.* 1984, Van de Straat *et al.* 1986, Bessems *et al.* 1996). It has been proven that NAPQI is the reactive electrophilic intermediate responsible for the observed toxicity of PAR (Albano *et al.* 1985, Van de Straat *et al.* 1988), although the semiqinone free radical *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) has been proposed as well (De Vries 1981). In the current paper, extensive attempts are described to experimentally identify one-electron-oxidized radical semiquinone imines of 3,5-disubstituted PAR analogues in enzymatic systems, i.e. horseradish peroxidase/H_2O_2 and rat liver microsomal P450. 3,5-Disubstituted PAR analogues with electron-donating alkyl groups as well as with electron-withdrawing halogen atoms were used. Furthermore, in an attempt to rationalize the observed ESR spectra, *ab initio* calculations were performed in the case of four fairly stable and experimentally detectable radicals of 3,5-disubstituted PAR analogues.

The results obtained with ESR detection provide substantial evidence that both the 3,5-dialkylated and the 3,5-dihalogenated PAR analogues are liable to single hydrogen abstraction on horseradish peroxidase/H_2O_2-catalysed oxidation. Moreover, the spectra obtained indicate that all radical species formed were highly likely a result from hydrogen atom abstraction from the phenolic group of the 3,5-disubstituted PAR analogues. If hydrogen atom abstraction were to take place from the nitrogen atom in the *N*-acetylamino side chain, spectra exhibiting broader hyperfine splittings would be expected, as nitrogen radicals usually cause triplets

with hyperfine splitting constants of > 14 G (Stolze and Mason 1991). Triplet spectra as wide as 28 G would therefore be anticipated in case of anilinyl radicals. The ESR spectra observed in this study, however, basically exhibited an intensity ratio of 1:2:1 or multiples thereof (typical of phenoxy radical) and did not exceed 20 G (oxidation of 3,5-diOCH_3-PAR and 3,5-diSCH_3-PAR).

The probability of the formation of oxygen-centred radicals over nitrogen-based radicals was determined using *ab initio* molecular orbital calculations. For all PAR analogues calculated and for PAR, the formation of phenoxy radicals was energetically strongly favoured over the formation of nitrogen based radicals (table 1). This result is in agreement with previous results where similar, although not identical, molecular orbital calculations were used to discriminate between phenoxy and anilinyl radical formation from PAR (Koymans *et al.* 1989). Secondary hydrogen atom abstraction was only found to be endothermic for the oxidation of the 3,5-difluoro-*N*-acetyl-*p*-amino-phenoxy radical (3,5-diF-NAPSQI). Formation of NAPQI and 3,5-disubstituted NAPQI's from NAPSQI and 3,5-disubstituted NAPSQI's, respectively, were all exothermic. These theoretical findings seem to correspond to the recent experimental findings that 3,5-dimethyl-NAPQI and 3,5-dichloro-NAPQI, but not 3,5-difluoro-NAPQI, are formed from the respective parent compounds by rat liver microsomal cytochrome P450 (Bessems *et al.* 1996). Moreover, it was found that the energy differences in both pathways, starting with the formation of a phenoxy or an anilinyl radical species (figure 11), were minimally influenced by the chemical nature of the 3,5-disubstituents (table 1).

We have presented experimental evidence for the formation of semiquinone imine radicals in horseradish peroxidase/H_2O_2-catalysed oxidations of PAR and 3,5-disubstituted PAR analogues. The results of the molecular orbital calculations indicated that formation of semiquinone radicals from all 3,5-disubstituted PAR analogues in this study was feasible with singlet oxygen as a model. Yet, free radicals as NAPSQI are known to be reactive chemical entities, for example they are liable to reaction with reducing agents such as NADPH (Keller and Hinson 1991), glutathione (Potter and Hinson 1987) and probably also protein thiols and lipids. Potter and Hinson (1987) reported that increasing concentrations of NADPH decreases the polymerisation of PAR (via NAPSQI) in a horseradish peroxidase/H_2O_2-system. It was therefore expected and shown here, despite the use of optimal ESR settings that were found with the horseradish peroxidase/H_2O_2-mediated radical formation for each of the 3,5-disubstituted PAR analogues, that these semiquinone imine-free radicals would be difficult to detect by ESR, once formed in microsomal incubations containing cytochrome P450, due to the presence of such free radical quenchers. In cytochrome P450-containing oxidative systems, either microsomal or reconstituted, no semiquinone imine radicals were observed. Moreover, ESR signals in horseradish peroxidase/H_2O_2 incubations vanished on addition of 0.5 mM NADPH or 1 mg/ml microsomal protein. From these and several other control experiments, it is concluded that cytochrome P450-catalysed oxidation of both PAR and the two series of 3,5-disubstituted analogues apparently does not result in detectable formation of free radicals.

The ESR spectra, observed for the radicals of the 3,5-dialkylated PAR analogues, could well be interpreted as caused by phenoxy radicals. Major hyperfine splittings are caused by β-CH_2 hydrogen atoms if present (e.g. for 3,5-diC_2H_5-PAR), by the 2,6-aromatic hydrogen atoms and by the *N*-acetyl hydrogen atoms. In the group of 3,5-dihalogenated analogues, for example, the ESR spectrum detected on oxidation

of 3,5-diF-PAR with horseradish peroxidase/H_2O_2 consisted mainly of a characteristic three-line pattern (intensity ratios 1:2:1) and a hyperfine splitting $a \approx$ 1.8 G, which is characteristic for two equivalent aromatic γ-hydrogen atoms at 2,6-positions (Juhl *et al.* 1991). The ESR spectrum observed for 3,5-diCl-PAR exhibited clear similarities with a spectrum reported for one-electron oxidized 2,5-dichloro-1,4-hydroquinone (Juhl *et al.* 1991). A three-line signal with intensity ratios of approximately 1:2:1 was seen as characteristic for two equivalent β- or γ-hydrogen atoms in an aromatic nucleus. Probably due to mesomerization, the hydrogen atoms either *ortho* or *meta* to the phenoxy oxygen are equal upon hydrogen atom abstraction from 2,5-dichloro-1,4-hydroquinone. Also the hyperfine splitting ($a \approx 2.2$ G) observed with 3,5-diCl-PAR compares well with the 1.9 G as published by Juhl *et al.* (1991). The less intense ESR signal with at least four peaks, which was superimposed on the main 1:2:1 signal, could not be assigned as yet. Moreover, the overall ESR signal was asymmetric (figure 7), most likely indicating the existence of more than one radical structure (including possibly a dimeric radical) since the aqueous incubation system is totally isotropic. With regard to the ESR signal observed on peroxidase/H_2O_2-catalysed oxidation of 3,5-diBr-PAR, it can only be concluded that the signal represents a phenoxy radical based on the width of the signal. As stated before, nitrogen based radicals generally cause much broader signals (at least about 25 G).

Lastly, calculated atomic spin populations were examined to rationalize the ESR spectra observed on incubation of 3,5-dialkylated and the 3,5-dihalogenated PAR analogues. As discussed earlier, all ESR spectra observed were highly likely based on phenoxy radicals. Calculated atomic spin populations (table 2a) predicted that the major part of the atomic spin population would reside on the phenoxy oxygen, which was in accordance with the experimental findings. More specifically, for the oxidation of 3,5-diCH_3-PAR, the calculated hyperfine splitting constants using the McConnell relationship ($a_{\chi H} = Q\rho$) (Heller and McConnell 1960) were mostly similar to the experimentally observed hyperfine splitting constants. In general, however, the predictive value of the calculations of the hyperfine splitting constants was rather low, based on the dissimilarity between the simulated (with calculated hyperfine splitting constants) and the experimental ESR spectra. The only calculated hyperfine splitting constants that are predictive throughout seem to be the hyperfine splitting constants caused by the aromatic hydrogen atoms. Possibly, the McConnell equation (1) only applies to aliphatic hydrogen atoms or γ-hydrogen atoms (CH_3-substituents in the phenoxy radical of 3,5-diCH_3-PAR). For example, for 3,5-diF-PAR with the McConnell equation a hyperfine splitting constant of about 4.45 G was calculated for the 3,5-diF-*N*-acetyl-*p*-benzosemiquinone imine radical (data not shown in table 4), whereas about 1.8 G was observed experimentally (figure 6a). An explanation for this disparity could be that the McConnell equation is not applicable to fluorine coupling. Furthermore, the hyperfine interactions between the unpaired spin and the acetylamino hydrogen are, due to the distance, expected to be mainly isotropic and are explained by a hyperconjugation mechanism between the π-system and the 1*s*-orbital of the acetylamino hydrogen (Wertz and Bolton 1972). Therefore, it remains unclear why the hyperfine splitting of the acetylamino hydrogen, as calculated here for 3,5-diCH_3 (table 4), did not predict the observed hyperfine splitting, as published by Fischer and Mason (1984).

In conclusion, it has been shown that the 3,5-disubstituted PAR analogues studied here are all liable to horseradish peroxidase/H_2O_2-catalysed oxidation to

phenoxy free radicals. This implies that horseradish peroxidase, a representative of the family of peroxidases of which there are several members in mammals (such as myeloperoxidase and prostaglandin synthase), can generate free radicals from compounds with various physicochemical properties. In our series of 3,5-di-substituted PAR analogues, lipophilicity, steric hindrance, electron donating and electron-withdrawing properties, and oxidizability seem only quantiative barriers, not qualitative barriers for the oxidation by a peroxidase. This could imply that for many more compounds (e.g. nephrotoxicants), radical formation might be toxicologically relevant (e.g. the kidney is rich in prostaglandin synthase). A few of the phenoxy radicals, i.e. the 3,5-dialkylated radicals, could be solved with respect to their ESR hyperfine splitting constants. The other radicals, as formed from 3,5-dihalogenated PAR analogues, 3,5-diOCH_3-PAR and 3,5-diSCH_3, however, could not be assigned accurately. It remains to be determined to what extent the observed radical formation is relevant as to the previously observed cytotoxicity of the 3,5-dihalogenated PAR analogues and the possible involvement of cytochrome P450-mediated radical formation (Bessems *et al.* 1997). Despite extensive attempts with specifically selected 3,5-disubstituted PAR analogues with varying radical stabilities, no radicals could be detected in microsomal or in reconstituted cytochrome P450 incubation systems. Indirect evidence is presented that this is either due to the presence of NADPH, thiol groups and lipids, or to a rapid abstraction of a second hydrogen atom in the active site of P450.

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Chapter 6 Rat liver microsomal cytochrome P450-dependent oxidation of 3,5-disubstituted analogues of paracetamol

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Rat liver microsomal cytochrome P450-dependent oxidation of 3,5-disubstituted analogues of paracetamol

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1. The cytochrome P450-dependent binding of paracetamol and a series of 3,5-disubstituted paracetamol analogues (R = -F, -Cl, -Br, -I, $-CH_3$, $-C_2H_5$, $-iC_3H_7$) have been determined with β-naphthoflavone (βNF)-induced rat liver microsomes and produced reverse type I spectral changes. $K_{s,app}$ varied from 0·14 mM for 3,5-diiC_3H_7-paracetamol to 2·8 mM for paracetamol.

2. All seven analogues underwent rat liver microsomal cytochrome P450-dependent oxidation, as reflected by the formation of GSSG in the presence of GSH. The GSSG-formation was increased in all cases upon pretreatment of rats by β-naphthoflavone (βNF) and was generally decreased upon pretreatment by phenobarbital (PB).

3. Rat liver microsomal cytochrome P450 as well as horseradish peroxidase catalysed the formation of 3,5-disubstituted NAPQI analogues from the corresponding parent compounds, as identified by UV-spectrophotometry of the NAPQI analogues and by GC/MS detection of the following GSH-conjugates: 2-glutathione-*S*-yl-3,5-dimethyl-1,4-dihydroxybenzene, 2-glutathione-*S*-yl-3,5-dichloro-paracetamol, and 2-glutathione-*S*-yl-3,5-dibromo-paracetamol.

4. In liver microsomal (βNF-induced) incubations, apparent K_m values, as determined for the cytochrome P450 catalysis-dependent oxidation of GSH, for seven 3,5-disubstituted paracetamol analogues (R = -F, -Cl, -Br, -I, $-CH_3$, $-C_2H_5$, iC_3H_7) varied from 0·07 to 0·64 mM. Paracetamol exhibited an apparent K_m of 0·73 mM. Apparent V_{max} values for the cytochrome P450 catalysis dependent oxidation of GSH varied from 0·66 nmol min^{-1} mg^{-1} protein for paracetamol to 3·0 nmol min^{-1} mg^{-1} protein for 3,5-dimethyl-paracetamol.

Introduction

Paracetamol (4′-hydroxyacetanilide, PAR) is a widely used over-the-counter drug with analgesic and antipyretic properties. After overdosage PAR has been shown to cause centrilobular hepatic necrosis and kidney damage in man and laboratory animals (Cobden *et al.* 1982, Prescott 1983, Hinson *et al.* 1990, Vermeulen *et al.* 1992). At normal dose levels, PAR is mainly glucuronidated and sulphated in the liver. However, low amounts of PAR are oxidized by the cytochrome P450 (CYP)-containing mixed-function oxidase system (MFO) into a potent cytotoxin, *N*-acetyl-*p*-benzoquinone imine (NAPQI), and into a non-toxic catechol, 3-hydroxyparacetamol (Hinson 1980, Hinson *et al.* 1980, Harvison *et al.* 1988). In the presence of reduced glutathione (GSH), NAPQI can either be reduced back to PAR or covalently linked to GSH to form a 3-glutathione-*S*-yl-paracetamol conjugate (Moldéus 1978, Van de Straat *et al.* 1986).

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Studies with purified rat liver microsomal cytochrome P450 showed that in comparison with CYP 2B1, the βNF-inducible CYP 1A1 predominantly catalyses NAPQI formation from PAR. In contrast, the phenobarbital (PB)-inducible CYP 2B1 primarily forms 3-hydroxyparacetamol (Harvison *et al.* 1988). In addition, rat liver microsomal CYP 1A2, 2E1, 3A1, and 3A2 also catalyse the oxidation of PAR to NAPQI (Pattern *et al.* 1993). Furthermore, NAPQI and 3-hydroxyparacetamol metabolite formation by different isoforms of cytochrome P450 have been suggested to be related to the nature of haem binding and ligand displacement (Van de Straat *et al.* 1987a, Myers *et al.* 1994).

In attempts to produce novel non-hepatotoxic analgesics, PAR has been modified by various mono- or di-substitutions at various aromatic positions. 3,5-Disubstitution (notably 3,5-dialkylation) appeared to be most successful in this regard (Dearden *et al.* 1980, Fernando *et al.* 1980, Harvison *et al.* 1986, Van de Straat *et al.* 1986, Van de Straat *et al.* 1987b, Barnard *et al.* 1993a, b, Bessems *et al.* 1995). In a series of 3,5-disubstituted PAR analogues, 3,5-diCH_3-PAR, 3,5-diOCH_3-PAR, and 3,5-diSCH_3-PAR, more strongly inhibited mouse brain cyclooxygenase activity *in vitro* and they were less cytotoxic in rat hepatocyte incubations when compared with PAR (Bessems *et al.* 1995). Another series of analogues, 3,5-disubstituted with -F, -Cl, -Br or -I, combined a lower cyclooxygenase inhibiting potency with a larger cytotoxic potential in freshly isolated rat hepatocyte incubations than PAR (Bessems *et al.* 1995). In line with these findings two recent studies of several ring-fluorinated analogues of PAR also presented an inverse relationship between analgesic activity and *in vivo* toxicity (Barnard *et al.* 1993a, b). In contrast, for several ring-methylated PAR analogues it had been shown that analgesic activity paralleled their hepatotoxicity (Harvison *et al.* 1986).

Since the hepatotoxicity of PAR is largely dependent on cytochrome P450-catalysed oxidation (Jollow *et al.* 1973, Hinson 1980, Vermeulen *et al.* 1992), we devised experiments to investigate the oxidative bioactivation of a series of novel 3,5-disubstituted PAR analogues. Since rat βNF-inducible liver microsomal cytochrome P450 is mainly involved in the bioactivation of PAR (Van de Straat *et al.* 1986, Harvison *et al.* 1988), the cytochrome P450-catalysed oxidation of the 3,5-disubstituted PAR analogues to their corresponding 3,5-disubstituted NAPQI derivatives was investigated with βNF-induced microsomal fractions.

The rate of cytochrome P450 catalysed oxidation of PAR to NAPQI is rather slow when compared with peroxidases (Moldéus *et al.* 1982). Therefore, horseradish peroxidase (HRP) in combination with hydrogen peroxide (H_2O_2), has been used as well to study the oxidation of PAR and its 3,5-dimethylated analogue (Fischer and Mason 1984). It was shown that, mainly depending on the concentration of H_2O_2, 3,5-dimethyl-PAR could undergo either a one- or two-electron oxidation to 3,5-dimethyl-*N*-acetyl-*p*-benzo-semiquinone imine (3,5-dimethyl-NAPSQI) or to 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine (3,5-dimethyl-NAPQI) respectively.

The aim of the present study was therefore to investigate the effects of 3,5-disubstitution, notably 3,5-dialkyl substitution and 3,5-dihalogen substitution of PAR on (1) the HRP/H_2O_2-dependent two-electron oxidation of PAR to the corresponding 3,5-disubstituted NAPQI analogues, (2) the binding of PAR to rat liver microsomal cytochrome P450, and (3) the rat liver microsomal cytochrome P450/NADPH-dependent two-electron oxidation of PAR to the corresponding 3,5-disubstituted NAPQI analogues. The various 3,5-disubstituted NAPQI analogues were synthesized for identification purposes and for determination of their stability.

Materials and methods

Chemicals

Paracetamol (PAR) and phenobarbital (PB) were obtained from Brocacef (Delft, The Netherlands). Oxidized glutathione (GSSG), GSSG reductase, horseradish peroxidase (HRP; type I approximately 80 U/mg), NADPH, *N*-ethylmaleimide (NEM) and nicotinamide were purchased from Sigma (St Louis, MO, USA), GSH and β-naphthoflavone (βNF) were from Janssen Chimica (Beerse, Belgium). Methyl iodide and ethyl iodide were from Aldrich (Brussels, Belgium). Catalase was from Boehringer GmbH (Mannheim, Germany), 1-ethynylpyrene was a kind gift of Dr W. L. Alworth (New Orleans, LA, USA) and 5,5′-dithiobis(2-nitrobenzoic acid) was from Merck (Darmstadt, Germany). 3,5-Dialkylated derivatives of PAR (R = $-CH_3$, $-C_2H_5$, $-i-C_3H_7$) were synthesized from their corresponding phenols according to Dearden and O'Hara (1978), 3,5-dihalogenated PAR derivatives (R = -F, -Cl, -Br, -I) were synthesized as described recently (Bessems *et al.* 1995). The 3-cysteine-*S*-yl-PAR conjugate was kindly provided by Sterling Winthrop (UK). All other chemicals were commercially obtained as analytical grades.

Synthesis of NAPQI and 3,5-disubstituted NAPQI analogues

NAPQI was synthesized by oxidation of PAR with silver(I)oxide (Ag_2O) according to the methods of Blair *et al.* (1980) and Dahlin and Nelson (1982). 3,5-Dimethyl-NAPQI was synthesized according to the procedure described by Fernando *et al.* (1980).

3,5-Dichloro-NAPQI was synthesized with silver(I)oxide as follows. 3,5-Dichloro-PAR (100 mg, 0·45 mmol) was dissolved in 15 ml $CHCl_3$. Freshly prepared silver oxide (200 mg, 0·9 mmol) was added and the mixture was subsequently stirred for 15 h at room temperature in a closed vial. The crude 3,5-diCl-NAPQI solution was filtered and centrifuged for 5 min to remove remaining Ag_2O and elemental silver. The resulting clear, yellow solution, was protected from light and stored at 4°C until use.

3,5-Difluoro-NAPQI was synthesized similarly by oxidizing 3,5-difluoro-PAR with freshly prepared Ag_2O. 3,5-Difluoro-PAR (11 mg, 0·06 mmol) was dissolved in 2 ml $CDCl_3$. After warming up in order to dissolve 3,5-difluoro-PAR, silver oxide (25 mg, 0·1 mmol) was added to the clear supernatant. This mixture was stirred for 15 h at room temperature in a closed vial. The crude 3,5-difluoro-NAPQI solution was filtered and centrifuged for 5 min in order to remove remaining Ag_2O and elemental silver. The resulting solution was protected from light and stored at 4°C.

3,5-Dibromo-NAPQI was synthesized analogously. 3,5-Dibromo-PAR (2·29 mg, 0·007 mmol) was dissolved in 0·75 ml $CDCl_3$. After addition of excess silver oxide the mixture was stirred for 0·5 h and centrifuged.

The purity and identity of the newly synthesized 3,5-disubstituted NAPQI analogues was assessed by tlc, UV-spectrophotometry, ^{1}H-nmr, and GC/MS.

Animals and preparation of microsomal fractions

Male Wistar rats (200–220 g; Harlan CPB, Zeist, The Netherlands) were housed in plastic cages in temperature (21–22°C) and humidity (60–65 %) controlled rooms with a 12-h light/dark cycle. Standard laboratory diet (Hope Farms, Woerden, The Netherlands) and tap water were provided *ad libitum*. Rats were starved overnight before isolation of the liver microsomal fractions. In the case of induction, the experimental animals were injected with βNF (80 mg/kg; dissolved in arachides oil) or PB (100 mg/kg; dissolved in saline) twice 48 and 24 h before decapitation. Under light diethyl ether narcosis, rats were decapitated and liver microsomal fractions were prepared by ultracentrifugation (Jefcoate 1978) and stored at −80°C. Protein concentrations were determined (Lowry *et al.* 1951). Before use, the microsomal fractions were rapidly thawed and kept on ice.

Spectral binding studies in rat liver microsomes

Spectral studies on haem ligand displacement from liver microsomal cytochrome P450 obtained from βNF-induced rats were performed with an Aminco DW-2a^{SB} UV/vis spectrophotometer as described (Schenkman *et al.* 1967). Microsomal fractions obtained from βNF-induced rats (2 mg/ml) in 50 mM K_2HPO_4/KH_2PO_4 (pH 7·4) including 0·1 mM EDTA, were divided between a sample and a reference cuvette. Aliquots of PAR and 3,5-dihalogenated PAR derivatives were dissolved in DMSO and added to the sample cuvette and equal volumes of DMSO were added to the reference cuvette. The final samples were mixed and allowed to equilibrate for 2 min before recording the difference spectrum. $\Delta A_{max} - A_{min}$'s were corrected for dilution, plotted against the final concentration of the 3,5-disubstituted PAR analogue and analysed by double reciprocal plots for the apparent spectral dissociation constants (K_s).

Microsomal and peroxidase catalysed two-electron oxidation reactions

Oxidation of PAR and the 3,5-disubstituted PAR analogues was studied in microsomal as well as peroxidase-catalysed incubations. Unless indicated otherwise, microsomal cytochrome P450 catalysed incubation conditions were 50 mM K_2HPO_4/KH_2PO_4 (pH 7·4), 1·0 mg/ml microsomal protein, 3 mM

$MgCl_2$, 2000 U/ml catalase and 0·3 mM PAR or a 3,5-disubstituted analogue (dissolved directly in buffer) in a final volume of 1 ml. Where applicable, 0·5 mM GSH was added to the preincubation mixture. After preincubation for 2 min at 37°C, typical incubations (10 min) were started by adding 0·3 mM NADPH. Catalase was added to the incubation mixtures to prevent oxidation of GSH by hydrogen peroxide. When used for analysis of GSSG formation, all results were corrected for GSSG formation in complete incubations without microsomal fractions. All concentrations stated are final concentrations upon incubation.

Typical incubations with HRP/H_2O_2 contained 0·15 mM PAR or a 3,5-disubstituted PAR analogue, 1·0 μg/ml HRP in 50 mM K_2HPO_4/KH_2PO_4 (pH 7·4) in a final volume of 1 ml. Reactions were started by adding 0·1 mM H_2O_2. In addition, the effect of adding 4 mM GSH, 1·5 mM bovine serum albumin, or 1 mg/ml microsomal protein on the peroxidase catalysed oxidation of 3,5-disubstituted PAR analogues was studied.

Ongoing reactions were either studied by UV-spectrophotometry or stopped after 10 min with 3 % $HClO_4$ for analysis of GSSG-formation or by putting the reaction tubes in ice-cold water for analysis of metabolite formation. Before analysis of GSSG formation, proteins were pelleted by centrifugation (10 min; 12000 *g*). For UV spectrophotometry, reference cuvettes contained the complete reaction mixture except substrate. HRP did not influence the UV absorbance of the various substrates.

Identification of NAPQI and 3,5-disubstituted NAPQI analogues

Two 3,5-disubstituted NAPQI analogues (3,5-diCH_3-NAPQI, representative for 3,5-dialkylated NAPQI analogues, and 3,5-diCl-NAPQI, regarded as representative for 3,5-dihalogenated NAPQI analogues), as well as NAPQI itself were primarily chosen for product identification purposes in HRP/H_2O_2 and microsomal incubations. NAPQI, 3,5-diCH_3-NAPQI as well as 3,5-diCl-NAPQI were found to be rather unstable in aqueous solutions and thus difficult to detect in incubations. To circumvent this problem, GSH (1·0 mM) was added to all HRP/H_2O_2 incubations as well as the cytochrome P450-catalysed incubations. The 3,5-disubstituted NAPQI analogues were subsequently detected as their glutathione-*S*-yl adducts by GC/MS after alkaline peralkylation (see below).

Analysis of sulphur-containing conjugates by GC/MS after alkaline peralkylation

Samples of HRP/H_2O_2 catalysed reactions or the microsomal incubations were subjected to a derivatization method as described by Slaughter and Hanzlik (1991) with a few modifications. First, by adding ascorbic acid (final concentration 1 mM) to the stopped reaction mixtures, the quinone imines were reduced to their respective *N*-acetylated *p*-aminophenols to prevent hydrolysis of the acetimide group during derivatization. For microsomal incubations, proteins were precipitated with the addition of 20 % trichloroacetic acid (w/v) followed by centrifugation. The NaOH-neutralized supernatant was used for the derivatization.

Of the samples, 0·5 ml was pipetted into 15 × 100-mm screw-cap (Teflon lined) glass reaction tubes and kept on ice. Then, 0·5 ml NaOH (8 M; N_2 purged) was added for cleavage of the glutathione carbon-sulphur bond by β-elimination. Finally, 0·5 ml ice-cold methyliodide (volatile and reactive) was added for *in situ* methylation of the thiol group. For the PAR incubations, ethyliodide was used in order to decrease the volatility of its derivatives. Nitrogen-flushed and tightly capped reaction tubes were immersed in a boiling water bath (100°C) for 1 h behind an explosion screen in a fume hood. Reaction tubes were then cooled on ice. Aliquots of 1·5 ml of the derivatization mixtures were extracted with *n*-pentane (2 × 3 ml), the pentane layer separated and slowly concentrated in an evaporation block (50°C). The residues were dissolved in 25 μl methanol and 1 μl samples were analysed by GC/MS.

NAPQI- and 3,5-disubstituted NAPQI-dependent GSH oxidation to GSSG

Supernatants of deproteinized microsomal incubations, were used for quantification of GSSG and for subsequent determination of several enzyme kinetic parameters. In order to examine the role of different isoforms of cytochrome P450, βNF-, PB- and uninduced (UT-) microsomes were used. To examine the role of βNF-inducible cytochrome P450s more specifically, 1-ethynylpyrene (dissolved in DMSO), a known inhibitor of CYP1A1 (Chan *et al.* 1993), was added to the incubation mixtures. Apparent K_m and V_{max} values were calculated from double reciprocal Lineweaver–Burke plots of the rate of GSH oxidation and the concentration of the substrates. To quantitate GSSG, 600-μl aliquots were taken from the supernatant of acid precipitated incubation samples and glutathione (GSH) was removed by alkylation (10 min) with 60 μl *N*-ethylmaleimide (NEM; 10 mM in reaction). Excess NEM was destroyed by alkaline hydrolysis essentially as described (Sacchetta *et al.* 1986). To adjust the pH for alkaline hydrolysis (final pH 11·2), 198 μl K_3PO_4/HCl buffer (3 M; pH 13) was added. After 10–15 min the samples were neutralized with 99 μl 30 % $HClO_4$ (v/v). Centrifugation at 12000 *g* removed precipitated $KClO_4$. In the clear supernatants, GSSG was quantified by flow-injection analysis as described by Redegeld *et al.* (1988). Calibration was performed by using standard solutions of GSSG. All values were corrected for GSSG formation in incubations without substrate.

Instrumentation

Proton nmr spectra were measured on a Bruker AC-200 spectrometer. GC/MS analyses were performed on a Hewlett Packard 5890 model GC and a Hewlett Packard 5971 MSD system set to scan between m/z 35 and 550. A CP-Sil capillary column (50 m) obtained from Chrompack B.V. (Middelburg, The Netherlands) was used. Unless otherwise stated, the operating conditions were 140 kPa (column head pressure), 250°C (split injector), electron energy 70 eV (EI/MS). The carrier gas was helium at a flow of about 3 ml/min. The GC oven temperature was kept at 60°C for 2 min and then increased (20°C/min) to 270°C. UV spectra were recorded in thermostated quartz cuvettes at 37°C on an Aminco DW-2a™ UV/vis spectrophotometer for microsomal incubations and a Philips PU 8720 UV/vis spectrophotometer for HRP/H_2O_2 oxidations.

Results

Synthesis of NAPQI and 3,5-disubstituted NAPQI analogues

Upon oxidation of PAR, 3,5-diCH_3-PAR and 3,5-diCl-PAR with silver oxide, respective NAPQI, 3,5-diCH_3-NAPQI and 3,5-diCl-NAPQI were formed in high yields (> 85 %; table 1). The ^{1}H-nmr and MS data of two known NAPQIs, NAPQI and 3,5-diCH_3-NAPQI (table 1), were similar to those reported previously (Blair *et al.* 1980, Fernando *et al.* 1980, Dahlin and Nelson 1982). As a representative of the 3,5-dihalogenated PAR analogues, structural analysis of 3,5-diCl-NAPQI was performed in detail. In the UV-spectrum of the synthetic 3,5-diCl-NAPQI, one absorption peak was discovered at λ_{max} 285 nm (table 1). Upon reduction of synthetic 3,5-diCl-NAPQI with sodium dithionite, a product was formed, which by UV spectrophotometry was indistinguishable from authentic 3,5-diCl-PAR. Judged from ^{1}H-nmr spectra, the oxidation of 3,5-diCl-PAR was complete. The aromatic protons of 3,5-diCl-NAPQI appeared at higher field (δ 7·19 ppm) when compared with the corresponding protons in 3,5-diCl-PAR (δ 7·45 ppm; table 1). The only resonance (δ 7·01 ppm; about 14 %) that could not be assigned to 3,5-diCl-NAPQI was identical to the ^{1}H-nmr resonance of authentic recrystallized 2,6-dichloro-1,4-benzoquinone (data not shown).

Upon GC/MS analysis of synthetic 3,5-diCl-NAPQI, in the total ion current chromatogram three peaks were observed with retention times of 6·99, 12·06 and 12·63 min respectively (figure 1). Mass spectra of these peaks all exhibited the characteristic isotopic mass pattern for two chlorine atoms (10:6:1). The peaks at retention times 6·99 min (15 area %) and 12·63 min (7 area %) corresponded to those of 2,6-dichloro-1,4-benzoquinone and 3,5-diCl-PAR respectively by comparison with retention times and mass spectra of authentic samples of the latter compounds (table 1). The peak at retention time 12·06 min (75 area %) was identified as 3,5-diCl-NAPQI.

For 3,5-diF-NAPQI and 3,5-diBr-NAPQI, both synthesized only in analytical quantities, ^{1}H-nmr and UV spectrophotometric data are also presented in table 1. All products appeared to be > 85 % pure upon synthesis, as judged from ^{1}H-nmr, and were used without further purification.

Spectral binding studies in rat liver microsomes

The binding of PAR and the seven 3,5-disubstituted PAR analogues to rat hepatic microsomal cytochrome P450 was measured by UV difference spectrophotometry. Upon addition of the 3,5-disubstituted analogues to solutions of βNF-induced rat liver microsomes, difference spectra with a peak of 422 nm and a trough at 387 nm were recorded, indicating a shift of the haem spin-equilibrium from high to low spin (Gibson and Tamburini 1984). 3,5-Dihalogen substitution of

Table 1. [1]H-nmr, MS and UV characterization of paracetamol, 3,5-dimethylated and 3,5-dihalogenated analogues of PAR, synthesized NAPQI, and the synthesized 3,5-disubstituted NAPQI analogues.

Compound	[1]H-nmr: δ in ppm[1]	MS: m/z (intens. %)[2]	GC[3] (min)	λ_{max}[4] (nm)	Yield[5] (%)
PAR	2·13 (s, 3H), 4·75 (s, 1H, OH or NH), 6·77 (d, 2H, $\mathcal{J}$ = 8 Hz), 7·31 (d, 2H, $\mathcal{J}$ = 8 Hz)	151 (28 %, $M^{+\cdot}$); 109 (100 %, $[M\text{-}COCH_3]^{+\cdot}$)	9·1	248	
NAPQI	2·29 (s, 3H), 4H-AB quartet, d 6·97, 6·62 ($\mathcal{J}$ = 10 Hz)	151 (37 %, $M^{+\cdot}$); 109 (100 %, $[M\text{-}COCH_3]^{+\cdot}$)	8·5	264	> 95
3,5-DiCH_3-PAR	2·11 (s, 3H), 2·21 (s, 6H), 4·6 (s, 1H, OH or NH), 7·06 (s, 2H)	179 (54 %, $M^{+\cdot}$); 137 (100 %, $[M\text{-}CH_2CO]^{+\cdot}$)	11·5	249	
3,5-DiCH_3-NAPQI	2·27 (s, 3H), 2·03 (s, 6H), 6·72 (s, 2H)	177 (85 %, $M^{+\cdot}$); 135 (15 %, $[M\text{-}CH_2CO]^{+\cdot}$)	9·0	274	> 95
3,5-DiF-PAR	2·14 (s, 3H), 5·02 (s, 1H, OH or NH), 7·13 (d, 2H, $\mathcal{J}_{FH}$ = 8 Hz)	187 (38 %, $M^{+\cdot}$); 145 (100 %, $[M\text{-}CH_2CO]^{+\cdot}$)	[7]	254	
3,5-DiF-NAPQI	2·21 (d, 3H, $\mathcal{J}_{FH}$ = 6 Hz), 6·68 (d, 2H, $\mathcal{J}_{FH}$ = 9 Hz)	nd[6]	nd	272	> 85
3,5-DiCl-PAR	2·14 (s, 3H), 5·67 (s, 1H, OH or NH), 7·45 (s, 2H)	219 (45 %, $M^{+\cdot}$ $^{35}Cl_2$); 177 (100 %, $[M\text{-}CH_2CO]^{+\cdot}$)	12·6	260	
3,5-DiCl-NAPQI	2·30 (s, 3H), 7·19 (s, 2H)	219 (34 %, $M^{+\cdot}$ $^{35}Cl_2$); 177 (100 %, $[M\text{-}CH_2CO]^{+\cdot}$)	12·1 75 %[8]	289	86
3,5-DiBr-PAR	2·14 (s, 3H), 5·72 (s, 1H, OH or NH), 7·64 (s, 2H)	309 (41 %, $M^{+\cdot}$ $^{79}Br^{81}Br$); 267 (100 %, $[M\text{-}CH_2CO]^{+\cdot}$)	[7]	nd	
3,5-DiBr-NAPQI	2·30 (s, 3H), 7·47 (s, 2H)	nd	nd		nd > 95

[1] Recorded in $CDCl_3$ (δ in ppm), internal standard was TMS.
[2] Partial mass spectra (EI-MS) using either GC or direct introduction of the samples.
[3] GC retention time.
[4] Recorded in potassium phosphate buffer (50 mM, pH 7·4). Compounds were predissolved in ethanol (not exceeding 1 %).
[5] Yields of the synthesized 3,5-dihalogenated NAPQI-analogues, as determined by [1]H-nmr.
[6] nd, not determined.
[7] Mass spectrum obtained by direct introduction of the sample.
[8] Total Ion Current, area %. Partial reduction on GC-column.

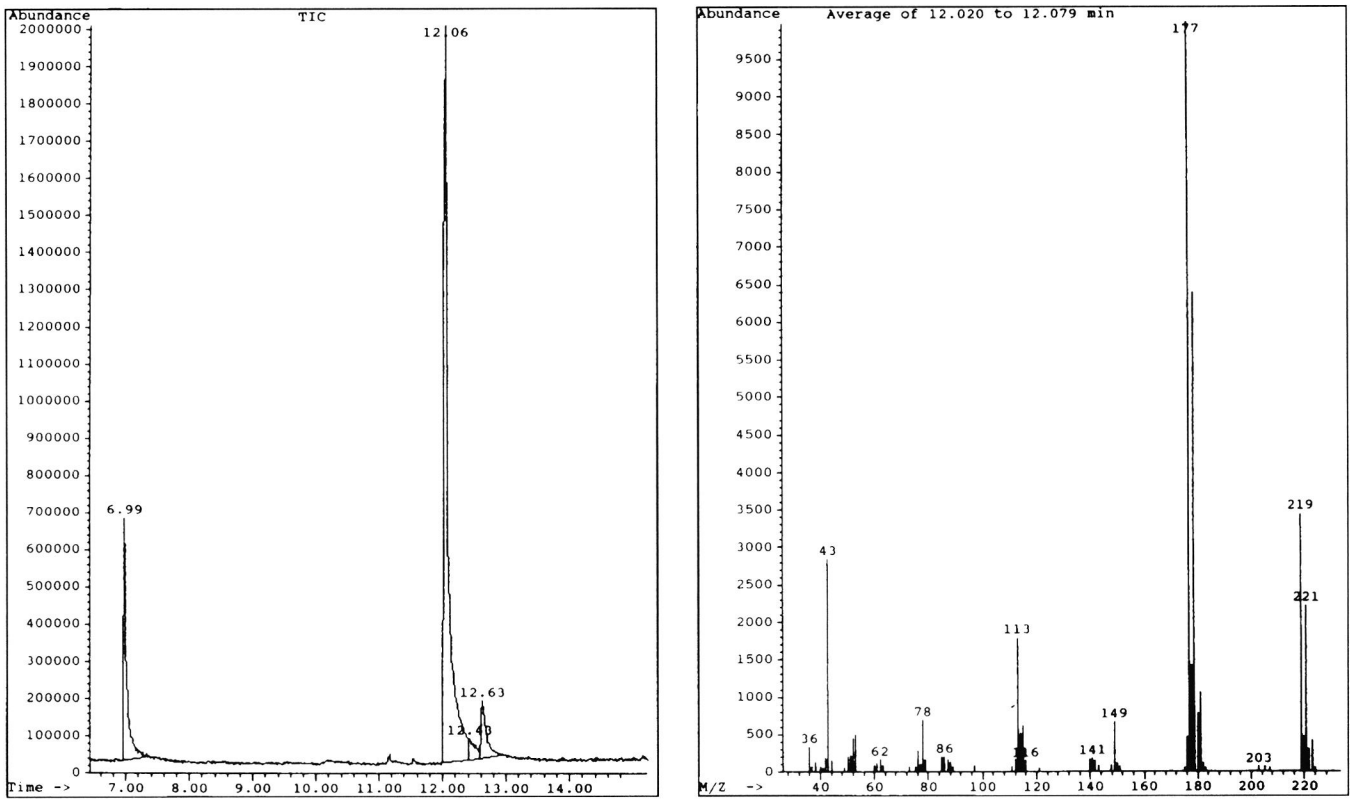


Figure 1. GC/MS analysis of synthetic 3,5-diCl-NAPQI. (a) Total ion chromatogram. (b) Mass spectrum of GC peak eluting at 12.06 min.

Table 2. Paracetamol and 3,5-disubstituted analogues: spectral dissociation and Michaelis–Menten constants with βNF-induced rat liver microsomal P450, and Σf's.

Compounds	$K_{s,app}$ [1]	$K_{m,app}$ [1]	V_{max} [1]	V_{max}/K_m	Σf [3]
PAR	2·8	0·73	0·66	0·9	0·408
3,5-DiCH$_3$-PAR	0·86	0·13	3·0	23·1	1·448
3,5-DiC$_2$H$_5$-PAR	0·27	0·07	1·7	24·3	2·486
3,5-Di*i*C$_3$H$_7$-PAR	0·14	0·21	1·8	8·6	3·526
3,5-DiF-PAR	0·19	0·64	0·82	1·3	0·888
3,5-DiCl-PAR	0·26	0·16	0·77	4·8	1·866
3,5-DiBr-PAR	0·26	0·10	0·85	8·5	2·268
3,5-DiI-PAR	[2]	0·07	1·7	24·3	2·892

[1] $K_{s,app}$ and $K_{m,app}$ are expressed in mM, V_{max} is in nmol min^{-1} mg^{-1} protein.
[2] No $K_{s,app}$ could be determined.
[3] Σf's were calculated according to the hydrophobic fragmental system developed by Rekker and Mannhold (1992).

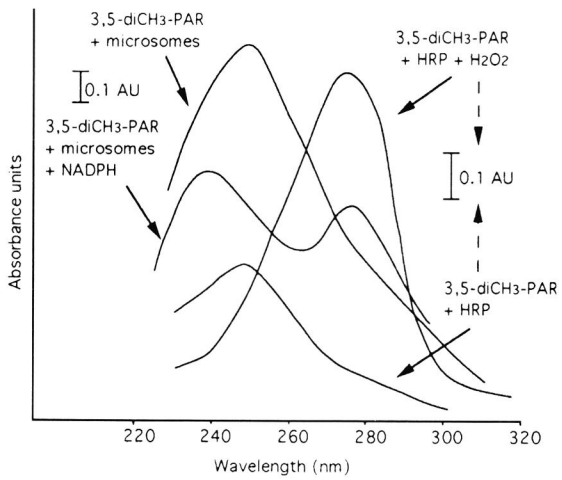


Figure 2. Cytochrome P450- and H_2O_2/peroxidase-mediated oxidation of 3,5-diCH$_3$-PAR as monitored by UV difference spectroscopy. The microsomal incubation was started by NADP$^+$ (0·1 mM), and contained βNF-microsomes (0·1 mg/ml), 3,5-diCH$_3$-PAR (0·25 mM), and an NADPH-regenerating system (0·5 mM glucose 6-phosphate; glucose 6-phosphate dehydrogenase 0·1 U/ml). The peroxidase incubation contained HRP (1 μg/ml), 3,5-diCH$_3$-PAR (25 μM) and H_2O_2 (100 μM).

PAR substantially decreased the $K_{s,app}$ for the binding of PAR to βNF-induced microsomal P450, from 2·8 to 0·19, 0·26 and 0·26 mM for R = -F, -Cl, and -Br respectively (table 2). The alkyl substituents decreased the apparent K_s values as well, namely to 0·86, 0·27 and 0·14 mM for R = -CH$_3$, -C$_2$H$_5$, and -*i*C$_3$H$_7$ respectively (table 2). A good correlation ($r = 0·938$) was observed between $K_{s,app}$ values and the estimated lipophilicity of PAR and the 3,5-disubstituted analogues, as calculated by summation of f (table 1) using the hydrophobic fragmental constant approach (Rekker and Mannhold 1992):

$$\log(1/K_{s,app}) = 0·425\,(\pm 0·079)\,\Sigma f - 0·481\,(\pm 0·174)$$
$$n = 6 \quad r = 0·938\, s = 0·184.$$

On statistical ground, 3,5-diF-PAR was rejected as being an outlier.

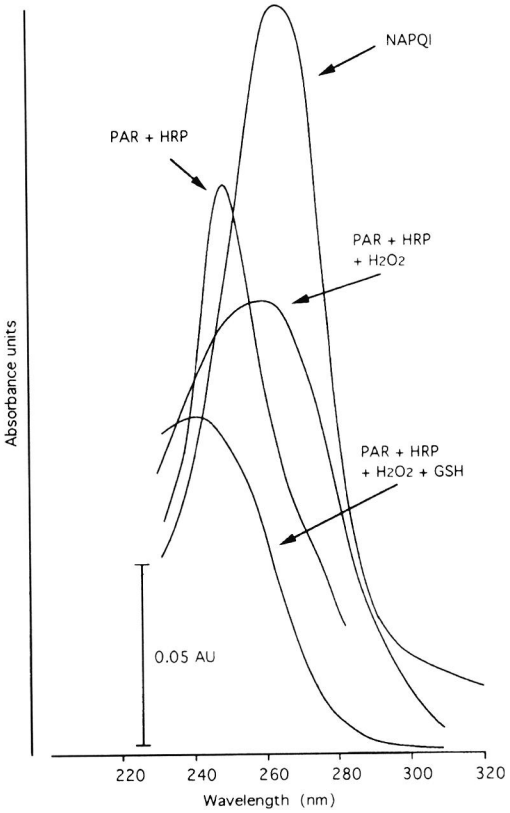


Figure 3. UV difference spectroscopy of synthetic NAPQI and of various peroxidase-mediated oxidation reactions of PAR. Additions were HRP (1 μg/ml), PAR (25 μM), H_2O_2 (200 μM) and GSH (4 mM).

UV-spectrophotometric analysis of microsomal and peroxidase catalysed two-electron oxidation

Rat liver βNF-induced microsomal cytochrome P450-catalysed oxidation of 3,5-diCH_3-PAR was studied first. The formation of a product with UV-absorbance characteristics similar to authentic *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (3,5-diCH_3-NAPQI) was observed (figure 2). A significant decay of the absorption of 3,5-diCH_3-PAR at 249 nm was accompanied by a simultaneous increase of a maximum absorbance at 274 nm. Formation of the peak at 274 nm was more rapid when an NADPH-regenerating system was used instead of NADPH. The absorption at 274 nm was abolished upon addition of GSH (4 mM) to the incubation mixture. Upon incubation of PAR, 3,5-diF-PAR, and 3,5-diCl-PAR with microsomal fractions of βNF-induced rat liver, no significant increases were observed at λ_{max}'s measured for the synthetic NAPQI (264 nm), 3,5-diF-NAPQI (272 nm), and 3,5-diCl-NAPQI (289 nm) respectively. Therefore, experiments studying the peroxidase-catalysed two-electron oxidation of PAR analogues were performed as well.

Horseradish peroxidase (HRP)/H_2O_2 catalysed oxidation of PAR and three representative 3,5-disubstituted PAR analogues (R = -CH_3, -F, -Cl) was studied by UV spectrophotometry. The HRP/H_2O_2 catalysed two-electron oxidation of 3,5-

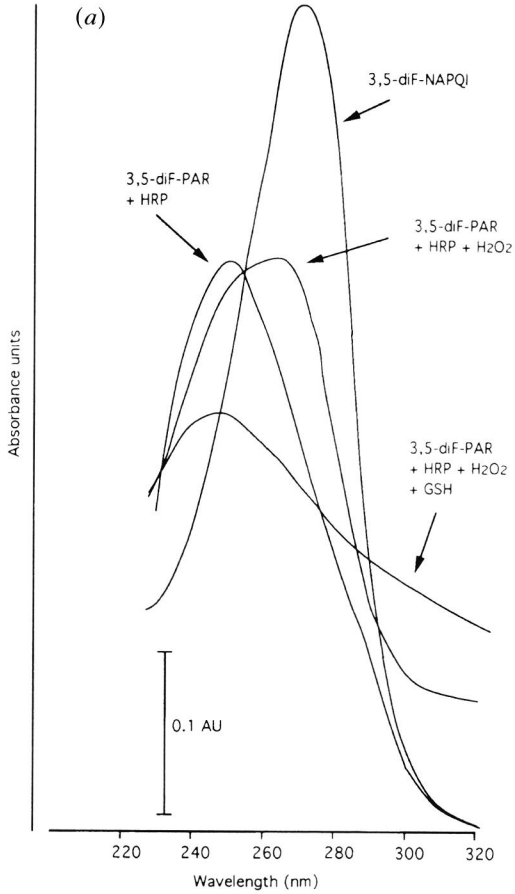


Figure 4(*a*). For legend see facing page.

diCH$_3$-PAR yielded *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (3,5-diCH$_3$-NAPQI). Decay of the absorption of 3,5-diCH$_3$-PAR at 249 nm was accompanied by a simultaneous increase at 274 nm, exhibiting a spectrum identical to that of authentic 3,5-diCH$_3$-NAPQI (figure 2). When 3,5-diCH$_3$-NAPQI was formed, and rat liver microsomes were added (1 mg/ml), the UV spectrum changed to a spectrum similar to that observed for 3,5-diCH$_3$-PAR. The same phenomenon was observed upon addition of an excess amount of bovine serum albumin (1·5 mM) or GSH (4 mM) to the sample cuvette (data not shown).

In a HRP/H_2O_2 incubation with PAR as a substrate, the decay of the PAR absorption at 248 nm was accompanied by a simultaneous increase in the absorption around 260 nm (figure 3) leading to a spectrum similar to authentic NAPQI. Upon addition to the incubation mixture of GSH (4 mM), the absorbance at 260 nm decreased. Comparable results were observed for 3,5-diF-PAR and 3,5-diCl-PAR. Upon incubation of 3,5-diF-PAR in the HRP/H_2O_2 system, the absorption of 3,5-diF-PAR at 254 nm decreased whereas the absorption at 272 nm, typical for *N*-acetyl-3,5-difluoro-*p*-benzoquinone imine (3,5-diF-NAPQI), increased (figure 4a). The resulting UV-absorbance spectrum was identical to that of synthetic 3,5-diF-NAPQI. Furthermore, upon addition of GSH (4 mM) the absorption at 272 nm

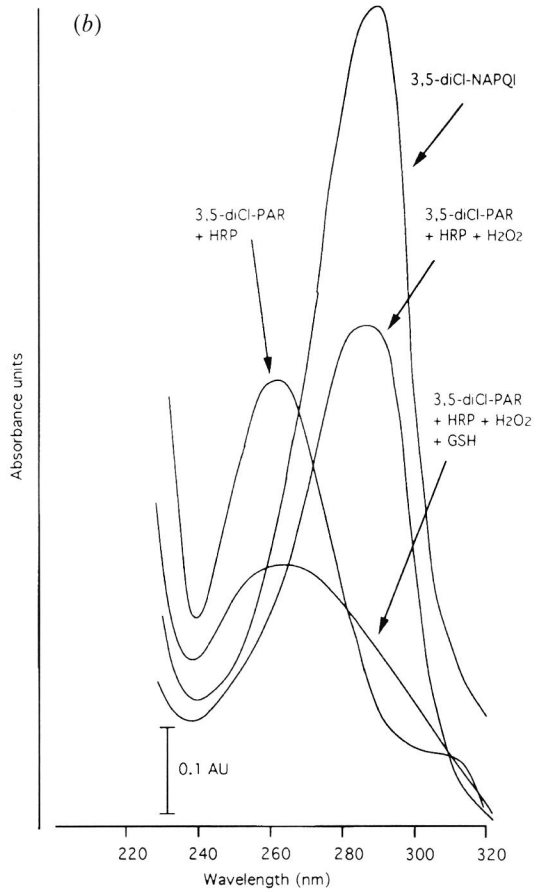


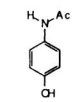
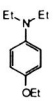
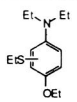
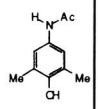
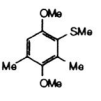
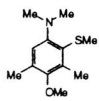
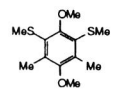
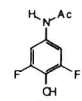
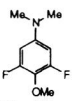
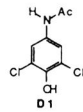
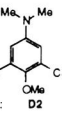
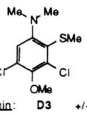
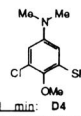
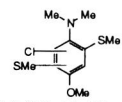
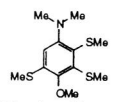
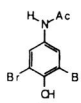
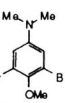
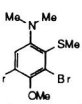
Figure 4. UV difference spectroscopy. (a) Synthetic 3,5-diF-NAPQI and the peroxidase-mediated oxidation of 3,5-diF-PAR; additions were HRP (1 μg/ml), 3,5-diF-PAR (100 μM), H_2O_2 (200 μM), and GSH (4 mM). (b) Synthetic 3,5-diCl-NAPQI and the peroxidase-mediated oxidation of 3,5-diCl-PAR; additions were HRP (10 μg/ml), 3,5-diCl-PAR (25 μM), H_2O_2 (100 μM), and GSH (4 mM).

decreased substantially. Decay of the absorption of 3,5-diCl-PAR at 260 nm in a HRP/H_2O_2 system was accompanied by a simultaneous increase at λ_{max} of 3,5-diCl-NAPQI at 289 nm (figure 4b). The resulting UV spectrum was identical to that obtained from chemically synthesized 3,5-diCl-NAPQI. Upon addition of GSH (4 mM) to the sample mixture in the cuvette, the absorption of 3,5-diCl-NAPQI at 289 nm decreased as well.

GC/MS analysis of microsomal and peroxidase catalysed two-electron oxidation productions

For PAR and four 3,5-disubstituted derivatives (R = -CH_3, -F, -Cl, and -Br), peroxidase and microsomal incubations in the presence of GSH were analysed by GC/MS for sulphur-containing metabolites. The incubation samples were derivatized by alkaline peralkylation and the results, including the GC/MS characteristics, are presented in table 3. Analysis of HRP/H_2O_2 incubations with PAR

Table 3. GC/MS analysis after alkaline peralkylation. HRP/H_2O_2 and microsomal incubations with PAR or 3,5-disubstituted analogues.[1,2]

Substrate	Derivative[3,4]	Products[3,4]			
 **A 1**	 7.7 min: **A2** +/+ 193 (48%,$M^{+\cdot}$); 178 (100%,$[M\text{-}CH_3]^{+\cdot}$); 164 (47%,$[M\text{-}C_2H_5]^{+\cdot}$)	 9.9 min: **A3** +/+ 253 (98%,$M^{+\cdot}$); 238 (52%,$[M\text{-}CH_3]^{+\cdot}$); 224 (100%,$[M\text{-}C_2H_5]^{+\cdot}$)			
 **B 1**	[5]	 8.5 min: **B3** +/+ 212 (100%, $M^{+\cdot}$); 197 (78%,$[M\text{-}CH_3]^{+\cdot}$)	 8.7 min: **B4** +/- 225 (100%,$M^{+\cdot}$); 210 (100%,$[M\text{-}CH_3]^{+\cdot}$); 195 (52%,$[M\text{-}C_2H_5]^{+\cdot}$)	 9.8 min: **B5** +/- 258 (100%,$M^{+\cdot}$); 243 (32%,$[M\text{-}CH_3]^{+\cdot}$)	
 **C 1**	 6.4 min: **C2** +/+ 187 (47%,$M^{+\cdot}$); 172 (100%,$[M\text{-}CH_3]^{+\cdot}$)	[6]			
 **D 1**	 8.6 min: **D2** +/+ $^{35}Cl_2$ 219 (33%,$M^{+\cdot}$); 204 (100%,$[M\text{-}CH_3]^{+\cdot}$)	 9.8 min: **D3** +/+ $^{35}Cl_2$ 265 (94%,$M^{+\cdot}$); 250 (100%,$[M\text{-}CH_3]^{+\cdot}$)	 10.1 min: **D4** +/- ^{35}Cl 231 (100%,$M^{+\cdot}$); 216 (96%,$[M\text{-}CH_3]^{+\cdot}$)	 10.4+11.3 min: **D5** +/- ^{35}Cl 277 (100%,$M^{+\cdot}$); 252 (88%,$[M\text{-}CH_3]^{+\cdot}$)	 11.8 min: **D6** +/- 289 (100%,$M^{+\cdot}$); 274 (50%,$[M\text{-}CH_3]^{+\cdot}$) 259 (81%,$[M\text{-}2CH_3]^{+\cdot}$)
 **E1**	 9.7 min: **E2** +/+ $^{79}Br^{81}Br$ 309 (38%,$M^{+\cdot}$); 294 (100%,$[M\text{-}CH_3]^{+\cdot}$)	 10.7 min: **E3** +/+ $^{79}Br^{81}Br$ 355 (100%,$M^{+\cdot}$); 340 (90%,$[M\text{-}CH_3]^{+\cdot}$)			

[1] HRP/H_2O_2 preincubation mixtures contained 50 mM K_2HPO_4/KH_2PO_4 (pH 7.4): HRP (10 μg/ml), PAR or a 3,5-disubstituted analogue (1 mM), and GSH (1.0 mM). Incubations were started by adding H_2O_2 (1 mM). Metabolism was H_2O_2-dependent. Microsomal preincubation mixtures contained 50 mM K_2HPO_4/KH_2PO_4 (pH 7.4): ßNF-microsomes (1 mg/ml), PAR or a 3,5-disubstituted analogue (2 mM), and GSH (1.0 mM). Incubations were started by adding NADPH (0.25 mM). All sulphur-containing metabolites detected were found upon NADPH-dependent microsomal oxidation of the substrates.

[2] PAR incubations and 3,5-disubstituted PAR analogue incubations were derivatized with ethyl iodide and methyl iodide respectively.

[3] GC/MS retention times (min) and partial mass spectra (m/z) with their relative intensities (%) are presented.

[4] Detection or absence of the derivative in HRP/H_2O_2 and/or microsomal incubations is indicated respectively by + and/or –.

[5] The permethylated substrate 3,5-diMe-PAR was not detected.

[6] No products were found that could be attributed to biotransformation of 3,5-diF-PAR.

Table 4. Inhibition of rat liver microsomal cytochrome P450-dependent GSSG formation in incubations containing a 3,5-dialkylated or a 3,5-dihalogenated PAR analogue.[1]

Compound	GSSG formation (nmol/min/mg protein)	
	DMSO[2]	DMSO + EP[3]
3,5-diCH_3-PAR	1·84	0·36
3,5-diC_2H_5-PAR	1·12	0·32
3,5-DiF-PAR	0·06	0·00
3,5-DiCl-PAR	0·15	0·03
3,5-DiBr-PAR	0·59	0·22
3,5-DiI-PAR	0·74	0·31

[1] Substrate concentrations were 0·5 mM (3,5-dialkylated PAR analogues) and 1·0 mM (3,5-dihalogenated PAR analogues). GSSG formation was determined upon a single incubation with rat liver microsomal fractions (1 mg/ml) in the presence of NADPH (1 mM) and GSH (0·5 mM).

[2] DMSO (1 %).

[3] 1-Ethynylpyrene (10 μM; dissolved in 1 % DMSO).

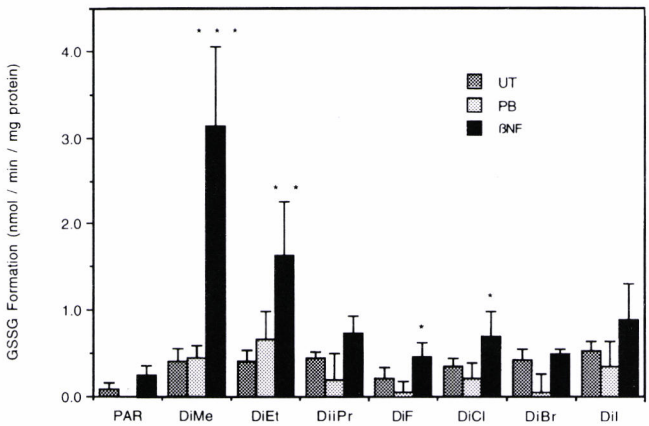


Figure 5. Effects of induction on the GSSG formation ($n = 3$; $\pm$SD) upon incubation of PAR and 3,5-disubstituted analogues (all 1 mM except 3,5-diI-PAR and 3,5-di*i*C_3H_7-PAR which were 0·25 mM) with rat liver microsomal fractions (1 mg/ml) in the presence of NADPH (1 mM) and GSH (0·5 mM). DiMe, 3,5-diCH_3-PAR; DiEt, 3,5-diC_2H_5-PAR; DiiPr, 3,5-di*i*C_3H_7-PAR; DiF, 3,5-diF-PAR; DiCl, 3,5-diCl-PAR; DiBr, 3,5-diBr-PAR; DiI, 3,5-diI-PAR. UT, βNF and PB are microsomes derived from animals that were untreated or induced with β-naphthoflavone or phenobarbital respectively. Student's t-test ($p \leqslant 0{\cdot}05$) statistics: * βNF-microsomes significantly different from PB microsomes; ** βNF-microsomes significantly different from UT microsomes; and *** βNF-microsomes significantly different from UT as well as from PB microsomes.

revealed a product (product A3) eluting at a retention time of 9·5 min with the following MS characteristics: m/z 253 ($M^{+\cdot}$), m/z 238 [M-CH_3]$^{+\cdot}$, and m/z 224 [M-C_2H_5]$^{+\cdot}$. Identical data, which were attributed to *N*,*N*-diethyl-3-thio-ethyl-4-ethoxyaniline, were obtained with a synthetic 3-cysteine-*S*-yl-conjugate of PAR was derivatized by alkaline peralkylation with ethyl iodide. Also upon microsomal oxidation of PAR product A3 could easily be detected using the same analysis. Upon analysis of the HRP/H_2O_2 incubation mixture with 3,5-diCH_3-PAR, three peaks were found (products B1, B2 and B3) that could be attributed to sulphur-containing derivatives of 3,5-diCH_3-PAR. Two of these products appeared to have lost their *N*-acetyl-group. In the microsomal incubation of 3,5-diCH_3-PAR only one of these three derivatives, i.e. product B3, was found.

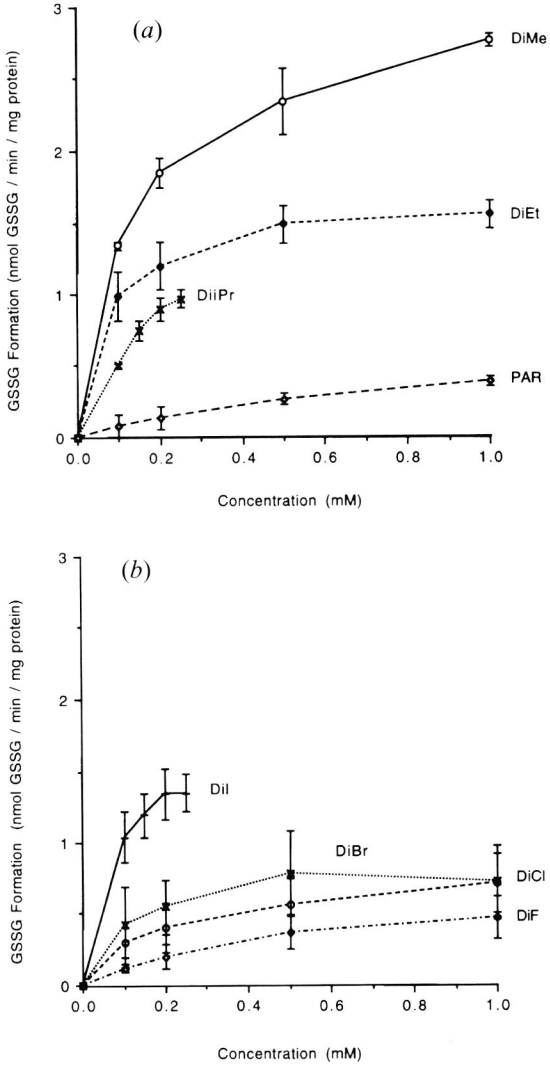


Figure 6. Formation of GSSG during incubation of various concentrations of PAR and 3,5-disubstituted analogues with hepatic βNF-microsomes in the presence of NADPH, GSH and oxygen. All values were corrected for GSSG formation in complete incubations without microsomes. (a) PAR and 3,5-dialkylated analogues: DiMe, 3,5-diCH_3-PAR; DiEt, 3,5-diC_2H_5-PAR; DiiPr, 3,5-di*i*C_3H_7-PAR. Values are the mean of three determinations ± SD. (b) 3,5-Dihalogenated PAR analogues: DiF, 3,5-diF-PAR; DiCl, 3,5-diCl-PAR; DiBr, 3,5-diBr-PAR; DiI, 3,5-diI-PAR. Values are the mean of four determinations ± SD.

Upon analysis of the microsomal and HRP/H_2O_2 incubation mixtures with 3,5-diF-PAR, no sulphur-containing derivatives were found. However, when 3,5-diCl-PAR was oxidized in the HRP/H_2O_2 system in the presence of GSH, three sulphur-containing metabolites, D3, D4 and D5, were found (table 3). In rat liver microsomal incubations of 3,5-diCl-PAR, only product D3 was found. Incubation of 3,5-diBr-PAR with HRP/H_2O_2 as well as with rat liver microsomes revealed the formation of only one sulphur-containing product (i.e. E3, table 3).

NAPQI- and 3,5-disubstituted NAPQI-dependent GSH oxidation to GSSG

The extent of two-electron oxidation of PAR and the 3,5-disubstituted analogues to the corresponding NAPQIs by rat liver microsomal fractions in the presence of GSH and NADPH was also investigated by measuring the oxidation of GSH to GSSG. Pretreatment of rats with PB or βNF differentially affected the liver microsomal oxidation of PAR and the 3,5-disubstituted analogues to corresponding NAPQI analogues. As shown in figure 5, βNF-induction increased the oxidation of all compounds, whereas induction by PB decreased it in most cases. For 3,5-diC_2H_5-PAR a significant difference was found between βNF-microsomes and uninduced (UT-) microsomes, with a GSSG formation of $1{\cdot}63 \pm 0{\cdot}62$ and $0{\cdot}42 \pm 0{\cdot}11$ nmol min^{-1} mg^{-1} protein respectively. For 3,5-diCH_3-PAR, the rates of GSSG-formation with βNF-microsomes were significantly different from those with PB- as well as UT-microsomes ($3{\cdot}14 \pm 0{\cdot}92$ versus $0{\cdot}44 \pm 0{\cdot}16$ and $0{\cdot}41 \pm 0{\cdot}14$ nmol min^{-1} mg^{-1} protein respectively). For 3,5-diF-PAR and 3,5-DiBr-PAR, significant differences were also observed in the microsomal oxidation as quantified by GSSG formation (figure 5). For 3,5-diF-PAR, the oxidation rates were $0{\cdot}46 \pm 0{\cdot}16$ and $0{\cdot}05 \pm 0{\cdot}13$ nmol min^{-1} mg^{-1} protein for βNF- and PB-microsomes respectively, and for 3,5-diBr-PAR $0{\cdot}51 \pm 0{\cdot}05$ and $0{\cdot}06 \pm 0{\cdot}20$ nmol min^{-1} mg^{-1} protein respectively. In addition, 1-ethynylpyrene substantially decreased the formation of GSSG in incubations with βNF-induced microsomes (table 4).

The GSSG-formation in rat liver microsomal incubations incubated with PAR and 3,5-disubstituted analogues was found to obey Michaelis–Menten kinetics (figure 6a and b). The apparent K_m, V_{max} and V_{max}/K_m values of PAR, the 3,5-dialkylated and 3,5-dihalogenated derivatives obtained with βNF-microsomes are presented in table 2.

Discussion

The aim of the present study was to investigate the two-electron oxidation of 3,5-disubstituted analogues of PAR to their corresponding 3,5-disubstituted NAPQI analogues in rat liver microsomes. 3,5-Disubstitution of PAR has been shown to affect differentially its biological activities. 3,5-Dialkylation decreases analgesic activity substantially (Dearden *et al.* 1980), whereas 3,5-difluorination has been shown to decrease the analgesic activity of PAR only slightly (Barnard *et al.* 1993b). Recently it was shown that the cyclooxygenase inhibiting capacity was also decreased by 3,5-dichloro-, 3,5-dibromo-, and 3,5-diiodo-substitution when compared with unsubstituted PAR (Bessems *et al.* 1995). The cytotoxicity in freshly isolated rat hepatocytes of 3,5-dihalogenated PAR analogues, however, was similar to that of PAR itself (Bessems *et al.* 1995); in contrast, 3,5-dialkylation of PAR has been shown to reduce the hepatotoxicity significantly (Van de Straat *et al.* 1987b). It is generally accepted that cytochrome P450 plays a vital role in the development of toxicity of PAR and the formation of NAPQI (Potter *et al.* 1973, Hinson 1980, Harvison *et al.* 1988, Patten *et al.* 1993). However, investigations as to the role of cytochrome P450 in the metabolism and the bioactivation of 3,5-disubstituted PAR analogues and the nature of possible reactive metabolites involved are limited. Therefore, in this study the effects of 3,5-disubstitution were investigated on their interaction with cytochrome P450, and on their oxidation by cytochrome P450 to the respective 3,5-disubstituted NAPQI analogues.

The 3,5-dihalogenated NAPQI analogues were synthesized by oxidation of the parent 3,5-dihalogenated PAR analogues in chloroform by silver oxide and the products appeared to be $> 85\%$ pure as judged from ^{1}H-nmr analysis. Synthetic 3,5-diCl-NAPQI was further analysed by GC/MS. The GC/MS analysis of the synthetical 3,5-diCl-NAPQI revealed two minor extra GC-peaks of which one (retention time 12·63 min; 7 area %) was assigned to 3,5-diCl-PAR and which may be due to reduction of this strong oxidant by traces of water in the sample or in the injector of the GC. The GC-peak, attributed to 3,5-diCl-NAPQI, exhibited a molecular ion at m/z of $[M+2]^{+\cdot}$. It is known, that quinones with a high redox potential may be reduced to hydroquinones by residual moisture in the inlet system and the ionization chamber of the mass-spectrometer (Zeller 1974). Therefore, 3,5-diCl-NAPQI was probably also reduced to 3,5-diCl-PAR. Both in a study of Dahlin and Nelson (1982), as well as in this study, a small $[M+2]^{+\cdot}$ ion was also detected in the mass spectrum of NAPQI. 3,5-DiCl-PAR, therefore, appeared to be absent in synthetic mixtures of 3,5-diCl-NAPQI as judged from ^{1}H-nmr. For the second additional GC-peak (retention time 6·99 min, 15 area %), both GC/MS and ^{1}H-nmr analyses pointed to the presence of an impurity of 2,6-dichloro-1,4-benzoquinone. This may be attributed to partial hydrolysis of 3,5-diCl-NAPQI (15% analogous to the hydrolysis of NAPQI to 1,4-benzoquinone previously observed (Dahlin and Nelson 1982).

UV-spectrophotometric analysis of all synthetic 3,5-disubstituted NAPQI analogues revealed blue-shifted λ_{max} values when compared with unsubstituted NAPQI. For NAPQI and 3,5-diCH_3-NAPQI, the ε's measured (data not shown) resembled previously reported values (Fischer *et al.* 1985). Upon dissolution of synthetical NAPQI and 3,5-diCH_3-NAPQI in potassium phosphate buffer shoulders were observed in the UV-absorption spectra, which might be explained by partial reduction to PAR and 3,5-diCH_3-PAR respectively and possibly to hydrolysis to the corresponding 2,6-disubstituted-1,4-benzoquinones, and/or radical coupling polymerization reactions (Dahlin and Nelson 1982).

In the present study, HRP/H_2O_2 was used as a model system for two-electron oxidation of 3,5-disubstituted PAR analogues to the corresponding NAPQI analogues. Preliminary studies with electron spin resonance (ESR) analysis revealed no formation of one-electron oxidized semiquinone imine (NAPSQI) species at the concentrations of HRP and H_2O_2 used in this study (data not shown). Formation of 3-hydroxy-PAR, a catechol, known to be a cytochrome P450-dependent metabolite of PAR (Hinson *et al.* 1980, Forte *et al.* 1984) has never been found in the presence of peroxidases. UV spectrophotometric studies with HRP/H_2O_2 confirmed the formation of NAPQI from PAR and 3,5-disubstituted NAPQI's from three 3,5-disubstituted analogues, 3,5-diCH_3-PAR, 3,5-diF-PAR and 3,5-diCl-PAR (figures 2–4). Upon addition of GSH to the incubations, the respective absorbances decayed, indicating disappearance of the quinone imine structures, caused either by a reduction or a conjugation reaction with GSH. Furthermore, several sulphur-containing derivatives were detected upon alkaline peralkylation of parallel HRP/H_2O_2 incubations with PAR and the 3,5-disubstituted analogues in the presence of GSH (table 3), thus supporting the occurrence of conjugation reactions with GSH. Since the products B4 and D3, found in incubations with 3,5-diCH_3-PAR and 3,5-diCl-PAR respectively contained sulphur substituents *ortho* to the nitrogen substituent, it was concluded that 3,5-disubstituted PAR analogues are liable to conjugation with GSH after oxidation to their respective 3,5-disubstituted

NAPQI analogues. Furthermore, upon analysis of incubations with 3,5-diCH_3-PAR, 1,4-dimethoxy-2-thiomethyl-3,5-dimethylbenzene (product B3) was found, indicating hydrolysis of 3,5-diCH_3-NAPQI to 2,6-dimethyl-1,4-benzoquinone, which subsequently conjugated to GSH at one of the unsubstituted ring carbon atoms. In the 3,5-diCH_3-PAR and the 3,5-diCl-PAR incubation mixture, di-sulphur-containing compounds (products B5 and D5; table 3) were found upon GC/MS analysis as well, indicating reoxidation of a monoglutathiolated product. Interestingly, in the 3,5-diCl-PAR incubation mixture, a derivative was found that had retained one chlorine substituent and the other one replaced by sulphur, as the result of a nucleophilic addition–elimination reaction.

As far as substrate binding to cytochrome P450 is concerned, structural requirements for substrates of βNF-inducible cytochrome P450s (Lewis *et al.* 1987) predict that PAR and the 3,5-disubstituted analogues will fit into the active site. A preliminary conformation analysis of the 3,5-disubstituted PAR analogues investigated in this study revealed each of them to have a coplanar acetamido moiety as well as a coplanar phenolic hydroxyl group in their lowest energy conformation (data not shown). Accordingly, the newly studied 3,5-substituted analogues interacted well with microsomal cytochrome P450 giving rise to reverse type I spectral changes (table 1). The observed spectral dissociation constants ($K_{s,app}$) of PAR and six of the 3,5-disubstituted analogues in βNF-induced rat liver microsomes were in the same range as reported previously for 3-mono- and 3,5-dialkylated PAR analogues (Van de Straat *et al.* 1986). However, the strength of haem binding varied considerably within this series of 3,5-disubstituted analogues of PAR. A strong correlation was found between the estimated lipophilicity and $\log(1/K_{s,app})$, indicating a hydrophobic interaction between the 3,5-disubstituted PAR analogues and cytochrome P450. Previously, it has been concluded, based on rates of metabolite formation, that the cytochrome P450-mediated oxidation of PAR and its 3-mono- and 3,5-dialkylated derivatives is primarily governed by other factors than lipophilicity and steric effects (Van de Straat *et al.* 1986).

PAR and all seven 3,5-disubstituted analogues investigated appeared to be oxidized during microsomal incubation in the presence of NADPH as deduced from the oxidation of GSH to GSSG. Monitoring of GSSG formation after oxidative biotransformations of PAR and 3,5-disubstituted analogues has been shown to provide a rapid and reliable answer to the question as to whether new PAR derivatives are liable to cytochrome P450-dependent oxidation to the corresponding NAPQIs (Van de Straat *et al.* 1986). Measuring oxidation of PAR to NAPQI by detecting NAPQI directly is known to be difficult (Harvison *et al.* 1988). For example, UV-detection of NAPQI is not feasible since NAPQI is easily reduced to PAR in NADPH-containing microsomal incubations (Dahlin *et al.* 1984). In the present study, however, the two-electron oxidation of 3,5-diCH_3-PAR to 3,5-diCH_3-NAPQI by rat liver microsomes, could be measured by UV-spectrophotometry (figure 2). Experimental evidence for the microsomal oxidation of PAR, 3,5-diCl-PAR and 3,5-diBr-PAR to NAPQI and the corresponding 3,5-dihalogenated NAPQI analogues, however, could be retrieved only from the detection of sulphur-containing metabolites with alkaline peralkylation and GC/MS (table 3). Except in the case of 3,5-diF-PAR, the sulphur-containing products detected supported the formation of the corresponding 3,5-dialkylated NAPQI and 3,5-dihalogenated NAPQIs respectively. Hydroxylated species, such as *ortho*- or *meta*-catechols would most likely have been detected by GC/MS analysis after alkaline

permethylation (Slaughter and Hanzlik 1991). No such products were found, however, in our studies. Therefore, it seems conceivable that the oxidation of GSH observed upon microsomal incubation with 3,5-dialkylated and 3,5-dihalogenated analogues of PAR, is caused mainly if not solely, by the formation of the corresponding 3,5-dialkylated and 3,5-dihalogenated NAPQI's by the microsomal cytochrome P450.

The involvement of the cytochrome P450-containing MFO system in the oxidation of PAR and its 3,5-disubstituted analogues to their corresponding NAPQI derivatives is supported by the binding and turnover data presented (table 2). Moreover, it is also deduced from the differential effects of the cytochrome P450 inducers βNF and PB on the cytochrome P450-mediated oxidation of all 3,5-disubstituted analogues of paracetamol investigated. Finally, the inhibition of cytochrome P450-dependent GSSG formation by 1-ethynylpyrene supported the dependency on βNF-inducible cytochrome P450s. The actual effects of induction on the cytochrome P450-mediated oxidation of the 3,5-disubstituted analogues investigated were similar to those reported earlier for oxidation of 3-monoalkylated and 3,5-dialkylated analogues of PAR: βNF (or 3-methylcholanthrene)-induction increased product formation, whereas after PB-induction product formation was decreased (Van de Straat *et al.* 1986). This suggests that PAR, as well as 3,5-dialkylated and 3,5-dihalogenated PAR analogues are oxidized by similar cytochrome P450 enzymes. Since 1-ethynylpyrene is known to be a specific inhibitor of rat CYP 1A1 (Chan *et al.* 1993), it seems that in βNF-induced rat liver, CYP 1A1 in particular is responsible for the oxidation of the 3,5-dialkylated and the 3,5-dihalogenated PAR analogues and the formation of reactive metabolites with oxidant capacities. Attempts to correlate $\log(1/K_{m,app})$ values as determined by formation of GSSG in microsomal incubations containing PAR or one of its 3,5-disubstituted analogues, with estimated lipophilicity (Σf; table 2) resulted in meagre statistics. Since the formation of GSSG is an end-point determination based on several enzymatic and non-enzymatic reactions, this is not unanticipated. However, a trend was observed that with increase of estimated lipophilicity $\log(1/K_{m,app})$ values increased also (data not shown).

In summary, in this study it was shown that four newly synthesized 3,5-dihalogenated and three 3,5-dialkylated PAR-analogues did bind to βNF-induced rat liver microsomal cytochrome P450, and binding correlated well with their estimated lipophilicity values. In addition it was shown that these 3,5-disubstituted PAR analogues were susceptible to HRP/H_2O_2-mediated oxidative biotransformation to 3,5-dihalogenated and 3,5-dialkylated NAPQI analogous, by trapping of these metabolites with GSH, and subsequent detection of sulphur-containing derivatives. Finally, as deduced from cytochrome P450-dependent GSSG formation upon incubation with the 3,5-disubstituted analogues of PAR, it was concluded that the 3,5-dihalogenated and 3,5-dialkylated PAR analogues were liable to cytochrome P450-mediated oxidative biotransformation to their respective 3,5-dihalogenated and 3,5-dialkylated NAPQI analogues, primarily by βNF-inducible cytochromes P450. The next question to be answered concerns the relevance of these results for understanding the cytotoxicity of 3,5-dihalogenated analogues (Bessems *et al.* 1995).

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SECTION IV CELLULAR EFFECTS AND PROTECTIVE ASPECTS IN PARACETAMOL CYTOTOXICITY

Chapter 7 Cytotoxicity of paracetamol and 3,5-dihalogenated analogues: Role of cytochrome *P*-450 and formation of GSH conjugates and protein adducts

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Cytotoxicity of Paracetamol and 3,5-Dihalogenated Analogues: Role of Cytochrome *P*-450 and Formation of GSH Conjugates and Protein Adducts

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Abstract—The effect of 3,5-dihalogenation of paracetamol (PAR) on the cytotoxicity in rat hepatocytes isolated from β-naphthoflavone pretreated, non-fasted rats, and the role of cytochrome *P*-450 in this regard, were studied. On incubation, 3,5-difluoro-PAR, 3,5-dichloro-PAR and 3,5-dibromo-PAR, as well as PAR, caused severe leakage of lactate dehydrogenase (LDH) which was preceded by a rapid concentration- and time-dependent depletion of intracellular glutathione (GSH). IC_{50} values, representing the concentration of compound that caused 50% GSH depletion after 30 min of incubation, varied from 0.1 to 0.5 mM. This LDH leakage and GSH depletion could be inhibited by 1-ethynylpyrene. In hepatocytes from uninduced rats, GSH depletion was much less prominent and the concomitant LDH leakage almost completely absent. HPLC analysis of soluble metabolites and gas chromatography–mass spectrometry analysis, after alkaline peralkylation of the protein fraction, revealed (a) that 3,5-dihalogenated PAR analogues were liable to structure-related detoxification by glucuronidation, and (b) analogous to PAR, a substantial amount of each 3,5-dihalogenated PAR analogue was bioactivated by cytochrome *P*-450, ultimately leading to GSH-conjugates as well as (for 3,5-dichloro-PAR and 3,5-dibromo-PAR), protein adducts at regio-specific aromatic positions. © *1997 Elsevier Science Ltd*

Abbreviations: BSA = bovine serum albumin; CYP = cytochrome *P*-450, 3,5-diBr-PAR = 3,5-dibromo-paracetamol; 3,5-diCl-PAR = 3,5-dichloro-paracetamol; 3,5-diF-PAR = 3,5-difluoro-paracetamol; EP = 1-ethynylpyrene; GSH = glutathione; GSSG = oxidized glutathione; γGT = γ-glutamyltranspeptidase; LDH = lactate dehydrogenase; NAPQI = *N*-acetyl-*p*-benzoquinone imine; βNF = β-naphthoflavone; βNF-hepatocytes = hepatocytes isolated from βNF-induced rats; PAR = paracetamol; TCA = trichloroacetic acid; UT-hepatocytes = hepatocytes isolated from non-induced rats.

INTRODUCTION

Paracetamol (acetaminophen, 4′-hydroxyacetanilide; PAR) is a widely used non-prescription analgesic and antipyretic drug. At normal dose levels the drug mainly undergoes sulfation and glucuronidation. It is known to be hepatotoxic in humans and various experimental animals on overdose (Anundi *et al.*, 1993; Prescott, 1983; Vermeulen *et al.*, 1992), presumably by cytochrome *P*-450 (CYP) dependent oxidation to the reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI). With rat liver microsomes and purified CYPs, it has been shown that the β-naphthoflavone (βNF)-inducible CYP form 1A1 is mainly involved (Harvison *et al.*, 1988). More recently, also rat liver CYP1A2, CYP2E1, CYP3A1, and α-naphthoflavone activated CYP3A2 were reported to catalyse the oxidation of PAR to NAPQI (Patten *et al.*, 1993).

NAPQI can both covalently bind to glutathione (GSH) and oxidize GSH. Analogously, NAPQI readily reacts with cysteine groups in proteins, leading to protein arylation (Hoffmann *et al.*, 1985) or oxidation (Albano *et al.*, 1985), leading to the formation of protein disulfides and GSH-protein mixed disulfides (Birge *et al.*, 1991; Kyle *et al.*, 1990). More and more, specific cytosolic proteins have been found to be arylated in mice and humans exposed to PAR, to an extent correlating with cellular damage for some proteins but not for other (Birge *et al.*, 1990, Hinson *et al.*, 1990 and 1995). Hepatic proteins that become arylated on *in vivo* exposure of mice to hepatotoxic doses of PAR include a cytosolic selenium-binding protein (Bartolone *et al.*, 1992, Pumford *et al.*, 1992) and a microsomal subunit of glutamine synthetase (Bulera *et al.*, 1995). Murine

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cytosolic proteins were recently found to be arylated as well on *in vivo* exposure to 3′-hydroxyacetanilide, the *m*-hydroxy isomer of PAR (Myers *et al.*, 1995). Covalent binding of NAPQI to the ϵ-amino lysine group of the Q-2 isoform of the microsomal thiol-protein oxidoreductase has recently been suggested to be involved as well in the toxicity mechanism of PAR in mice (Holtzman, 1995).

Taking these mechanisms into consideration, there have been many efforts to prevent paracetamol-induced hepatotoxicity by modification of the structure of paracetamol (Harvison *et al.*, 1986; Van de Straat *et al.*, 1986). Three 3,5-dialkylated analogues were found to be far less toxic than paracetamol in hepatocytes, corroborating with the fact that no irreversible GSH depletion nor covalent protein binding occurred. These analogues were, nevertheless, shown to undergo CYP-dependent oxidative biotransformation (Van de Straat *et al.*, 1986). More recently, however, 3,5-dihalogenated analogues, were found to possess cytotoxic properties similar to PAR in incubated freshly isolated rat hepatocytes (Bessems *et al.*, 1995). The 3,5-dihalogenated PAR analogues were also shown to be liable to microsomal CYP-dependent two-electron oxidation to the corresponding 3,5-disubstituted NAPQI analogues (Bessems *et al.*, 1996). In contrast to 3,5-dialkylated PAR analogues (Van de Straat *et al.*, 1986), the 3,5-dihalogenated PAR analogues were found to be conjugated to sulfur-containing nucleophiles in the presence of a microsomal CYP bioactivation system (Bessems *et al.*, 1996).

In this study, the cytotoxic properties and metabolite profiles of three different 3,5-dihalogenated PAR analogues were determined; in addition, the role of CYP and, more specifically, the CYP1A subfamily in rat hepatocytes, the intracellular depletion of GSH, the formation of conjugates of GSH, and protein adduct formation were investigated.

MATERIALS AND METHODS

Chemicals and enzymes

Collagenase (type B), arylsulfatase, and β-glucuronidase (from *Escherichia coli*, solution in glycerine, 50%, v/v, pH 6, 200 units/ml) were obtained from Boehringer (Mannheim, Germany). PAR was obtained from Brocacef (Delft, The Netherlands). GSH and βNF were purchased from Janssen Chimica (Beerse, Belgium), HEPES and DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] from Merck (Darmstadt, Germany). GSH-reductase (type III, from bakers' yeast), oxidized glutathione (GSSG), NADPH, *N*-ethylmaleimide (NEM), nicotinamide, bovine serum albumin (BSA), acivicine, and γ-glutamyltranspeptidase (γGT; EC 2.3.2.2; Type II, 25 units/mg) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Methanol (HPLC grade) was from Rathburn. 1-Ethynylpyrene (EP) was a gift from W.L. Alworth (New Orleans, LA, USA). Methyl iodide and ethyl iodide were from Aldrich (Brussels, Belgium). 3,5-Difluoro-, 3,5-dichloro- and 3,5-dibromo-PAR were synthesized as described recently (Bessems *et al.*, 1995). All other chemicals were commercially obtained at analytical grade.

Animals and isolation of hepatocytes

Male Wistar rats (200–250 g) from Harlan CPB (Zeist, The Netherlands) were used for all experiments and housed in plastic cages with wood chips at a controlled temperature of 22°C and 50% relative humidity. The animals had free access to food (standard laboratory diet from Hope Farms, Woerden, The Netherlands) and tap water. For induction, βNF (80 mg/kg, dissolved in arachidis oil) was injected ip once, 24 hr before use.

Hepatocytes were isolated from non-fasted rats by two-step collagenase perfusion of the liver, essentially as described by Seglen (1973). The preperfusion and perfusion solutions were buffered at 37°C at pH 7.4 and pH 7.6, respectively, with 10 and 100 mM HEPES buffer. The total length of the perfusion was approximately 15–20 min. Collagenase was washed out with Hanks'–HEPES buffer (37°C) supplemented with 1.5% (w/v) BSA (pH 7.6), but containing 10 mM lactate and 1 mM pyruvate instead of glucose. Non-parenchymal cells were removed by centrifugation using the same washing buffer at 0°C. Aliquots of freshly isolated cells were immediately counted with a haemocytometer in 0.4% trypan blue solution containing 0.9% NaCl. Cell suspensions obtained by this method usually contained more than 90% viable cells.

Hepatocyte incubations and determination of cytotoxicity

Before the start of incubation, all compounds (eventually including EP or acivicine), were dissolved by ultrasonification in Hanks'–HEPES buffer (37°C) supplemented with 1.5% (w/v) BSA (pH 7.6), but containing 10 mM lactate and 1 mM pyruvate instead of glucose. EP (final concentration 10 μM) was added in order to study the role of CYP1A isoforms in the cytotoxicity, and acivicine (0.25 mM) was added in order to prevent breakdown of GSH conjugates. Solutions were diluted to 6.5 ml to give the final concentrations in 7-ml incubations that are indicated in the Figures. Hepatocytes were incubated in screw-capped plastic scintillation vials and flushed with carbogen (95% O_2/5% CO_2) on addition of the hepatocyte suspension and every time that samples were taken. Incubations were performed on a temperature-controlled (37°C) metal platform, shaking horizontally clockwise (90 oscillations/min) and started by addition of 0.5 ml of the hepatocytes to give 1×10^6 cells/ml.

At the times indicated, 0.5 ml aliquots were removed. Viable cells were reharvested by gentle

centrifugation (50 ***g*** for 5 min). Lactate dehydrogenase (LDH) leakage in the resultant supernatant was assayed as described by Moldéus *et al.* (1978), using a Philips PU-8720 UV/vis spectrophotometer, and 100% LDH activity was obtained after lysis of the cells by addition of Triton X-100. The remaining supernatant was frozen at − 20°C and used within 1 wk for the determination of extracellular GSSG.

Intracellular GSH and extracellular GSSG

1 ml 6.5% (w/v) trichloroacetic acid (TCA) was added to the reharvested cells and, following centrifugation, (3500 ***g*** for 15 min), a 0.5-ml aliquot of the supernatant was used for the estimation of intracellular GSH, as described by Saville (1958). Values were expressed as percentage of the zero time incubations.

For the determination of extracellular GSSG, samples of the supernatant medium fraction were thawed and deproteinized with 20% TCA (w/v) followed by centrifugation. Aliquots of 600 μl were taken from the supernatant, GSH was removed and GSSG was determined by an enzymatic method after that of Redegeld *et al.* (1988) and modified as described recently (Bessems *et al.*, 1996).

Analysis of metabolites in cytosol and extracellular medium

For analysis of metabolites formed, separate 0.25-ml aliquots were taken during the incubation and adjusted to 20% (w/v) TCA for protein precipitation followed by centrifugation. Supernatants were diluted 1 in 5 and 40 μl was injected onto two RP-C_{18} glass tube cartridge columns (3 × 100 mm each; particle size 5 μm; Chrompack) in series, and metabolites were separated by gradient elution using a Waters Associates M-600A pump equipped with a 440 UV detector (set at 250 nm). The modified gradient system (Howie *et al.*, 1977; Moldéus, 1978) consisted of component A (water–methanol–acetic acid; 89:10:1, by vol) and component B (methanol) where component B was increased (2% per min) for 50 min and was kept at 100% for another 15 min (flow rate 0.4 ml/min). Qualitative recording was performed at 200 mV or 20 mV (Kipp recorder); peak integration was performed with a Shimadzu C-R1B integrator. For peak identification, samples were pretreated either with β-glucuronidase or arylsulfatase for 16 hr or with γGT for 24 hr (samples of incubations without acivicine) (Nakamura *et al.*, 1987; Te Koppele *et al.*, 1986). Semiquantitative determination of the metabolites was accomplished using the parent compounds (PAR or 3,5-dihalogenated analogue) as a standard, since the molar extinction coefficients of the PAR and its metabolites are essentially the same (Howie *et al.*, 1977), and this was assumed to be valid also for the 3,5-dihalogenated PAR analogues and their respective metabolites.

Alkaline peralkylation of sulfur-containing protein adducts and analysis by GC/MS

Samples of hepatocyte incubations, taken after 4 hr of incubation, were subjected to alkaline-induced fragmentation of *S*-arylcysteines and conversion to *S*-methyl derivatives, as described by Slaughter and Hanzlik (1991) with a few modifications. Incubation samples were transferred to derivatization tubes and adjusted to 20% TCA (w/v). The precipitates were collected by centrifugation and washed once with water, resuspended again in 0.5 ml water and used for alkaline peralkylation and gas chromatography–mass spectrometry (GC/MS) analysis. On ice, 0.5 ml NaOH (8 M; N_2 purged) was added to induce C(β)-*S* bond cleavage, followed by 0.5 ml ice-cold methyliodide (volatile and reactive) for conversion of the resulting thiolate-moieties to *S*-methyl derivatives. For the PAR incubations, ethyliodide instead of methyliodide was used in order to decrease the volatility of its derivatives. Nitrogen-flushed and tightly capped reaction tubes were immersed in a boiling water-bath (100°C) for 1 hr behind an explosion screen in a fume hood and then cooled on ice. After extraction with *n*-pentane (2 × 3 ml) and concentration, the residues were dissolved in 25 μl methanol and 1-μl samples were analysed by GC/MS (Bessems *et al.*, 1996).

GC/MS analyses were performed on a Hewlett Packard 5890 GC and a Hewlett Packard 5971 MSD system set to scan between *m/z* 35 and *m/z* 550. A CP-Sil capillary column (50 m) from Chrompack B.V. (Middelburg, The Netherlands) was used. Unless otherwise stated, the operating conditions were 140 kPa (column head pressure), 250°C (split injector) and the electron energy 70 eV (EI/MS). The carrier gas was helium at a flow of about 3 ml/min. The GC oven temperature was kept at 60°C for 2 min and increased to a final temperature of 270°C (20°C/min).

RESULTS

Cytotoxicity and effects on the glutathione status

Addition of 0.3 mM of each of the three 3,5-dihalogenated PAR analogues to hepatocytes freshly isolated from βNF-induced rats (βNF-hepatocytes), had effects on the viability of the cells similar to those of addition of 0.3 mM PAR (Fig. 1a). Loss of viability was measured as LDH leakage, whereas early deleterious effects were determined by measuring the intra- and extracellular concentrations of glutathione, all parameters in comparison with blank incubations or zero time incubations. Intracellular glutathione in the reduced form (GSH) in exposed cells was already significantly depleted after 1 hr of incubation (Fig. 1b). The initial GSH depletion induced by PAR- and 3,5-dihalogenated PAR analogues was followed by a significant leakage of LDH from the hepatocytes in all four cases (Fig. 1a). Moreover, the time-depen-

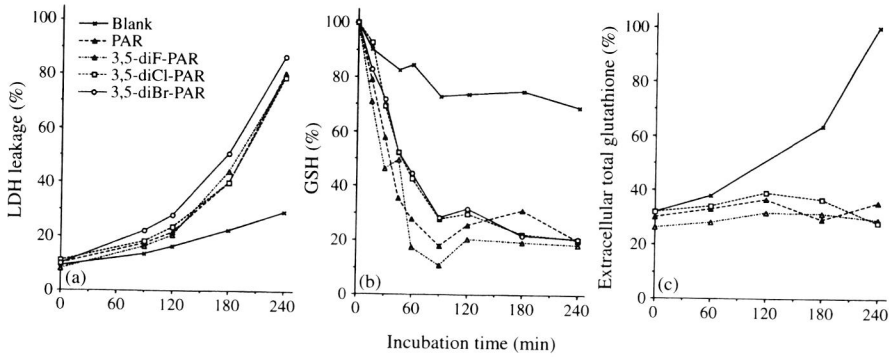


Fig. 1. (a) Cytotoxicity (LDH leakage), (b) intracellular GSH depletion, and (c) excretion of glutathione (mainly GSSG) in freshly isolated rat hepatocytes from βNF-induced rats on incubation with 0.3 mM PAR, 3,5-diF-PAR, 3,5-diCl-PAR or 3,5-DiBr-PAR. Incubations were performed in rotating vials using 10^6 cells per ml of incubate as described in Materials and Methods. Each data point is the average of two separate determinations in separate but parallel incubation vials. Each graph represents one hepatocyte isolation and incubation experiment typical of four. Intracellular GSH is relative to that of the zero time incubation. Extracellular total glutathione is presented as a percentage of that in the 4 hr blank incubation.

dent depletion of GSH (and the leakage of LDH) on incubation of the hepatocytes with PAR and each of the three PAR analogues was found to be concentration dependent (shown only for PAR and 3,5-diCl-PAR in Fig. 2). The increase of extracellular total glutathione in blank incubations was counteracted by PAR and, to the same extent, by the 3,5-dihalogenated PAR analogues (Fig. 1c). IC_{50} values at 30 min, estimated from semilogarithmic plots and representing the concentration of the compound under investigation causing 50% GSH depletion on 30 min of incubation, were 0.1, 0.3, 0.5 and 0.5 mM, for 3,5-diF-PAR, 3,5-diCl-PAR, 3,5-diBr-PAR and PAR, respectively (Table 1). These IC_{50} values roughly correlate with the extent of LDH leakage (Table 1).

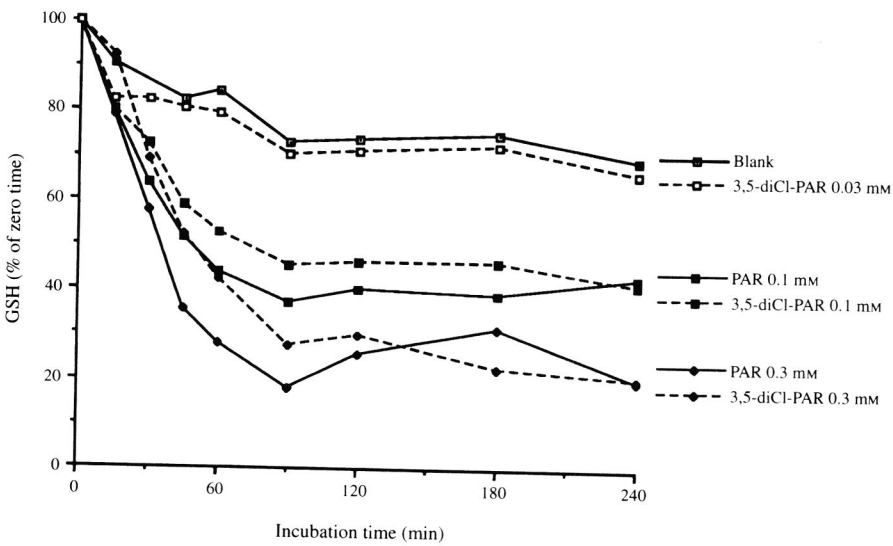


Fig. 2. Time-dependent intracellular GSH depletion in freshly isolated hepatocytes from βNF-rats in the presence of a concentration range of PAR or 3,5-diCl-PAR. Results represent one experiment (with parallel incubations) typical of four as explained in the legend of Fig. 1.

Table 1. Effects of βNF-induction and co-incubation with EP on LDH leakage, during incubation for 3 hr with PAR and 3,5-dihalogenated PAR analogues, in freshly isolated rat hepatocytes, and their IC_{50} values for GSH depletion*

	LDH (%)			IC_{50} (mM§)
	UT†	βNF‡		βNF
Compound	− EP	− EP	+ EP	− EP
Blank	9	11	14	–
PAR	11	50	13	0.5
3,5-diF-PAR	9	61	12	0.1
3,5-diCl-PAR	8	57	18	0.3
3,5-diBr-PAR	8	51	ND	0.5

ND = not determined

*Data represent one typical experiment from two.
†UT-hepatocytes were incubated with 1 mM PAR and 3,5-dihalogenated PAR analogues.
‡βNF-hepatocytes were incubated with 0.3 mM PAR and 3,5-dihalogenated PAR analogues. Where indicated, 10 μM EP was co-incubated.
§Concentration of compound causing 50% GSH depletion in βNF-hepatocytes on 30 min incubation.

In UT-hepatocytes (hepatocytes isolated from non-induced rats) incubated with 0.1 or 0.3 mM PAR, 3,5-diF-PAR, 3,5-diCl-PAR, or 3,5-diBr-PAR for up to 3 hr, no GSH depletion or LDH leakage was observed. Only UT-hepatocytes incubated with 1.0 mM concentrations of PAR and the three dihalogenated PAR analogues caused a slight (although not statistically significant) depletion of GSH (data not shown). LDH leakage was completely absent during 3 hr of incubation of UT-hepatocytes (Table 1), indicating an important role of induced isoforms of CYP in the cytotoxicity of the compounds under investigation. The involvement of CYP1A enzymes was indicated by the potentiating effect of βNF-induction and by the inhibitory effect of co-incubation with 10 μM of EP on LDH leakage in incubations of βNF-hepatocytes with PAR, 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR (Table 1). EP was found to counteract the GSH depletion that was due to incubation with PAR and 3,5-dihalogenated analogues: the GSH contents of the hepatocytes incubated with PAR and the 3,5-dihalogenated analogues were depleted only to about 55% in the presence of EP. Remarkably, EP itself decreased the intracellular GSH content as well, by an extra 14% in blank incubations (Table 2).

Metabolite profiles in cytosol and extracellular medium

Deproteinized hepatocyte incubation samples, containing the extracellular incubation medium and cytosol of viable and of dead cells, were analysed by HPLC. The results, as presented in Table 3, indicate that the HPLC gradient programme was well suited for the separation of several metabolites of the 3,5-dihalogenated PAR analogues and also of PAR. On HPLC analysis of deproteinized hepatocyte samples, after incubation with PAR, 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR, apart from the parent compounds, glucuronide conjugates as well as GSH-conjugates were found. Furthermore, several additional peaks, specific for the compounds incubated (PAR and each of three 3,5-dihalogenated analogues) were observed, although these were always minor when compared with the assigned peaks. Because these additional peaks were not sensitive to treatment with β-glucuronidase, arylsulfatase or γ-GT, they could not be assigned. The areas of the substrate peaks in sulfatase-treated split-samples did not change to any measurable extent, indicating that sulfate conjugates, if formed at all, were of minor importance. As the HPLC column was equilibrated with 10% methanol, it may be that the sulfate conjugate of PAR, which is known to elute well before the glucuronide conjugate of PAR, had already been eluted together with other hydrophillic compounds. The chromatogram for the incubation with 3,5-diCl-PAR is presented in Fig. 3 as a representative of all the HPLC chromatograms. The estimated relative amounts of metabolites are presented in Table 3. After 3 hr of incubation, about 11% of PAR was recovered as the PAR-glucuronide and about 4% as PAR-glutathione conjugate. Analysis of incubations with 3,5-diF-PAR revealed that about 23% was recovered as a glucuronide and about 2% as a glutathione conjugate. In the case of 3,5-diCl-PAR, about 37% glucuronidation and 3% GSH-conjugate formation were observed. For 3,5-diBr-PAR, these values were 45 and 5%, respectively, where the latter was estimated from the area of the cysteinyl–glycine conjugate peak that appeared in the chromatogram on treatment with γ-glutamyl-transpeptidase. No peak for the glutathione conjugate of 3,5-diBr-PAR could be assigned directly, possibly because of co-elution with the parent

Table 2. Effects of co-incubation with EP on the GSH depletion induced by PAR and 3,5-dihalogenated PAR analogues in βNF-hepatocytes*

	GSH (%)†	
Incubation	− EP	+ EP
Blank	74	60
PAR	23	55
3,5-diF-PAR	30	55
3,5-diCl-PAR	29	58

*Typical GSH contents were determined in a single incubation with βNF-hepatocytes freshly isolated from rat.
†Intracellular GSH after 2 hr expressed as a percentage of the zero time incubation.

Table 3. HPLC analysis of metabolic products in freshly isolated rat hepatocytes incubated (3 hr) with PAR and 3,5-dihalogenated analogues

Compound	HPLC peaks*			
	R	R-Gluc†	R-SG‡	R-CysGly§
PAR	85 (12)	11 (7)	4 (16)	4 (10)
3,5-diF-PAR	75 (25)	23 (13)	2 (21)	2 (14)
3,5-diCl-PAR	60 (29)	37 (25)	3 (26)	2 (22)
3,5-diBr-PAR	50 (32)	45 (27)	‖(27)	5 (24)

*Values are areas expressed as percentages of total area of unchanged parent compound (R), glucuronide (R-Gluc), and GSH conjugate (R-SG), respectively. Retention times (min) in parentheses.

†HPLC peak that disappeared on injection of split-samples treated with β-glucuronidase.

‡Assigned to the peak in the HPLC chromatogram of hepatocyte mixtures in which acivicine had been coincubated. This peak was absent in chromatograms of hepatocyte incubations without acivicine that had been treated with γGT before HPLC analysis.

§Assigned to the peak that appeared in the chromatogram on injection of γGT-treated samples of hepatocyte incubations in which no acivicine has been coincubated. ‖Co-elution of the GSH-conjugate with the substrate 3,5-diBr-PAR.

3,5-diBr-PAR. As shown in Fig. 4, during 4 hr βNF-hepatocyte incubation in the presence of 0.5 mM 3,5-diCl-PAR, the recovery of 3,5-diCl-PAR as (1) unmodified 3,5-diCl-PAR, (2) its glucuronide or (3) its glutathione conjugate, was more than 90%. When comparing PAR and the three 3,5-dihalogenated PAR analogues, it appeared that glucuronide formation increased in the order PAR, 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR. Conjugation with GSH did not vary to any great extent for PAR and the 3,5-dihalogenated analogues and was always 5% (for 3,5-diBr-PAR as judged from cysteinyl-glycine conjugate detection) or less.

Analysis of protein sulfur adducts by GC/MS

As conjugation of reactive intermediates, presumably 3,5-dihalogenated NAPQI analogues, to proteins could be involved in the cytotoxicity mechanism of the 3,5-dihalogenated PAR analogues, cellular proteins were analysed for the formation of such conjugates. Previously, an alkaline peralkylation derivatization method, followed by GC/MS analysis, was described for the detection of soluble sulfur conjugates formed in rat liver microsomal incubations of PAR and 3,5-dihalogenated PAR analogues in the presence of GSH (Bessems *et al.*, 1996). Several sulfur adducts were found in the acid-precipitated and washed protein fraction of freshly isolated rat hepatocytes when PAR, 3,5-diCl-PAR or 3,5-diBr-PAR were incubated (Table 4). More precisely, one sulfur-bound perethylated PAR derivative, which probably reflects a 3′-cysteinyl protein adduct, was found in βNF-hepatocytes incubated with PAR (compound **A1a** in Table 4). However, in principle, the 2′-cysteinyl protein adduct (that would have resulted in compound **A1b**) cannot be fully excluded by this method.

In the pelleted protein fraction of hepatocytes incubated with 3,5-diF-PAR, no products were found on alkaline permethylation that could be assigned to products derived from a protein sulfur adduct of a reactive metabolite of 3,5-diF-PAR. On alkaline permethylation of the protein fraction of 3,5-diCl-PAR exposed βNF-hepatocytes, one product was found that could be attributed to a compound containing one chlorine and two thiomethyl-groups

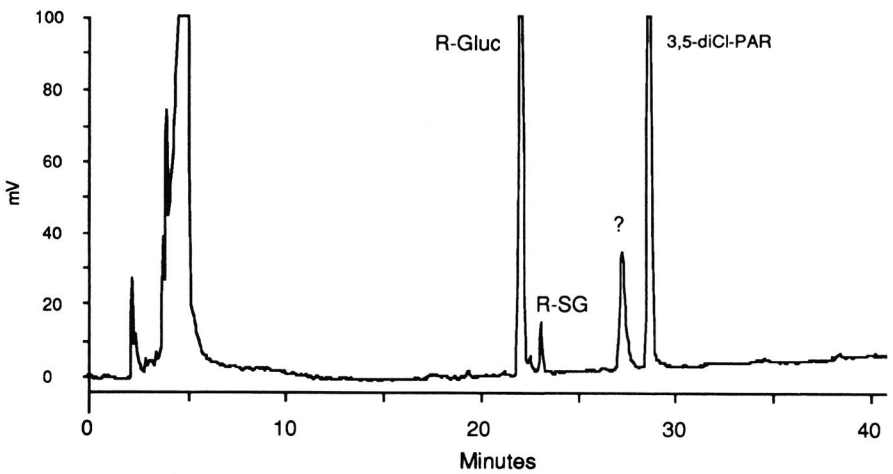


Fig. 3. HPLC analysis of results of incubation (3 hr) of freshly isolated hepatocytes with 3,5-diCl-PAR (0.5 mM). Deproteinized samples were diluted 1 in 5 and 40 μl was injected. Metabolites were assigned by comparison with chromatograms of glucuronidase, sulfatase or γGT 'postincubated samples'. R-Gluc = glucuronide conjugate; R-SG = glutathione conjugate; ? = unknown metabolite.

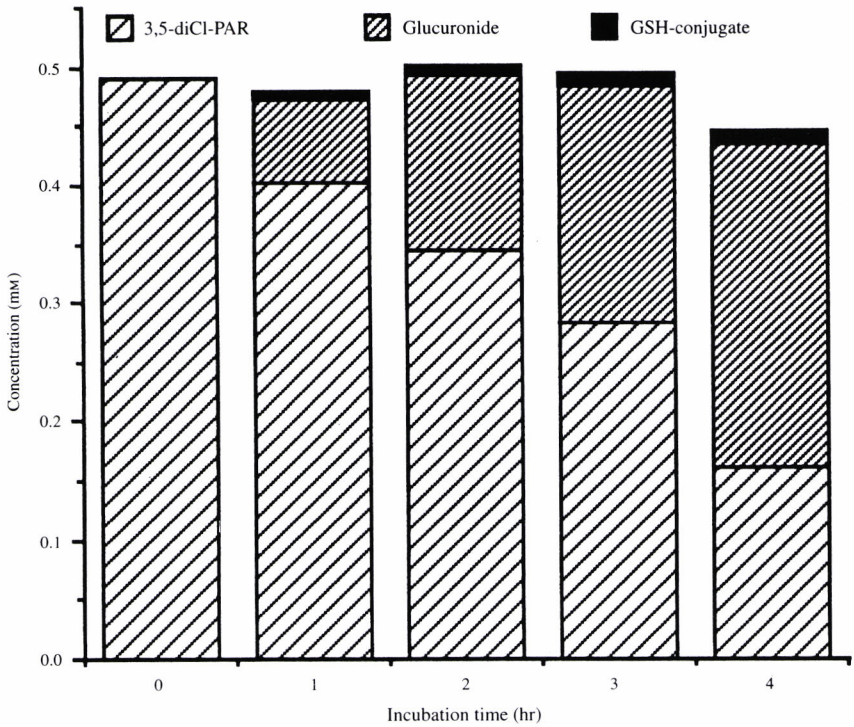


Fig. 4. Time course of the concentration of 3,5-diCl-PAR, its glutathione conjugate and its glucuronide conjugate, in the complete mixture of a single incubation of βNF-hepatocytes with 3,5-diCl-PAR (0.5 mM), as analysed by HPLC.

(compound **C1**). Since the mass spectrum applies to two possible structural isomers, both possibilities are presented in Table 4 (structures **C1a** and **C1b**). Incubating 3,5-diBr-PAR with βNF-hepatocytes revealed the formation of two sulfur adducts (derivatized to **D1** and **D2**), of which one had lost one bromine, probably on the addition-elimination reaction with a thiol group. The amount of structure **D1** was greatly in excess of that of structure **D2**. All results presented here should be regarded as qualitative, since no attempt was made to quantify protein sulfur adduct formation.

DISCUSSION

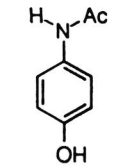
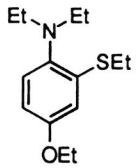
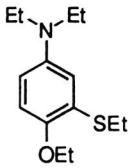
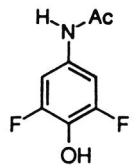
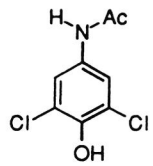
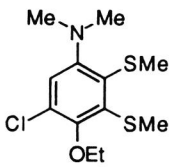
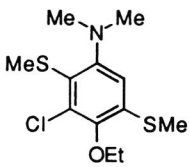
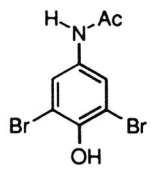
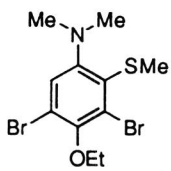
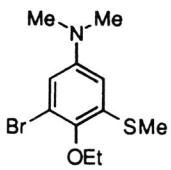
Cytotoxicity

The aim of the study described here was to investigate the cytotoxicity of three 3,5-dihalogenated PAR analogues (3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR), with respect to the role of CYP, conjugative metabolism and formation of protein adducts. The results indicate that, corroborative previous findings, freshly isolated liver parenchymal cells of non-fasted rats that have been pretreated once with βNF (80 mg/kg) are a good model with which to study the cytotoxicity of PAR and 3,5-dihalogenated analogues (Bessems *et al.*, 1995). Depriving the experimental animals of food, prior to isolation of the hepatocytes, does not seem to be necessary. Moreover, it has the disadvantage of possible interference of an increased level of CYP2E1 or a diminished concentration of cell constituents such as GSH (Liu *et al.*, 1993; Price *et al.*, 1987).

The results obtained in this study show that the cytotoxic capacity of 3,5-diF-PAR, 3,5-diCl-PAR and 3,5-diBr-PAR, in freshly isolated rat hepatocytes, is of the same order of magnitude as that of PAR itself. Exposure to 0.3 mM of all compounds caused severe LDH leakage in freshly isolated hepatocytes. This contrasts with results found on incubation of similar rat hepatocytes with PAR analogues that are 3,5-dialkylated, for which hardly any cytotoxicity has been reported (Porubek *et al.*, 1987; Van de Straat *et al.*, 1986). In some experiments in the present study, hepatocytes were exposed to 0.1–1.0 mM 3,5-dimethyl-PAR but in none of these was significant cytotoxicity found (data not shown).

With respect to CYP isoforms involved in the

Table 4. GC/MS analysis of acid-precipitated protein after alkaline peralkylation of isolated rat hepatocytes incubated with PAR and 3,5-dihalogenated analogues*

Compounds	Derivatized adducts(†)		
 PAR	 A1a	or  A1b	9.9 min: 253 (98%,$M^{+\cdot}$); 238 (52%,$[M-CH_3]^{+\cdot}$); 224 (100%,$[M-C_2H_5]^{+\cdot}$)
 3,5-diF-PAR	(‡)		
 3,5-diCl-PAR	 C1a	or  C1b	11.3 min: ^{35}Cl 277 (100%,$M^{+\cdot}$); 252 (88%,$[M-CH_3]^{+\cdot}$). ^{37}Cl 279 (100%,$M^{+\cdot}$); 254 (88%,$[M-CH_3]^{+\cdot}$).
 3,5-diBr-PAR		 D1	10.7 min: $^{79}Br^{79}Br$ 353 (100%,$M^{+\cdot}$); 338 (90%,$[M-CH_3]^{+\cdot}$). $^{79}Br^{81}Br$ 355 (100%,$M^{+\cdot}$); 340 (90%,$[M-CH_3]^{+\cdot}$). $^{81}Br^{81}Br$ 357 (100%,$M^{+\cdot}$); 342 (90%,$[M-CH_3]^{+\cdot}$).
		 D2	10.2 min: ^{79}Br 275 (100%,$M^{+\cdot}$); 260 (60%,$[M-CH_3]^{+\cdot}$). ^{81}Br 277 (100%,$M^{+\cdot}$); 262 (60%,$[M-CH_3]^{+\cdot}$).

*Rat liver hepatocytes were incubated for 4 hr with PAR, 3,5-diF-PAR, 35-diCl-PAR or 3,5-diBr-PAR. A TCA-pelleted protein of 1 ml samples was derivatized and analysed by GC/MS as described (Bessems *et al.*, 1996). PAR incubations and 3,5-dihalogenated PAR analogue incubations were derivatized with ethyl iodide and methyl iodide, respectively.

†Postulated structure, GC retention times, and partial mass spectrum (m/z) with relative intensities of fragments. No important additional fragmentations, but the losses of $-CH_3$ or $-C_2H_5$ that were noted are presented.

‡No covalent adducts were found that could be attributed to biotransformation of 3,5-diF-PAR.

cytotoxicity of PAR and 3-mono-alkylated analogues, the involvement of βNF-inducible CYPs has been shown in previous studies (Van de Straat *et al.*, 1986). In order to substantiate the role of CYP1A isoforms CYP in the cytotoxicity observed for the 3,5-dihalogenated PAR analogues in the present study, 10 μM EP was coincubated. Recently it was shown that, at 1 μM concentration, EP is a suicide inhibitor of CYP1A1 but not of CYP2B1 in rat liver microsomal incubations (Hopkins *et al.*, 1992). Thus, the significant inhibitory effect of EP, as observed here in hepatocytes (Table 1), strongly indicates that it is mainly CYP1A1 that is involved in the cyto toxicity observed, since βNF is reported to induce predominantly CYP1A1 (70–75%) and to a minor extent, CYP1A2 (Guengerich *et al.*, 1982), and since no toxicity was observed in UT-hepatocytes. These findings are in line with previous findings in rat liver microsomes regarding the involvement of rat CYP1A isoforms in the oxidative biotransformation of 3,5-dihalogenated PAR analogues (Bessems *et al.*, 1996).

Glutathione status

Preceding LDH leakage, severe depletion of GSH was observed in freshly isolated hepatocytes on incubation with either one of three 3,5-dihalogenated PAR analogues (Figs 1 and 2). GSH depletion was counteracted by EP as well for PAR, 3,5-diF-PAR and for 3,5-diCl-PAR. EP itself was found to cause an extra decrease of about 14% in the GSH content of the hepatocytes. This may be due to chemical reactivity between GSH and the ethynyl moiety of EP, since EP may reach intracellular concentrations of up to 1 mM. The extent of GSH depletion as found in this study, suggests that, as well as reversible oxidation of GSH to GSSG (as with 3,5-dialkylated analogues in previous studies; Van de Straat *et al.*, 1987), with 3,5-dihalogenated PAR analogues irreversible depletion of GSH is also involved, similar to recent findings in microsomal incubations (Bessems *et al.*, 1996). As shown by HPLC analysis, formation of GSH-conjugates was indeed observed in hepatocyte incubations with PAR and either one of three 3,5-dihalogenated PAR analogues (Table 3; Fig. 3). The intracellular depletion of GSH, as caused by exposure of hepatocytes to PAR or a 3,5-dihalogenated analogue, was reflected by diminished excretion of total glutathione into the extracellular medium. Cellular excretion of total glutathione in blank incubations of freshly isolated hepatocytes has been previously reported to be inhibited by exposure to PAR, although no rationale for this observation was presented (Willson *et al.*, 1991). It may be, however, that exhaustion of GSH in the cell will diminish excretion of glutathione, since the viable hepatocyte will try to maintain the common GSH/GSSG ratio. Regarding the cellular efflux of total glutathione, EP was found to restore total glutathione excretion to only a minor extent, and only in the incubations with PAR and 3,5-diCl-PAR (data not shown). It is not yet known why there are no more obvious effects of EP on cellular excretion of glutathione by hepatocytes exposed to PAR and one of three 3,5-dihalogenated analogues.

Lipid peroxidation

Another phenomenon, which has been discussed regularly as being involved in the cytotoxicity of PAR, is lipid peroxidation (Albano *et al.*, 1983). Preliminary experiments, involving exposure of hepatocytes to 0.5 mM PAR, 3,5-diF-PAR, 3,5-diCl-PAR or 3,5-diBr-PAR, revealed that lipid peroxidation did occur but only to a very limited extent (data not shown), especially when compared with previous findings regarding PAR in similar rat hepatocyte incubations (Van de Straat *et al.*, 1987). A possible explanation might be the increased sensitivity to oxidative stress in hepatocytes that were isolated from fasted as opposed to fed rats as used here, as fasting may lower the ATP content and thus the normal physiology of the cell. Furthermore, it has been concluded in several studies that lipid peroxidation does not have a causal role in loss of cell viability induced by PAR (Donatus *et al.*, 1990; Garrido *et al.*, 1991; Van de Straat *et al.*, 1987). The curcumin, for example, was found to protect against PAR-induced lipid peroxidation, without protecting against LDH leakage and GSH depletion (Donatus *et al.*, 1990). Another explanation might be a possible antioxidant activity of the 3,5-dihalogenated PAR analogues, as has been shown before for PAR and for 3-monoalkylated and 3,5-dialkylated PAR analogues (Van de Straat *et al.*, 1988).

HPLC analysis

A uniform HPLC method was developed for metabolite analysis of hepatocyte incubations with PAR and one of three 3,5-dihalogenated PAR analogues with emphasis on conjugates formed with either glucuronic acid or GSH. No detailed analysis of conjugates formed with sulfate was performed because the rate of sulfation has previously been found to be only about 20% of the glucuronidation in hepatocytes incubated with PAR (Moldéus and Gergely, 1980). Furthermore, emphasis on sulfate conjugates would have greatly increased the duration of HPLC analysis, since sulfates were expected to be the most hydrophilic class of conjugates (Howie *et al.*, 1977; Moldéus, 1978).

In this study, the GSH conjugate of PAR eluted later than PAR itself, which is in agreement with previous findings (Howie *et al.*, 1977; Moldéus, 1978). In contrast, with the 3,5-dihalogenated PAR analogues it was observed that all metabolites, including the GSH conjugates, eluted before the parent compound, a phenomenon that in general may be expected on reversed-phase chromatographic analysis (Table 3). Moreover, the appearance of cysteinyl–glycine conjugates in HPLC-analyses of γGT-treated split-samples, in quantities analogous

to those of the GSH conjugates, is striking (Table 3).

Semiquantitatively, formation of the glucuronide conjugate of PAR was estimated to be about 180 nmol per 10^6 cells in 3 hr, which is comparable to 50 nmol per 10^6 cells in 1 hr as presented by Moldéus for hepatocyte incubation with PAR (Moldéus, 1978). From the results presented in Fig. 4 and Table 3, it is obvious that, for 3,5-dihalogenated PAR analogues, quantitatively, the glucuronidation route is the most important in hepatocyte incubations; conjugation to GSH was shown to be only a minor route. The relative importance of glucuronidation over GSH conjugation for 3,5-diF-PAR is in agreement with recent findings *in vivo*, where about 55% of the dose of 3,5-diF-PAR was found to be glucuronidated (Barnard *et al.*, 1993).

Protein sulfur adducts

Analysis of the hepatocyte incubations with alkaline peralkylation and GC/MS revealed measurable protein sulfur adduct formation on incubation of rat hepatocytes with PAR, 3,5-diCl-PAR and 3,5-diBr-PAR (Table 4), analogous to the results of murine microsomal and *in vivo* experiments with PAR studying protein adduct formation by GC/MS analysis, in which a 3-cysteine-*S*-yl adduct was identified (Hoffmann *et al.*, 1985). Next to depletion of intracellular GSH, covalent protein adduct formation might be an important factor in the mechanism of toxicity. The relative importance of the formation of protein sulfur adducts, however, remains to be clarified, since these experiments did not distinguish between cytosolic, microsomal or mitochondrial protein. For PAR and its non-toxic regio-isomer, 3′-OH-acetanilide, it was found that total covalent protein binding *in vivo* was approximately equal whereas PAR and 3′-OH-acetanilide bound more extensively to mitochondrial and microsomal proteins, respectively (Tirmenstein and Nelson, 1989). Moreover, the finding that for 3,5-diF-PAR, no protein sulfur adducts were found in this study, despite extensive LDH leakage, makes this series of 3,5-dihalogenated PAR analogues valuable for further investigation of the relative importance of GSH depletion and of protein adduct formation in the toxicity mechanism.

Conclusion

Toxicity in freshly isolated rat hepatocytes on exposure to 3,5-diF-PAR, 3,5-diCl-PAR or 3,5-diBr-PAR, was shown to be comparable to the cytotoxicity of PAR and also to be mediated by CYP isoforms of the CYP1A subfamily. Furthermore, cytotoxicity was preceded by a rapid, time-associated and concentration-dependent depletion of GSH, of the same order of magnitude for PAR and each of three 3,5-dihalogenated analogues. With regard to the formation of metabolites, primarily glucuronidation was found to be important and structure dependent. Furthermore, GSH conjugates as well as protein sulfur adducts were detected.

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Chapter 8 Mechanism of protection of ebselen against paracetamol-induced toxicity in rat hepatocytes

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MECHANISM OF PROTECTION OF EBSELEN AGAINST PARACETAMOL-INDUCED TOXICITY IN RAT HEPATOCYTES

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Abstract—The protective effect of ebselen (PZ 51), an anti-inflammatory agent, on paracetamol-induced (1 mM) cytotoxicity in hepatocytes freshly isolated from β-naphthoflavone-pretreated rats was studied. At a concentration of 50 μM added simultaneously with paracetamol, ebselen prevented paracetamol-induced leakage of lactate dehydrogenase (LDH) almost completely and lipid peroxidation (LPO) and depletion of glutathione (GSH) substantially. These protective effects were even more pronounced at 100 μM concentration of ebselen. When added to the hepatocytes 1 hr before paracetamol, 50 μM of ebselen also prevented LDH leakage, LPO and GSH depletion. Reverse addition of paracetamol and ebselen did not result in protection. Simultaneous incubation of 100 μM ebselen and paracetamol inhibited GSH conjugation of paracetamol by more than 50%, however, without any effect on glucuronidation and sulfation of paracetamol. Ebselen was shown not to react directly with paracetamol nor to inhibit cytochrome P450 activity measured as 7-ethoxycoumarin *O*-deethylase (ECD) activity in the hepatocytes. At mixing, synthetic ebselen selenol and synthetic *N*-acetyl-*p*-benzoquinone imine (NAPQI) were shown to form paracetamol and ebselen diselenide. No indication was found for the formation of an ebselen–paracetamol conjugate upon reacting synthetic NAPQI and synthetic ebselen selenol. Reduction of NAPQI, the reactive metabolite of paracetamol, by ebselen selenol is discussed in terms of the mechanism of cytoprotection.

Key words: ebselen; anti-inflammatory agent; paracetamol; cytotoxicity; mechanism of protection; reactive intermediate; ebselen selenol

Paracetamol (acetaminophen, 4′-hydroxyacetanilide) is a commonly used and safe analgesic drug, which upon overdose is known to cause centrilobular hepatic necrosis [1–3]. At normal dose levels the drug mainly undergoes sulfation and glucuronidation in man and most other mammalian species. At higher doses, however, paracetamol is increasingly metabolized into a reactive metabolite, NAPQI‡ [4], by rat liver cytochrome P450. In *in vitro* studies it had been shown that the β-NF-inducible cytochrome P450 form 1A1 is mainly involved [5]. Recently, also the P450 enzymes 1A2 and 2E1 in man [6], 3A1 and 3A2 in rat liver microsomal incubations and 3A4 in Hep G2 cells expressing human cytochrome P450 [7] were reported to be importantly involved in bioactivation of paracetamol. NAPQI possesses both electrophilic and oxidant characteristics. As a consequence, it can deplete intracellular GSH and protein thiol groups by alkylation [8] and oxidation [9] which can lead to the formation of mixed disulfides [10, 11]. These events are subsequently giving rise to changes in the cellular calcium homeostasis [12–14], LPO [15, 16], loss of mitochondrial respiratory function [17], and finally to cell death. Taking these mechanisms into consideration there have been many efforts to prevent paracetamol-induced hepatotoxicity either by interference with biochemical processes involved [18] or by modification of the structure of paracetamol [15]. Extensive knowledge of the molecular mechanisms of paracetamol-induced cytotoxicity makes paracetamol an interesting and useful model toxin to study effects and mechanisms of cytoprotective agents [3].

Ebselen (PZ 51; 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), is a seleno-organic compound with low reported toxicity [19, 20], presumably because elemental selenium is not bioavailable as such. Ebselen is undergoing clinical trials for the treatment of various liver diseases and as an anti-inflammatory drug [21]. Ebselen has been demonstrated to have direct antioxidant [22] and thiol-dependent peroxidase-like activity [23, 24]. During the latter activity peroxides are inactivated by a metabolic reaction cycle in which a selenenyl sulfide, a hypothetical intermediate ebselen selenol, diselenide and selenenic acid anhydride intermediates are

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‡ Abbreviations: α-NF, α-naphthoflavone; β-NF, β-naphthoflavone; CDNB, 1-chloro-2,4-dinitrobenzene; EC, 7-ethoxycoumarin; ECD, ethoxycoumarin *O*-deethylase; GSH, glutathione; GSSG, disulfide form of glutathione; LDH, lactate dehydrogenase; LPO, lipid peroxidation; NAPQI, *N*-acetyl-*p*-benzoquinone imine.

formed through chemical reactions with GSH [25, 26] and synthetic thiols such as dithioerythreitol [27] and *N*-acetyl-L-cysteine [28]. Recently, the formation of a selenol derivative of ebselen in reaction with thiols was proven [29] by trapping the ebselen selenol with CDNB. Ebselen was shown to inhibit adenosinediphosphate-$FeSO_4$ induced LPO in isolated hepatocytes [27] and to prevent diquat induced toxicity in freshly isolated hepatocytes, which is thought to be mediated by oxygen radicals formed by redox-cycling [28].

Sodium selenite, another selenium-containing compound, has recently been shown to protect against nephrotoxicity of cisplatin [30] as well as against the toxicity of paracetamol induced in rat hepatocytes [31]. Earlier, protective effects of sodium selenite consumption against LPO after paracetamol intoxication in mice and rats, respectively, were demonstrated [16]. Recently, the nephrotoxicity of cisplatin was reported to be alleviated in mice and rats also by ebselen [32]. Baldew *et al.* [32] assumed the protective effects of ebselen and sodium selenite on cisplatin toxicity occur via ebselen selenol, a product of a reaction between ebselen and thiols for which only recently solid experimental evidence has been presented [29].

This led us to investigate whether ebselen, like sodium selenite, could protect against the various toxicity mechanisms induced by paracetamol and, moreover, to investigate the possible role of a selenol in such a protection. For reasons indicated above, hepatocytes freshly isolated from β-naphthoflavone-induced rats were selected as a test system.

MATERIALS AND METHODS

Biological materials. Collagenase B, β-glucuronidase, arylsulfatase and pyruvate were obtained from Boehringer (Mannheim, Germany). BSA, GSH and GSSG reductase, NADPH and γ-glutamyltranspeptidase (γ-GT) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bio-Rad Protein Assay kit was obtained from Bio-Rad Laboratories GmbH (München, Germany).

Chemicals. Paracetamol was purchased from Brocacef (Maarssen, The Netherlands), CDNB and EC were from Sigma. 7-Hydroxycoumarin and β-NF were from Janssen Chimica (Beerse, Belgium) and α-NF was from Aldrich-Europe (Beerse, Belgium). Ebselen was a generous gift of Rhône-Poulenc Nattermann (Cologne, Germany).

Ebselen diselenide [2,2′-diselenobis(benzanilide)] was synthesized, recrystallized and analysed by ^{1}H-NMR according to Engman and Hallberg [33]. Ebselen selenol (*N*-phenyl-2-carboxamidobenzeneselenol) was prepared by reduction of ebselen diselenide as described by Cotgreave *et al.* [29]. Synthesis of ebselen selenol was confirmed by GC–MS after derivatization by diazomethane. 2-Methylselenobenzanilide (characteristic selenium isotopic distribution; molecular ion at m/z 291 with fragment ions at m/z 276 and m/z 199) was formed [34].

NAPQI was prepared in dichloromethane with silver oxide as described by Huggett and Blair [35]. The stability and the purity of NAPQI was checked by ^{1}H-NMR and GC–MS.

Reactions between ebselen, paracetamol and their metabolites. Routinely, the stability of ebselen selenol was assessed by aromatic S_N2-substitution with CDNB as described by Cotgreave *et al.* [29]. Furthermore, this reaction between CDNB and the selenol was used to determine remaining quantities of ebselen selenol after various reactions. For the ebselen-dinitrobenzene conjugate we developed an HPLC analysis method based on reversed phase chromatography. Two RP-C_{18} cartridge columns in series (3 × 100 mm each; particle size 5 μm; Chrompack, Middelburg, The Netherlands) were used and were eluted with 1% H_3PO_4/acetonitrile (60/40) at 0.4 mL/min.

To investigate possible direct reactions between ebselen (50 μM) and paracetamol (50–350 μM) both compounds were incubated in potassium phosphate buffer (pH 7.4). UV/vis absorption between 280 and 380 nm was followed spectrophotometrically. The reactions between NAPQI and ebselen selenol (respectively metabolites of the parent compounds paracetamol and ebselen) were studied by mixing ethyl acetate solutions of NAPQI (50 and 100 μM) and selenol (50 μM).

Animals and isolation of hepatocytes. Male Wistar rats (220–240 g) from Harlan CPB (Zeist, The Netherlands) were used in the present study. They were housed in humidity (50%) and temperature (22°) controlled rooms with a 12 hr lighting cycle. Food and water were provided *ad libitum*. The rats were pretreated twice intraperitoneally with β-NF (80 mg/kg dissolved in arachides oil) at 48 and 24 hr before isolation of the hepatocytes, to induce cytochrome P450 1A1 thus making the hepatocytes more susceptible to paracetamol-induced cytotoxicity [13]. Rats were fasted overnight before isolation of the hepatocytes. The liver parenchymal cells were isolated by a two-step collagenase perfusion method essentially according to the procedure described by Seglen [36] as modified by Nagelkerke *et al.* [37]. The viability of the hepatocytes obtained was usually between 94 and 98% as judged by trypan blue exclusion.

Incubation of hepatocytes. Freshly isolated hepatocytes (1.5–2 × 10^6 cells/mL) were incubated in 7.0 mL of Hanks' HEPES buffer (pH 7.6), containing 1.5% BSA. The cells were incubated in plastic scintillation vials in a rotary shaker (140 rpm) equilibrated at 37° under a 95% oxygen and 5% carbon dioxide atmosphere for 15 min. Then paracetamol, dissolved in physiological saline, or ebselen, dissolved in DMSO were added (concentrations of paracetamol and ebselen are given in the legends). The maximal concentration of DMSO in incubation medium was 0.3% (v/v) and equal amounts of DMSO were added to the control incubations. At the start of the incubations and during 3 hr thereafter, 0.4 mL samples were taken for measuring LDH leakage and cellular GSH levels and 0.5 mL samples for measuring LPO.

At time 0 and 3 hr, 0.5 mL cell suspensions were taken for analysis of paracetamol metabolites by HPLC as described by Howie *et al.* [38]. When measuring ECD activity in the hepatocytes [39],

2 mL cell suspensions were preincubated with 1 mM paracetamol and/or 50 μM ebselen. Then ECD assays were initiated by adding EC dissolved in Hanks' HEPES buffer to a final concentration of 100 μM. After 30 min, incubations were stopped by placing the vials into liquid nitrogen.

Assays. The cytotoxicity of paracetamol was determined as LDH leakage from the hepatocytes into the medium [40]. Samples of cell suspensions (0.4 mL) were centrifuged at 100 *g* for 3 min. Of the supernatant, 0.2 mL samples were taken for the assay of LDH leakage, whereas the cell pellet was used for measuring intracellular GSH levels using the method described by Redegeld *et al.* [41]. Also lipid peroxidation (LPO) was determined in the cell pellet by measuring the formation of products reacting with 2-thiobarbituric acid essentially as described by Haenen and Bast [42], without the addition of butylated hydroxytoluene.

ECD activities were determined by measuring the formation of 7-hydroxycoumarin during 30 min of incubation with 7-ethoxycoumarin using a Perkin–Elmer fluorescence spectrophotometer with excitation and emission wavelengths of 368 and 456 nm, respectively, as described by Edwards *et al.* [39]. Protein contents were measured by a Bio-Rad protein assay.

Analysis of paracetamol metabolites. Metabolites of paracetamol were analysed by an HPLC system consisting of a Millipore Waters Model 510 pump, a Kontron Uvikon 725 ultraviolet detector (set at 250 nm), a Kipp & Zonen BD 40 recorder, and two RP-C_{18} glass tube cartridge columns (3 × 100 mm each; particle size 5 μm; Chrompack) in series. The mobile phase consisted of 1% aqueous acetic acid–methanol–ethyl acetate (90:15:0.1) and the flow rate was 0.4 mL/min. All samples were deproteinized by 12.5% trichloroacetic acid. For peak identification and quantification, samples were incubated with β-glucuronidase or arylsulfatase for 16 hr [43] or with γ-GT for 24 hr [44].

All results were expressed as means ± SD. Statistical significance was tested by Student's *t*-test.

RESULTS

Cytotoxicity of paracetamol

Using LDH leakage as a parameter, 1 mM of paracetamol was found to be cytotoxic in freshly isolated hepatocytes from β-NF-pretreated rats (Fig. 1). Paracetamol strongly and consistently induced LDH leakage from the hepatocytes, namely from 8.8 ± 3.3% before to 59.6 ± 6.9% after 3 hr incubation (Fig. 1a). Paracetamol also induced extensive LPO, measured as 2-thiobarbituric acid reactive materials in the hepatocytes over the same time period (Fig. 1b). Addition of paracetamol resulted in depletion of intracellular GSH levels to 19.2 ± 1.1% of the zero time levels after 1 hr of incubation, and subsequently to 5.2 ± 1.5% after 3 hr of incubation (Fig. 1c). The paracetamol-induced increase of LDH leakage and LPO in the hepatocytes consistently appeared 1 hr later than the decrease of intracellular GSH levels.

Cytotoxicity of ebselen

Ebselen has a maximum absorbance at 324 nm. Therefore, it was firstly examined whether ebselen itself might interfere with the assay of LDH leakage involving measurement of changes of NADH concentrations at 340 nm. It appeared that ebselen did not disturb the assay of LDH leakage in the concentration range between 50 and 200 μM.

As shown in Fig. 2a, up to a concentration of 100 μM, ebselen was not toxic to hepatocytes. However, at concentrations of 150 and 200 μM, ebselen caused a slight increase of LDH leakage from the hepatocytes after 1 hr incubation. At 50 and 100 μM concentrations ebselen did not significantly influence spontaneous LPO (Fig. 2b) and GSH depletion (Fig. 2c) as seen in control incubations with hepatocytes from β-NF-pretreated rats. Therefore and because of the limited solubility of ebselen in aqueous suspensions, 50 and 100 μM concentrations of ebselen were chosen for testing protective effects against paracetamol-induced cytotoxicity in the hepatocytes.

Protective effects of ebselen against paracetamol-induced cytotoxicity in hepatocytes

When added simultaneously with paracetamol to hepatocytes from β-NF-pretreated rats, 50 μM ebselen significantly prevented the paracetamol-induced LDH leakage from the hepatocytes (Fig. 1a). Ebselen also significantly protected against the paracetamol-induced LPO (Fig. 1b) and it delayed the paracetamol-induced GSH depletion (Fig. 1c). After 1 and 3 hr of incubation of the hepatocytes with 1 mM paracetamol and 50 μM ebselen, intracellular GSH levels decreased to 49.4 ± 9.2% and 11.1 ± 2.6% of zero time levels, respectively. Increasing the concentration of ebselen to 100 μM almost completely prevented cellular LDH leakage and LPO induced by paracetamol. GSH levels only decreased to 81.2 ± 13.9% of zero time levels after 1 hr of incubation and to 30.7 ± 10% after 3 hr of incubation (Fig. 1c).

In order to find out whether there exists a time dependency of the protection by ebselen against the paracetamol-induced toxicity, the protection of ebselen was also examined when added 1 hr before or after paracetamol (Table 1). Added 1 hr before paracetamol, 50 μM ebselen still alleviated paracetamol-induced LDH leakage, LPO and GSH depletion in the hepatocytes (Table 1). However, when ebselen (50 μM) was added 1 hr later than paracetamol, it did not protect anymore against LDH leakage, LPO and intracellular GSH depletion due to paracetamol (Table 1).

Effects of ebselen on paracetamol metabolism

During 3 hr of incubation, approximately 35% of 1 mM paracetamol was metabolized by the hepatocytes to only three well-known metabolites which were detected by HPLC, i.e. the glucuronide, the sulfate and the GSH conjugate. As shown in Table 2, 100 μM ebselen added simultaneously with 1 mM paracetamol to hepatocytes had no statistically significant effect on the formation of the glucuronide and sulfate conjugates of paracetamol. However, it significantly decreased the formation of the GSH conjugate of paracetamol (which forms about 5% of total paracetamol metabolism) to less than about

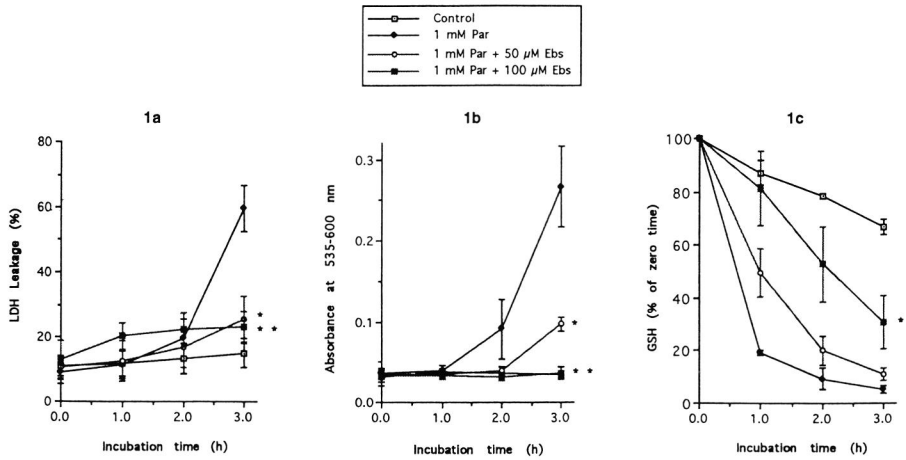

Fig. 1. Effects of paracetamol (1 mM) and paracetamol plus concomitantly added ebselen (50 or 100 μM) on LDH leakage (a), LPO (b) and GSH depletion (c) in freshly isolated hepatocytes from β-NF-pretreated rats during incubation. Par: paracetamol; Ebs: ebselen. Results are presented as mean values ± SD of four experiments. *$P < 0.05$, **$P < 0.01$.

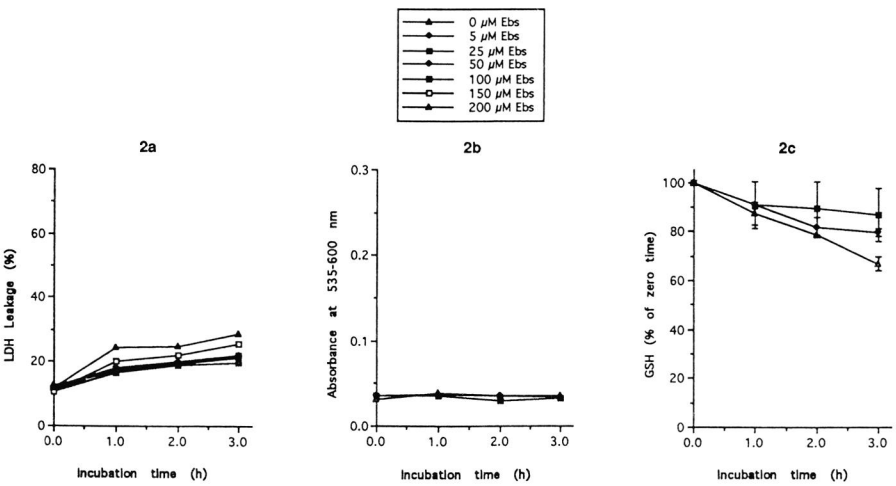

Fig. 2. Cytotoxicity of ebselen measured as LDH leakage (a) and effects of ebselen on LPO (b) and GSH levels (c) in hepatocytes from rats pretreated with β-NF. During the incubation, 0.2 mL samples of the cell suspensions were taken at every hour for measuring LDH leakage from the hepatocytes, 0.4 and 0.5 mL for GSH levels and LPO, respectively. LDH leakage is expressed as % of total activity in the cells. LPO was measured as TBA-reactive material at 535–600 nm. The results are presented as mean values ± SD of four separate experiments.

Table 1. Time dependency of the effect of ebselen on paracetamol-induced toxicity in freshly isolated hepatocytes

Treatment	LDH leakage (%)		Lipid peroxidation		GSH levels (%)	
	t = 0 hr	t = 3 hr	t = 0 hr	t = 3 hr	t = 0 hr	t = 3 hr
Control	12.9 ± 3.2	19.3 ± 7.1	7.0 ± 7.5	9.3 ± 5.4	100	93.4 ± 3.2
Par (1 mM)	12.2 ± 5.2	63.3 ± 19.4	5.0 ± 6.0	208.3 ± 72.7	100	2.6 ± 0.7
Par + Ebs*	13.0 ± 2.4	30.0 ± 13.5§	5.3 ± 3.8	65.5 ± 45.8§	100	7.7 ± 5.1
Par + Ebs†	12.1 ± 2.3	47.3 ± 8.0§	5.7 ± 5.0	83.7 ± 72.7	100	6.2 ± 2.0
Par + Ebs‡	11.8 ± 4.6	75.5 ± 18.4	4.0 ± 4.6	134.3 ± 81.9	100	3.1 ± 1.6

The final concentrations of paracetamol (Par) and ebselen (Ebs) in incubation mixtures were 1 mM and 50 μM, respectively. Rats were pretreated with β-NF as described in Materials and Methods. LPO was measured as absorbance at 535–600 nm $\times 10^{-3}$. The results are presented as means ± SD (N = 3).

* Paracetamol and ebselen were added concomitantly; †ebselen was added 1 hr before paracetamol; ‡ ebselen was added 1 hr later than paracetamol.

§ $P < 0.05$ as compared with paracetamol alone.

Table 2. The effect of ebselen on paracetamol metabolism in hepatocytes freshly isolated from β-NF-pretreated rats during 3 hr incubation

Treatment	Paracetamol	Paracetamol conjugates		
		Glucuronide	Sulfate	Glutathionyl
Par	100 ± 9	100 ± 37	100 ± 20	100 ± 22
Par + Ebs	108 ± 8	81 ± 11	103 ± 29	41 ± 20*

Par: 1 mM paracetamol; Par + Ebs: 1 mM paracetamol + 100 μM ebselen. Individual values are given as percentage of the quantity recovered in the reference incubation with paracetamol solely. All data are presented as means ± SD (N = 4).

* $P < 0.001$.

50% of the original amount. In the presence of 100 μM ebselen, total biotransformation of paracetamol was not changed significantly, since still some 30% of 1 mM paracetamol was metabolized by the hepatocytes (Table 2).

Effect of ebselen on ECD activity

The cytotoxicity of paracetamol is thought to be mediated by NAPQI, the formation of which is catalysed by various forms of cytochrome P450. ECD activity reflects the activity of a number of cytochromes P450, some of which (e.g. 1A1) are responsible for the conversion of paracetamol to NAPQI [5]. Therefore, the effect of ebselen on the *in situ* ECD activity in hepatocytes was examined. Firstly, the effect of DMSO (solvent for ebselen) was checked. ECD activities in the hepatocytes were 26.5 ± 4.8 (no preincubation), 26.9 ± 6.8 (preincubated with DMSO for 1.5 hr) and 29.0 ± 13.3 (preincubated with DMSO for 2.5 hr) nmol/min/mg protein (Fig. 3; control bars). This indicates no significant influence of DMSO during preincubation on the ECD activity. ECD activity in hepatocytes was completely inhibited by the presence of α-NF (50 μM) upon 30 min of incubation (results not shown), indicating that ECD activity as assayed can be inhibited (positive control).

Neither ebselen (50 μM) nor paracetamol (1 mM) significantly influenced ECD activity in the hepatocytes, even when incubated with hepatocytes for

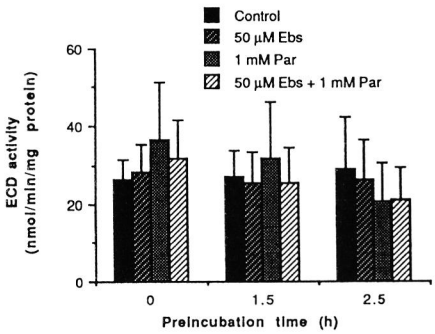

Fig. 3. Effects of ebselen and paracetamol on ECD activity in freshly isolated hepatocytes from β-NF-pretreated rats. Before the ECD assays started the hepatocytes were preincubated with 0.5% DMSO (control), 50 μM ebselen and/or 1 mM paracetamol for 0, 1.5 and 2.5 hr. ECD assays were started by adding EC (100 μM) to the hepatocytes and the incubation continued for another 30 min. The formation of EC was measured fluorimetrically with excitation and emission wavelengths set at 368 and 456 nm, respectively. The results are presented as mean values ± SD of six experiments.

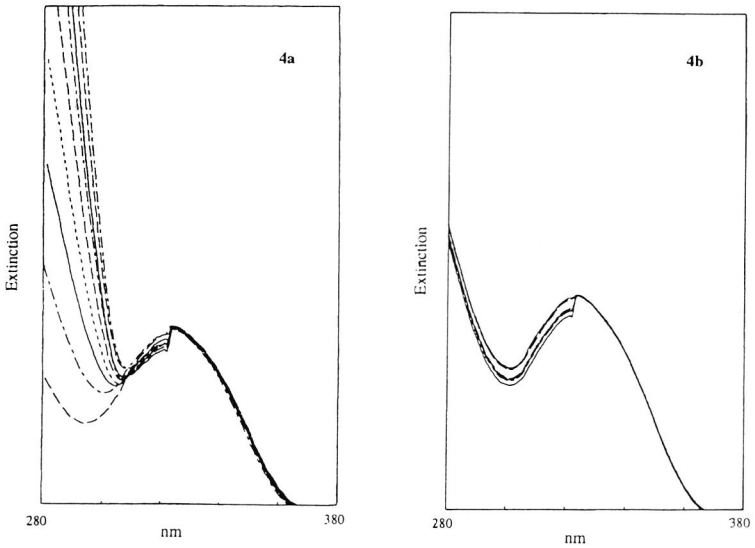

Fig. 4. UV/vis spectra of ebselen in the presence or absence of paracetamol. Incubations were carried out at 37° in potassium phosphate buffer (pH 7.4). Time dependency (a): ebselen (50 μM) and paracetamol (50 μM) were incubated for 30 min and during the incubation the UV/vis spectra were recorded every 2 min. Concentration dependency (b): ebselen (50 μM) was incubated with 50, 100, 150, 200, 250, 300 and 350 μM paracetamol, respectively. After incubation for 2 min the UV/vis spectra were recorded.

1.5 and 2.5 hr before the ECD assay was started (Fig. 3). Even when ebselen (50 μM) and paracetamol (1 mM) were added simultaneously, the ECD activity was not significantly different from incubations with paracetamol alone.

Reactions between ebselen, paracetamol and metabolites

Ebselen is known to react rapidly with the thiol group of GSH and other biological thiols to give a selenenyl sulfide, which in the presence of excess thiols is rapidly converted into the diselenide of ebselen [25, 26]. With UV/vis spectroscopy and HPLC it was investigated whether ebselen reacts directly with paracetamol in buffer. Besides an aromatic absorption at 280 nm, ebselen has a maximum absorption at 324 nm representing the five-membered isoselenazol ring. Addition of paracetamol in concentrations varying from 50 to 350 μM to 50 μM ebselen, increased the absorption at 280 nm without any change at 324 nm (Fig. 4a). Incubation of 50 μM paracetamol and 50 μM ebselen for 30 min did not change the absorption of ebselen at 324 nm (Fig. 4b). HPLC analysis of the latter reaction mixture revealed only two peaks with retention times of 2.7 and 4.9 min, respectively, which were identical to those of separately injected paracetamol and ebselen, indicating that ebselen did not react directly with paracetamol.

When NAPQI (50 μM) and ebselen selenol (50 or 100 μM), both in ethyl acetate, were mixed, HPLC analysis revealed four peaks after "trapping" of unreacted ebselen selenol with CDNB (Fig. 5). One peak coeluted with authentic paracetamol (2.7 min), the second with unreacted CDNB (4.9 min), the third (7.1 min) with authentic ebselen dinitrobenzene conjugate (reflecting trapped ebselen selenol) and the last peak with authentic ebselen diselenide (13.0 min). No peak of a conjugated product of NAPQI and ebselen selenol was detected.

DISCUSSION

The aim of the present study was to investigate whether ebselen possesses protective activities against paracetamol-induced cytotoxicity in freshly isolated rat hepatocytes and furthermore to study the underlying mechanisms of protection. The mechanisms by which paracetamol causes hepatic necrosis upon overdose involve metabolic conversion of the drug into a reactive metabolite, NAPQI, primarily by 3-methylcholanthrene and β-NF-inducible forms of cytochrome P450. NAPQI, the presumed toxic metabolite of paracetamol, has been shown to irreversibly deplete cellular GSH, to bind covalently to protein thiol groups and to induce an oxidative stress in hepatocytes manifesting itself in oxidative depletion of protein thiol levels, LPO and

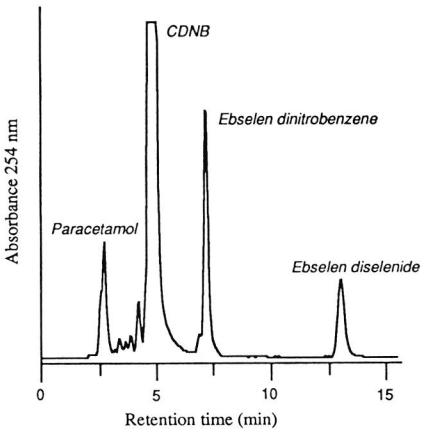

Fig. 5. HPLC analysis at reaction of ebselen selenol and NAPQI. Paracetamol (2.7 min), CDNB (4.9 min), ebselen dinitrobenzene (7.1 min), ebselen diselenide (13.0 min).

disturbances of e.g. the cellular Ca^{2+}-homeostasis [3, 13, 45].

The present results show that cell death caused by paracetamol occurred 2–3 hr after addition of paracetamol (1 mM) to hepatocytes isolated from β-NF-pretreated rats (Fig. 1). The cytotoxicity of paracetamol observed in this study corresponds well with that previously reported [13, 15], and indicates that this system under the applied circumstances is useful as a test system for cytoprotective effects. During the first 2 hr paracetamol was apparently converted into NAPQI, immediately causing GSH depletion and subsequently LPO and cell death (Fig. 1). GSH plays an important role in the detoxification of electrophilic reactive intermediates, active oxygen species and other radicals. Hydrogen peroxide and lipid hydroperoxides are reduced by GSH peroxidase, in a process in which GSH is oxidized to GSSG. Furthermore, GSH plays a role in the reduction of protein disulfides and protein mixed disulfides under the formation of GSSG [11].

Ebselen has been shown to be a potential anti-inflammatory drug [19] and to possess antioxidant activity [23]. In addition, ebselen possesses GSH peroxidase-like activity, using hydrogen peroxide and lipid hydroperoxides as substrates [23, 25]. In the present study, at concentrations of 50 and 100 μM, ebselen showed no toxicity to freshly isolated hepatocytes from β-NF-pretreated rats. When added concomitantly with paracetamol to the hepatocytes, ebselen at 100 μM completely prevented paracetamol-induced cytotoxicity, as indicated by LDH leakage (Fig. 1a) and LPO (Fig. 1b), yet, it only partly inhibited GSH depletion induced by paracetamol (Fig. 1c). These results support the concept that a moderate GSH depletion by paracetamol does not necessarily cause cell death [13, 15]. Ebselen (50 μM) added 1 hr before paracetamol also could prevent paracetamol-induced cytotoxicity. However, added 1 hr after paracetamol 50 μM of ebselen could not protect the hepatocytes anymore. A concentration of 100 μM ebselen decreased paracetamol GSH conjugate formation in the hepatocytes more than 50% (Table 2). In contrast, no statistically significant effect on formation of paracetamol glucuronide and sulfate conjugates was seen. This suggests that ebselen prevents the paracetamol-induced depletion of cellular GSH, and afterwards, LPO and cell death, by inhibiting the conjugation of paracetamol to GSH.

The mechanisms for the inhibition of the formation of a paracetamol–GSH conjugate by ebselen may be at least 5-fold:

(1) Ebselen inhibits cytochrome P450 and hence decreases the formation of NAPQI from paracetamol. Ebselen has been shown to disrupt rat hepatic microsomal electron transport chains, e.g. the one catalysed by NADPH-dependent cytochrome P450 reductase [46]. Furthermore, ebselen has been shown to convert microsomal cytochrome P450 to cytochrome P420 [47], presumably by interaction with a sulfhydryl group. In this study the effect of ebselen on ECD activity of cytochrome P450 was investigated in hepatocytes from β-NF-pretreated rats. The ECD activity in control hepatocyte incubations (containing 0.5% DMSO) was in the same order of magnitude as the activity shown by Edwards *et al.* [39]. The ECD activity in our hepatocytes could be completely inhibited by 50 μM α-NF. Ebselen however, at 50 μM, did not significantly influence the ECD activity in the freshly isolated hepatocytes (Fig. 3). The aforementioned inhibitory effects of ebselen on the microsomal mixed function oxidase system and/or purified NADPH-dependent cytochrome P450 reductase obviously are not relevant in a more integrated test system such as the hepatocyte which contains GSH. Protection mechanism (1) thus does not seem to be relevant in rat hepatocytes.

(2) Ebselen protects as a direct, thiol-independent antioxidant or radical scavenger. Our results show that ebselen significantly reduced paracetamol-induced LPO. However, it is still questionable, whether radical-mediated LPO is causally involved in cell death by paracetamol. The antioxidant curcumin for example has been published to protect against paracetamol-induced LPO but not cell death [48]. On the other hand, flavones can protect hepatocytes against the toxicity of paracetamol by antioxidant activity [18]. A direct, radical scavenging antioxidant activity of ebselen as observed e.g. for halogenated peroxyl radicals [22] cannot be excluded beforehand as a mechanism for the observed prevention of paracetamol-induced cell death.

(3) Ebselen protects via thiol-dependent GSH peroxidase-like activity. Since in this study ebselen showed a significant protection against paracetamol-induced LPO (Fig. 1), it is conceivable that GSH peroxidase-like activity may also be responsible for this phenomenon. The inhibitory effect of ebselen on paracetamol-induced LPO could be based on removal of hydrogen peroxide which was assumed by several authors to be involved in this form of

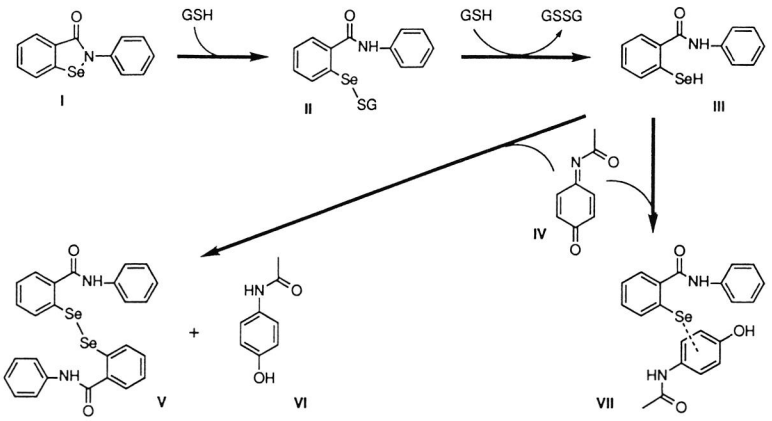

Fig. 6. Proposed mechanism of protection against paracetamol-induced cytotoxicity by ebselen. The route to compound VII is very unlikely, based on the results of this study. I, ebselen; II, selenenylsulfide; III, ebselen selenol; IV, NAPQI; V, ebselen diselenide; VI, paracetamol; VII, ebselen–paracetamol conjugate.

LPO [45, 49]. At investigating the protective effects of ebselen on paracetamol-induced LPO, Harman *et al.* [49] suggested the GSH peroxidase-like activity of ebselen to be mainly involved. However, care should be taken when comparing their results with our data because freshly isolated mouse hepatocytes were used by these authors. The fact that ebselen does not exhibit GSH peroxidase-like activity in GSH depleted hepatocytes [27] favors a contribution of the latter mechanism (3) over the GSH-independent protection mechanism (2). When adding ebselen 1 hr after paracetamol, ebselen did not protect the hepatocytes anymore against paracetamol-induced cytotoxicity (Table 1). The reason for this may be the fact that insufficient GSH remained to bioactivate ebselen to ebselen selenol. This ebselen selenol is assumed to be an intermediate in the formation of ebselen diselenide, which has been published to be responsible for the GSH peroxidase-like action of ebselen [25, 28].

(4) Ebselen or an ebselen metabolite reacts directly with the remaining paracetamol (after glucuronidation and sulfation), thus decreasing the formation of NAPQI from paracetamol. In the present study we showed however, that ebselen does not react with paracetamol directly (Fig. 4). Furthermore, HPLC analysis revealed no reaction products upon mixing paracetamol and synthetic ebselen selenol.

(5) Ebselen or a metabolite of ebselen reacts with NAPQI. Two possible reactions as depicted in Fig. 6 may occur. An important metabolite of ebselen *in vitro* in the presence of thiols is ebselen selenol as has been postulated [25] and recently demonstrated [29]. Also, *in vivo* experiments (the urinary excretion of an Se-glucuronide of ebselen) support the formation of ebselen selenol [34]. In our experiments in which synthetic ebselen selenol and NAPQI were reacted in ethyl acetate in the absence of GSH, it was shown that only ebselen diselenide and paracetamol were formed. This indicates a simple reduction of NAPQI by ebselen selenol (Fig. 6). Moreover, we have shown in an aqueous system (unpublished results) that ebselen itself reduced NAPQI to paracetamol only in the presence of GSH which is necessary to activate ebselen to ebselen selenol. Importantly, in a direct reaction between NAPQI and ebselen selenol no selenium-containing conjugate of paracetamol could be identified (Fig. 6). Similar analysis of hepatocyte incubations failed to detect any such conjugate (results not shown). Apparently, reduction of NAPQI by ebselen selenol is much more efficient than conjugation between the nucleophilic selenol and NAPQI. In line with a decrease of the NAPQI concentration in the hepatocyte due to reduction by ebselen selenol, less paracetamol GSH conjugate was found to be formed (Table 2).

Summarizing, we have shown that at a concentration of 50 μM, ebselen protects rat hepatocytes almost completely against cell death and partly against LPO and GSH depletion induced by paracetamol. At a concentration of 100 μM, the protective effect of ebselen was even more complete and highly significant for GSH depletion. The mechanism of protection may be the GSH peroxidase-like activity of ebselen, as postulated by Harman *et al.* [49] recently, assuming hydrogen peroxide to be involved in paracetamol-induced cytotoxicity. The results of this study however, strongly indicated that a reaction between ebselen selenol and NAPQI may also be responsible. In contrast to the reaction between CDNB and ebselen selenol recently described [29], we were not able to detect any nucleophilic substitution product between NAPQI and ebselen selenol in a chemical system. Therefore

conjugation between these intermediates is not a likely mechanism of protection in the hepatocytes. Ebselen selenol obviously more efficiently reduces NAPQI back to paracetamol instead of forming a substitution product. We are currently working on the kinetics of this unexpected mechanism of protection against paracetamol-induced toxicity. Further studies have to be carried out as to the relevance of this protection mechanism for *in vivo* toxicity of paracetamol.

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SECTION V OVERALL SUMMARY

Chapter 9 Summary, conclusions and future perspectives

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

General introduction and aim of the research project

Paracetamol (PAR, acetaminophen, 4'-hydroxyacetanilide) is used widely as an over the counter drug with analgesic and antipyretic properties (Thomas, 1993). Although regarded in general to be safe at therapeutic doses, PAR is known to cause severe hepatic and renal damage upon overdose. For several decades, numerous attempts have been described to increase the benefit/risk ratio of PAR (Vermeulen *et al.*, 1992). Molecular pharmacological investigations revealed that the beneficial properties are probably dependent mechanistically on decreased synthesis of prostaglandins by inhibition of prostaglandin endoperoxide synthase (Thomas, 1993). As the phenolic hydroxyl group in PAR is an important structural requisite for the pharmacological properties and hydrogen abstraction from this -OH group seems to be involved in the inhibition of prostaglandin endoperoxide synthase, the redox potential of the -OH group is likely to be an important parameter when studying structural modification of PAR in order to improve its analgesic action.

With respect to the molecular toxicological aspects of PAR, it has been known since long that in biotransformation reactions, often referred to as phase II conjugation reactions, PAR is linked with uridine-5'-diphosphoglucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) with concomitant formation of watersoluble conjugates. In the, with respect to PAR, minor reactions by prostaglandin endoperoxide synthase (PGES) and microsomal cytochrome P450-dependent mixed-function oxidases (MFO; monooxygenase or peroxydase activities), often referred to as phase I metabolic transformation reactions, PAR is oxidised in order to introduce a reactive group for subsequent phase II conjugation. With respect to PGES, this phase I functionalisation reaction presumably (*in vitro* observations) results in the formation of the free radical species *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) as well as *N*-acetyl-*p*-benzoquinone imine (NAPQI). P450-dependent oxidation results in the formation of 3-hydroxyparacetamol (3-OH-PAR) and, toxicologically more relevant, *N*-acetyl-*p*-benzoquinone imine (NAPQI). In a succeeding phase II detoxification reaction this oxidative metabolite NAPQI is conjugated with glutathione (GSH). However, at high intracellular concentrations of PAR in the hepatocyte as a consequence of an overdose, increased NAPQI formation exhausts the cellular GSH-pool. This results in covalent modification of enzymatic proteins as well as impairment of the cellular capacity to keep essential thiol groups in enzymes in their reduced state (Vermeulen *et al.*, 1992).

At the start of the research, of which the results are described in this dissertation, various attempts to decrease the hazardous properties of PAR with concurrent retainment or improvement of its pharmacological properties by structural modification had been described (Vermeulen *et al.*, 1992). Efforts were mainly aimed at prevention of GSH-depletion as caused by conjugation with NAPQI. Mostly, the basic structure of PAR was modified by mono- and disubstitution by alkyl-groups at various

aromatic positions (Harvison *et al.*, 1986; Van de Straat *et al.*, 1986; Porubek *et al.*, 1987; Van de Straat *et al.*, 1987a). Especially 3,5-dialkylation of PAR resulted, with retainment of phase I oxidation, in significant decrease or even prevention of hepatotoxicity in male DAP mice (Van de Straat *et al.*, 1986; Van de Straat *et al.*, 1987a). However, unfortunately some general toxicity at other sites than the liver was observed upon *in vivo* administration of the mono- and dialkylated anlogues which was not totally unanticipated as they were designed specifically for avoiding hepatotoxicity (unpublished results). The pharmacological consequences did not receive much attention so far.

In this thesis, the aim was broadened. We set out to investigate whether, with retainment of analgesic activities, structural modification of PAR could be used primarily to modulate phase I biotransformation reactions with special emphasis on one of the activities of the cytochrome P450-dependent mixed-function oxidase (MFO) system. This system exhibits monooxygenase and peroxidase activity, oxygen reductase activity and substrate reductase activity (Goeptar *et al.*, 1995). Although in enzyme catalysed transformation reactions of PAR and of metabolites, more than one of these activities may be relevant (Van de Straat *et al.*, 1987b), the investigations described in this dissertation concentrated on the monooxygenase and peroxidase activity.

As increased microsomal cytochrome P450-dependent oxidation of the phenolic -OH group at high intracellular concentrations is producing larger quantities of a highly reactive metabolite, the redox potential of PAR and analogues is probably as important with respect to the hazardous properties as with respect to its pharmacological functioning. In order to simplify various investigations, the plant enzyme horseradish peroxidase was used as a model enzyme with one-electron oxidative (or single hydrogen abstraction) properties towards PAR, exhibiting similarities with the pharmacologically relevant PGES as well as with the toxicologically relevant P450. Actually, the P450-catalysed two-electron oxidation (or double hydrogen abstraction) of PAR to NAPQI is not a classical monooxygenase-reaction as no oxygen rebound is taking place (Koymans *et al.*, 1989). Instead, a second hydrogen atom is abstracted resulting in an overal dehydrogenation. In contrast, the P450-catalysed formation of 3-OH-PAR, a minor metabolite of PAR as mentioned before, is an authentic monooxygenase reaction.

As mentioned above, disubstitution of PAR with electron-donating alkyl-groups adjacent to the phenolic hydroxyl appeared to have only a minor influence on microsomal P450-dependent oxidation (Van de Straat *et al.*, 1986). Modulation of the electronic properties of the hydroxyl group by introducing electron-withdrawing substituents, next to electron-donating groups other than alkyl-moieties, could possibly result in differential oxidation by P450. However, the NAPQI derivatives that are likely to be formed could also exhibit various properties with respect to subsequent phase II conjugation reactions, due to modification of the electronegativity of the site for nucleophylic GSH attack. Thus, extension of the modifications of PAR was not only anticipated to result in mechanistic tools for the investigation of phase I bioactivation but also of phase II detoxification.

Review on molecular toxicological and biochemical aspects

A comprehensive review of the molecular toxicological and biochemical investigations into the mechanism of PAR-dependent cell damage and the possibilities of chemoprotection and of structural modification to improve the benefit/risk ratio of PAR with special emphasis on the last decade is presented in **Chapter 2**. Albeit also renal toxicity is described the review is dealing mainly with hepatotoxicity aspects upon intake of large amounts of PAR at once as this is the most prominent potential risk of PAR.

Significant species differences with respect to susceptibility to PAR-dependent hepatic and renal toxicity (mice and hamsters are relatively sensitive whereas rat, rabbit and guinea pig are rather resistant) are described to be reflected by the ratio of urinary excretion of toxication pathway-related versus detoxication pathway-related metabolites, i.e. glutathione-pathway metabolites versus the sulphate and the glucuronide conjugate of PAR. In combination with species differences in phase II biotransformation enzymes, variance in hepatic phase I oxidative enzymes is importantly correlated with sensitivity of PAR-toxicity. In mice and hamsters, phase I biotransformation seems to be mostly limited to the P450 enzymes CYP2E1 and CYP1A2, both activating P450s. In contrast, rats seem to exhibit a broad scala of P450s that are active in oxidation of PAR as well as inactivation of PAR to NAPQI and 3-OH-PAR, respectively. In addition, some rat P450s such as CYP2B1 exhibit significant regioselective catalytic oxidation in favour of 3-OH-PAR. Although the sensitivity of the human species has not been compared in detail with that in other species, in man, probably CYP3A4 is mainly involved in oxidative biotransformation of PAR at therapeutic intake whereas CYP2E1 becomes increasingly involved at high intake levels. Acute renal toxicity is largely dependent on species and gender specific activation of PAR by renal CYP2E1 with male mice being most sensitive. Additional species differences in the other phase I biotransformation group of enzymes, i.e. the peroxidases (such as PGES), may cause species selective formation of radical metabolites from PAR via *p*-aminophenol. With respect to the mechanism of oxidation of PAR to NAPQI by P450s, a direct two-electron oxidation mechanism is most likely. A single hydrogen abstraction mechanism, resulting in a phenoxy radical intermediate (NAPSQI), and followed by a second hydrogen abstraction or hydroxyl radical recombination, resulting in NAPQI and 3-OH-PAR, respectively, has been suggested but lacks experimental proof.

Conjugation of NAPQI with sulfhydryl groups (of GSH and proteins) via 1,4-Michael addition was described to provide a relatively inert thioether bond. A possible mechanisms of targetting of NAPQI to extrahepatic tissues sites distinct from the site of formation may be the formation of a relatively unstable *ipso*-adduct at the C1-carbon (to which the *N*-acetyl group is linked). NAPQI could be released from an *ipso*-adduct, either with GSH or protein, at sites distinct from the endoplasmatic reticulum or even at extracellular molecular targets. Thus, cytoplasmatic, mitochondrial, plasma membrane as well as nuclear proteins could be covalently modified as such in the first hours after PAR-intoxication, designated as Stage I of toxicity. Formation 3-(cystein-*S*-yl)paracetamol protein adducts, as even found in

plasma of PAR-intoxicated patients, probably exibits a dominant mechanistic role in acute hepatic necrosis. These PAR-arylated proteins appear time- and dose-dependently in liver fractions mice upon administration of PAR and the adduct levels in serum correlate with serum ALT levels. Many of these proteins that have been shown the last decade to become arylated, catalyse important biochemical events and may exhibit cellular signalling functions. The major alkylated proteins, of which the actual functions remain to be elucidated, are designated as the 55 to 58 kDa 'acetaminophen-binding proteins' may act as scavengers of reactive metabolites of xenobiotics with oxidant properties or even exhibit a nuclear signalling function (see Figure 1).

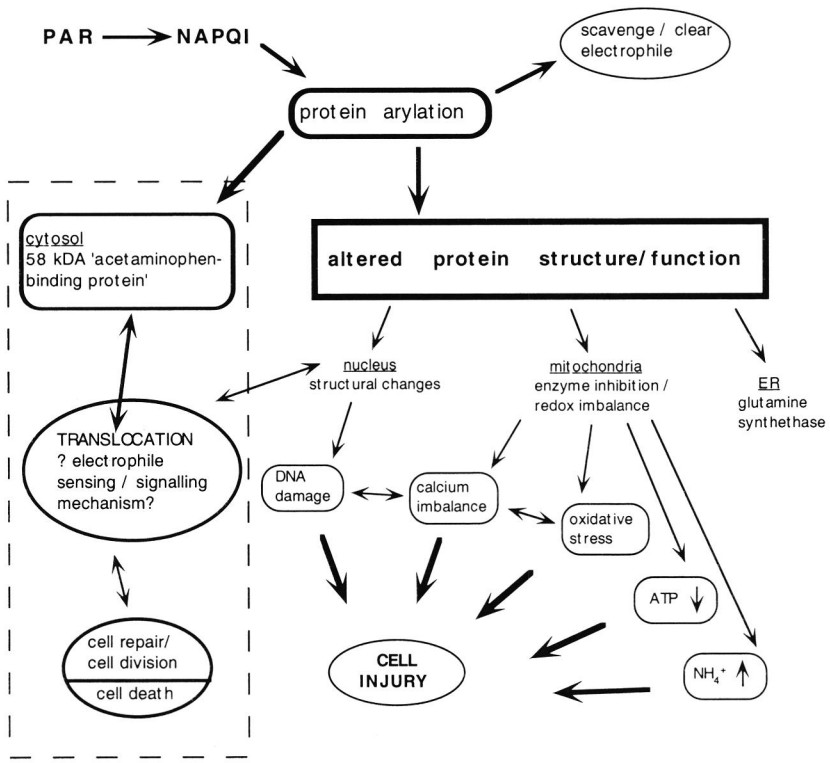

Figure 1: Proteins with scavenging effects or even nuclear signalling function. Adapted from Cohen and Khairallah (1997)

An emerging number of *in vitro* results as well as epidemiological reports were described that point to all kinds of nuclear effects. The most prominent findings are impaired DNA repair and even DNA-adduct formation at low target concentrations. In addition to necrosis, also apoptosis may be involved in some stages of the highly

integrated process of PAR-induced toxicity. It remains to be established, however, what the relevance as well as the possible consequences of the *in vitro* findings as well epidemiological findings are for PAR-intoxicated patients as well as humans taking PAR at therapeutic levels.

It was envisaged in the last decade that in the multistage process of PAR-poisoning many extracellular events emerged upon administration of a toxic dose of PAR to mice after the first hours (Stage I) and spread in liver tissue. These events, designated as Stage II phenomena, include excretion of growth factors, inflammatory mediators and reactive oxygen species by hepatocytes as well as non-parenchymal cells such as the Kupffer cells. Some of these mediators stimulate tissue repair whereas others provoke tissue damage. As of yet it is not clear whether a relation exists between the nuclear effects and these Stage II occurrings. However, these findings may provide interesting leads for clinical treatment of acute hepatic failure.

It was described that with the exception of *N*-acetylcysteine, the most widely used antidote, many agents that have been investigated mainly in experimental animals for modulation of liver toxicity of PAR are of little value in the clinic as they are to be administered before (chemoprevention) PAR. Components to be administered concomitantly with PAR (chemoprotection) could provide protection. A new guide might be to protect susceptible molecular targets by stimulation or improvement of the functioning of the 'acetaminophen-binding proteins', e.g. by adding selenium containing compounds to PAR-tablets. In addition, as most primary damage (Stage I) often has taken place before admittance to a hospital, also, as indicated above, intervention in Stage II processes might be a promising lead for clinical treatment of PAR-intoxication.

Lastly, structural modification was described as a lead for numerous investigations into improvement of analgesic and safety properties of PAR. A new lead for structural modification of PAR in order to obtain a safer analgesic substance should combine two traces. The analgesic lead prescribes an acetanilide with an -OH or eventually an $-NH_2$ at *para*-position and the *N*-acetyl group 'coplanar'. The 'toxicologically safe' lead prescribes substituents *ortho* to the -OH or maybe $-NH_2$ group that are not liable to a rapid addition-elimination reaction by GSH after formation of a corresponding NAPQI analogue. In addition, the substituents themselves should not be inert to biotransformation since this could direct biotransformation to *N*-deacetylation. Substitution by alkyl groups via an ether or thioether bond might be fullfilling both analgesic and safety requirements.

In general, it is concluded that notwithstanding the huge amount of investigations on chemoprevention, on chemoprotection and on various analogues of PAR, very few clinically useful results have been obtained. However, the benefits of the ever increasing knowledge on the moleculair mechanisms of the PAR-dependent toxicity are evident. Mechanism-based development of chemoprotective agents and progress in the development of safer structural analogues may be expected. In addition, it was argued that understanding of the molecular toxicological aspects of the model-toxicant PAR is important in the comprehension of toxicity-mechanisms of many clinically relevant and clinically non-relevant chemical substances. Perception of these molecular and

biochemical mechanisms may help in the development of improved methods for early treament of intoxications as well as refined methods in toxicological risk assessment of chemicals.

Experimental work

In **Chapter 3**, the physicochemical (oxidation potential), pharmacological (inhibition of cyclooxygenase, one of the activities of PGES) and toxicological properties (cytotoxicity) of a new series of six 3,5-disubstituted analogues of PAR were examined and compared to the properties of PAR and 3,5-dimethylparacetamol, an existing analogue. The effect of disubstitution adjacent to the hydroxyl group was studied in order to establish possible structure-activity relationships. Oxidation of the phenolic hydroxyl group of PAR and the analogues studied is likely involved in the analgesic action of paracetamol as well as in its toxification by cytochrome P450 (Fig. 2).

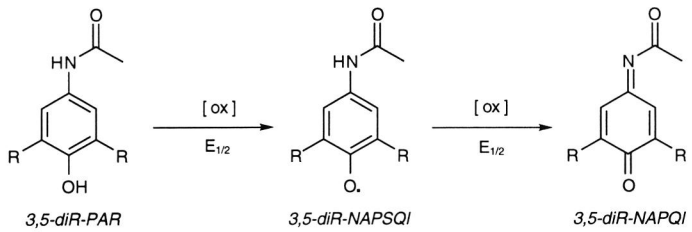

Figure 2: Two subsequent hydrogen abstractions from paracetamol

3,5-Substituents with electron-donating capacities (R = $-CH_3$, $-OCH_3$, $-SCH_3$) decreased the electrochemical half-wave oxidation potential substantially by 0.07 V to 0.16 V when compared to PAR, improved the mouse brain cyclooxygenase inhibiting capacity of PAR and inhibited the cytotoxicity of PAR, when measured as leakage of lactate dehydrogenase from freshly isolated rat hepatocytes, almost completely.

Electron-withdrawing halogen substituents (R = -F, -Cl, -Br or -I) increased the oxidation potential by 0.04 V to 0.06 V when compared to PAR and decreased the cyclooxygenase inhibiting capacity. In agreement with this, the *in vivo* analgesic activity of the 3,5-dihalogenated analogues in mice was lower when compared to PAR. Most 3,5-dihalogen substituents (R = -F, -Cl or -Br) diminished the cytotoxicity of PAR slightly. The fourth electron-withdrawing substituent (R = -I), however, strongly lowered the cytotoxicity of PAR in this test system.

In conclusion, a superior cyclooxygenase inhibitory potency of derivatives of PAR 3,5-disubstituted with electron-donating groups (R = $-CH_3$, $-OCH_3$, $-SCH_3$) seemed to correlate with a lower oxidation potential and a lower cytotoxicity. Therefore, 3,5-disubstituted analogues with electron-donating substituents might be safer analgesics than PAR itself with respect to hepatotoxicity at high intake levels. The opposite probably applies mostly to analogues of PAR with electron-withdrawing

substituents (R = -F, -Cl, -Br or -I) at the 3- and 5-positions in the aromatic nucleus (Bessems *et al.*, 1995).

In **Chapter 4**, results on alternative purification methods for rat liver microsomal CYP1A1, CYP2E1 and P450-RED were described. By using High Performance (HP) HiLoad ion-exhange chromatography columns in an FPLC (fast protein liquid chromatography)-system the benefits of classical preparative open-end scale chromatography were combined with the convenience of programmable HPLC equipment. Preliminary results indicated important improvements with respect to the classical time-consuming purification starting mostly with octylamino Sepharose hydrophobic interaction chromatography.

A pilot scale CYP1A1 purification procedure was performed using liver microsomes of rats, pretreated with ß-naphthoflavone (ßNF) and 0.3% Emulgen 911 for solubilisation. For further removal of non-CYP1A1 proteins, only HP Q-Sepharose ion-exchange chromatography was performed. After removal of Emulgen 911 by hydroxyapatite chromatography CYP1A1 was apparently pure as judged by SDS-PAGE and Western blotting using anti-rat CYP1A1.

In the large scale CYP2E1 purification procedure, proteins were solubilised from microsomes of pyrazole-treated rats with 0.4% Emulgen 911. Anionic proteins were removed by retention on High Performance (HP) Q-Sepharose. CYP2E1 emerged in the pass-through fraction and was subsequently applied to a HP S-Sepharose cation-exchange column. Hydroxyapatite chromatography with unconventional gradient elution removed further impurities and detergent. Although the overall revovery was minor, the final sample appeared almost pure with only one impurity observed (45 kDa). The ultimate specific content and specific activity of the CYP2E1 were 3.6 nmol mg^{-1} protein and 0.37 nmol min^{-1} $nmol^{-1}$ P450, respectively (*p*-nitrophenol hydroxylation). In addition, NADPH-dependent cytochrome P450 reductase (P450-RED) was isolated parallel with the CYP2E1 purification on HP Q-Sepharose. Subsequent affinity chromatography on a 2',5'-ADP-Sepharose column ultimately resulted in apparently pure P450-RED. The ultimate purified fraction contained 2.9 nmol with a specific content of 3.1 nmol mg^{-1} protein (purification factor 28).

In **Chapter 5**, the behaviour of PAR and analogues in phase I biotransformation reactions was investigated. The exact mechanism of the P450-catalysed formation of NAPQI from PAR was not proven, be it a direct two-electron oxidation (abstraction of two hydrogen atoms) or a sequential mechanism of two successive single hydrogen atom abstractions with a free radical intermediate. Therefore, the potential formation of free radicals, e.g. structural analogues of NAPSQI, during enzyme catalysed oxidation of eight 3,5-disubstituted analogues of PAR was investigated. A simple peroxidase system (horseradish peroxidase) as well as rat liver microsomal cytochrome P450-containing systems were used (Fig. 3).

Radicals were detected by electron spin resonance (ESR) equipment upon incubation of PAR and 3,5-disubstituted analogues (R = $-CH_3$, $-C_2H_5$, $-tC_4H_9$, $-OCH_3$, $-SCH_3$, -F, -Cl, and -Br) with horseradish peroxidase (HRP) in the presence of hydrogen

peroxide (H_2O_2). However, no radicals were detected in rat liver cytochrome P450-containing microsomal or reconstituted systems in which rat liver CYP1A1 or CYP2E1 (for which PAR is known to be a substrate) were present. Moreover, as observed by quenching of the ESR-signals, the phenoxy radicals in the HRP/H_2O_2 incubations were neutralised by the addition of NADPH as well as boiled microsomes.

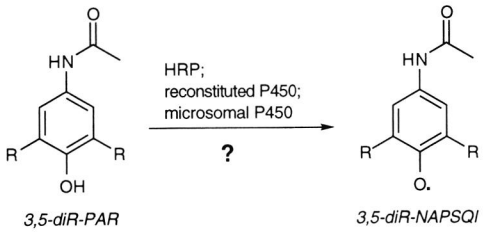

Figure 3: Single hydrogen abstraction from paracetamol

Initial analysis of the observed ESR spectra in HRP/H_2O_2 incubations, revealed all radical species to be phenoxy radicals (NAPSQI analogues), based on the absence of dominant nitrogen hyperfine splittings. In order to rationalise these ESR spectra, hydrogen atom abstraction of PAR and four of the 3,5-disubstituted derivatives (R = -CH_3, -OCH_3, -F, and -Cl) was modelled using *ab initio* calculations. The calculations indicated that for all compounds studied an initial hydrogen atom abstraction from the phenolic hydroxyl group is favoured by approximately 125 kJ/mol over an initial hydrogen atom abstraction from the acetylamino nitrogen atom, and that after hydrogen abstraction from the phenolic hydroxyl group, the unpaired electron remains predominantly localised at the phenoxy oxygen atom (± 85 %).

The experimental findings of phenoxy radicals in HRP/H_2O_2 incubations paralleled the computational findings on the substrates. The failure to experimentally detect phenoxy radicals in cytochrome P450-catalysed oxidation of any of the eight 3,5-disubstituted PAR analogues indicates at least that the quantity of these radicals remains below the detection level of the ESR-analysis. This could indicate that phenoxy radicals do not leave the active site of the P450 involved at all or at best in very low quantities. In addition, the reducing effects that agents like NADPH and protein thiol groups have on phenoxy radicals rather than the physical instability of the respective radicals might prevent detection by ESR (Bessems *et al.*, 1998).

In **Chapter 6**, the rat liver microsomal cytochrome P450-dependent binding and oxidation of various derivatives of PAR to e.g. NAPQI analogues was investigated. The P450-dependent binding of PAR and a series of 3,5-disubstituted paracetamol analogues (R = -F, -Cl, -Br, -I, -CH_3, -C_2H_5, -iC_3H_7) has been determined with ß-naphtoflavone (ßNF)-induced rat liver microsomes by using UV/Vis difference spectroscopy. All analogues studied appeared to produce reverse type I spectral changes

with apparent spectral dissociation constants ($K_{s,app}$) varying from 0.14 mM for 3,5-di*i*C_3H_7-paracetamol to 2.8 mM for paracetamol. Moreover, although only few PAR analogues were studied, a preliminary quantitative structure-activity relationship (QSAR) could be established. A good correlation (r = 0.938) was observed between $K_{s,app}$ values and the estimated lipophilicity of PAR and the 3,5-disubstituted analogues, as calculated by summation of the designated *f*-values using the hydrophobic fragmental constant approach (Rekker and Mannhold, 1992).

In addition, all seven PAR analogues underwent rat liver microsomal cytochrome P450 (P450)-dependent oxidation, as reflected by the formation of GSSG in the presence of GSH and NADPH. The GSSG-formation was increased in all cases upon pretreatment of rats by ß-naphthoflavone (ßNF) and was generally decreased upon pretreatment by phenobarbital (PB), indicating the importancy of P450 enzymes of the CYP1A subfamily. However, no solid SAR could be established for the P450-catalysed oxidative biotransformation of the PAR analogues studied, probably because this catalytical reaction was only measured indirectly by determination of GSSG and not of NAPQI analogues formed directly. Thus, more than one rate constant is involved and the changed physicochemical properties in the 3,5-disubstituted analogues may exhibit a dissimilar influence on both rate constants (Fig. 4).

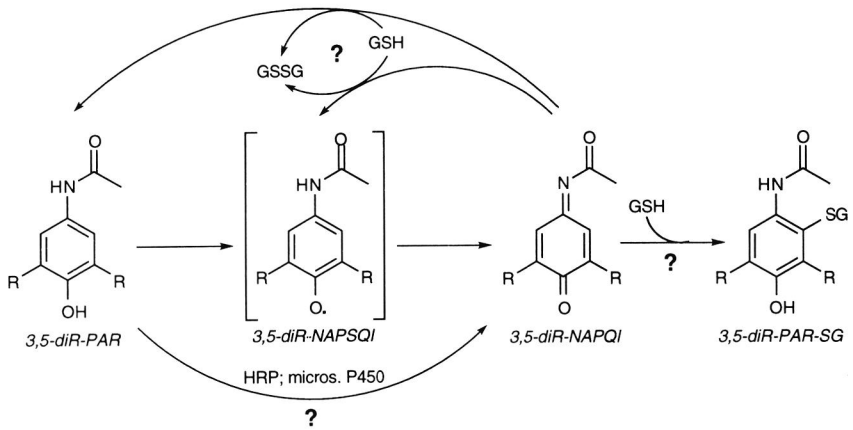

Figure 4: Oxidation of paracetamol in various systems followed by reactions with GSH

In order to study the horseradish peroxidase (HRP) as well as rat liver microsomal P450 catalysed oxidation of 3,5-disubstituted analogues of PAR in more detail, several disubstituted NAPQI analogues were synthesised, i.e. with R = -CH_3, -F, -Cl and -Br. Furthermore, an analytical method for the detection of thioethers was devised in order to determine GSH-conjugates of 3,5-disubstituted PAR analogues in incubations both with HRP and microsomal P450. Both enzyme systems (HRP and microsomal P450) appeared to catalyse the formation of 3,5-disubstituted NAPQI analogues from the

corresponding parent compounds, as identified by UV-spectrophotometry and by GC/MS detection of the following GSH-conjugates: 2-glutathione-*S*-yl-3,5-dimethyl-1,4-dihydroxybenzene, 2-glutathione-*S*-yl-3,5-dichloro-PAR, and 2-glutathione-*S*-yl-3,5-dibromo-PAR. Interestingly, the formation of 2-glutathione-*S*-yl-3,5-dimethyl-1,4-dihydroxybenzene, a glutathione conjugate of a hydroquinone, was indicative for hydrolysis of the complete *N*-acetyl moiety. It remains to be investigated, however, where in the sequence of reactions as depicted in the above picture, this hydrolysis took place.

In liver microsomal (ßNF-induced) incubations, apparent K_M values (Michaelis constant), as determined for the cytochrome P450 catalysed oxidation of GSH, for seven 3,5-disubstituted paracetamol analogues (R = -F, -Cl, -Br, -I, $-CH_3$, $-C_2H_5$, and $-iC_3H_7$) varied from 0.07 mM to 0.64 mM. Paracetamol exhibited an apparent K_M value of 0.73 mM. Apparent V_{max} values for the cytochrome P450 catalysis dependent oxidation of GSH varied from 0.66 nmol min^{-1} mg^{-1} protein for PAR to 3.0 nmol min^{-1} mg^{-1} protein for R = $-CH_3$ (Bessems *et al.*, 1992; Bessems *et al.*, 1996).

In **Chapter 7**, the effect of 3,5-dihalogenation of PAR on cytotoxicity including the role of enzymes of the cytochrome P450 1A subfamily (CYP1A), is described. Freshly isolated hepatocytes from ß-naphthoflavone pre-treated, non-fasted rats were incubated for several hours. Upon incubation at ≥0.3 mM, 3,5-disubstituted analogues (R = -F, -Cl and -Br) as well as PAR caused severe leakage of lactate dehydrogenase (LDH) which was preceeded by a rapid, concentration and time dependent depletion of intracellular glutathione (GSH). IC_{50}-values, representing the concentration of compound that caused 50% GSH depletion after 30 min of incubation, varied from 0.1 to 0.5 mM. In order to study more specifically the role of one specific cytochrome P450 enzyme, i.e. CYP1A1, in these phenomena, hepatocytes were co-incubated with 1-ethynylpyrene as a specific inhibitor of rat liver CYP1A1. The LDH leakage and GSH depletion appeared to be inhibited by 1-ethynylpyrene. In hepatocytes from uninduced rats, GSH depletion was much less prominent and the concomitant LDH leakage almost completely absent (Fig. 5).

In addition, HPLC analysis of soluble metabolites and GC/MS analysis after alkaline peralkylation of the protein fraction, revealed two phenomena. Analogously to PAR, a substantial amount of each 3,5-dihalogenated PAR analogue seems to be bioactivated by P450, ultimately leading to GSH-conjugates as well as, for R = -Cl and -Br, protein adducts at regio-specific aromatic positions. For R = -Cl, an unspecified disulfhydryl protein conjugate was found with one chlorine atom replaced. For R = -Br, two monosulfhydryl protein conjugates were found, in one of which the bromine atom was replaced whereas in the other conjugate both bromine atoms were still present. Moreover, all three 3,5-dihalogenated PAR analogues studied (R = -F, -Cl and -Br) were liable to structure-related detoxification by glucuronidation with increased conjugate formation at enlargement of the halogen substituents (Bessems *et al.*, 1992; Bessems *et al.*, 1997).

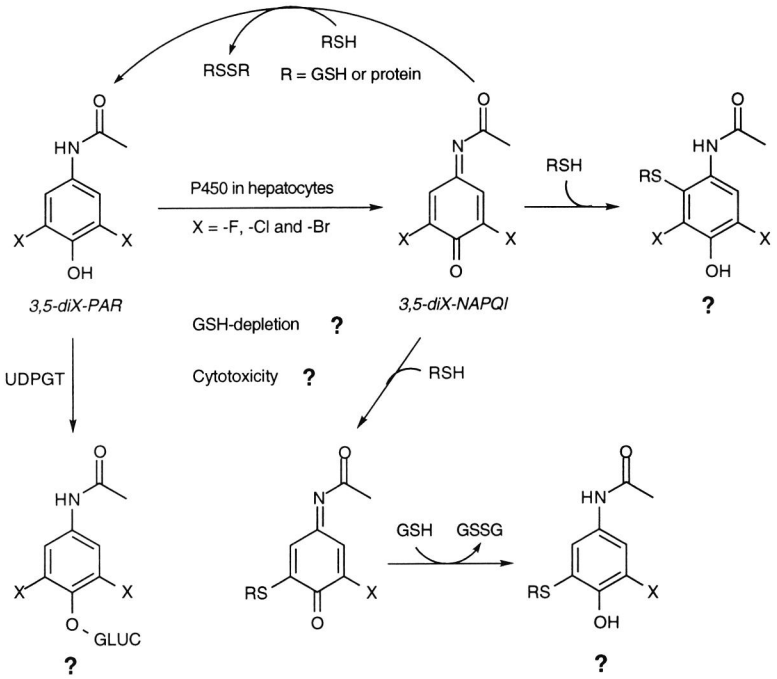

Figure 5: Oxidation of paracetamol in hepatocytes followed by reactions with GSH and protein thiol groups

In **Chapter 8**, possibilities of interfering in PAR-mediated toxicity by chemoprotectioin, i.e. without structural modification was investigated in an *in vitro* system. Mechanistic aspects of the protective effect of ebselen, an anti-inflammatory agent, on PAR-induced (1 mM) cytotoxicity in hepatocytes freshly isolated from ß-naphthoflavone-pretreated rats were studied (Fig. 6).

At a concentration of 50 µM, ebselen, added simultaneously with PAR, prevented PAR-induced leakage of lactate dehydrogenase (LDH) almost completely and lipid peroxidation (LPO) and depletion of glutathione (GSH) substantially. These protective effects were even more pronounced at 100 µM concentration of ebselen. When added to the hepatocytes 1 h before PAR, 50 µM of ebselen also prevented LDH leakage, LPO and GSH depletion. Reverse addition of PAR and ebselen did not result in protection. Simultaneous incubation of 100 µM ebselen and PAR inhibited GSH conjugation of PAR by more than 50%, however, without any effect on glucuronidation and sulfation of paracetamol.

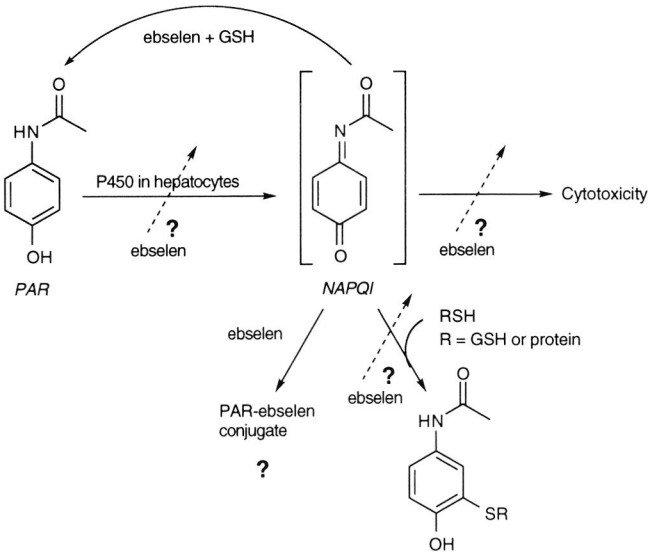

Figure 6: Protection of ebselen against paracetamol-induced toxicity in hepatocytes. Potential mechanisms.

Further attempts to investigate the protective mechanism revealed that ebselen did not react directly with PAR or inhibit cytochrome P450 activity measured as 7-ethoxycoumarin *O*-deethylase (ECD) activity in the hepatocytes. Lastly, the potential role of a ring-opened selenol derivative of ebselen, a potentially physiogically relevant product of ebselen and GSH was studied. At mixing, synthetical ebselen selenol and synthetical *N*-acetyl-*p*-benzoquinone imine (NAPQI) were shown to form PAR and ebselen diselenide. No indication was found for the formation of an ebselen-PAR conjugate upon reacting synthetical NAPQI and synthetical ebselen selenol. The possible reduction of NAPQI by ebselen selenol was discussed in terms of the mechanism of cytoprotection. In short, ebselen selenol was hypothesized to be a more efficient reductant than GSH which could cause a faster reduction of NAPQI. It was hypothesized with the current knowledge that this could lead to prevention of covalent adduct formation at sites distinct from the P450 in the endoplasmatic reticulum. Another or additional explanation might be that since ebselen selenol is expected to be more lipophylic than GSH, it could reach higher concentrations in the lipophylic environment of the endoplasmatic reticulum (Li *et al.*, 1994).

Conclusive remarks

The aim of the research of which the experimental results are described in this thesis was to investigate the molecular mechanisms of P450-dependent oxidative biotransformation of PAR in relation to its hepatotoxicity and to investigate

possibilities of protection against it by chemoprotection and by structural modification, if possible with retainment of pharmacological activity.

Although a comprehensive mechanistic explanation for the observed differences with respect to the analgesic properties and the toxicity cannot be presented in detail at the moment, it seems that the present investigations into structural modification of PAR described in this thesis was quite fruitful. Evidence has been obtained that the bulkiness as well as the electronic properties of substituents *ortho* to the phenolic -OH group are important determinants in binding to active sites of specific phase I as well as probably also of phase II biotransformation enzymes. For lipophylicity and microsomal P450 this was shown by the establishment of an, albeit preliminary, quantitative structure-activity relationship (QSAR).

The electronic properties are probably important factors for ultimate enzyme catalysis, both for prostaglandine synthase, for phase I biotransformation (cytochrome P450 but also peroxidase) and for phase II conjugation reactions (glucuronyltransferase, glutathione *S*-transferase etc.). Substitution *ortho* to the -OH groups with electron-donating substituents seems to improve the analgesic properties, whereas the opposite seemingly holds true for electron-withdrawing substituents. This was indicated by measuring cyclooxygenase inhibition which probably involves one-electron oxidation of this -OH group. Furthermore, all of the 3,5-disubstituted PAR analogues that were studied (R = $-CH_3$, $-C_2H_5$, $-tC_4H_9$, $-OCH_3$, $-SCH_3$, -F, -Cl, and -Br) in a peroxidase system as a model for monooxygenase and peroxidase reactions were liable to single hydrogen atom abstraction as revealed by the detection of *ortho*-substituted phenoxy radicals upon ESR-analysis. These findings were substantiated for R = $-CH_3$, $-OCH_3$, -F and -Cl by computational findings that predicted phenoxy free radicals are to be formed. Unfortunately, no reliable QSAR's could be established with respect to prostaglandin synthase (anti-cyclooxygenase activity), due to the small number of PAR analogues studied.

No free radicals were detected with ESR in rat liver cytochrome P450-containing microsomal or reconstituted systems in which rat liver CYP1A1 or CYP2E1 (for which PAR is known to be a substrate) were present. The failure to experimentally detect phenoxy free radicals in cytochrome P450-catalysed oxidation of any of the eight 3,5-disubstituted PAR analogues studied, even of those analogues that provide very stable phenoxy radicals, indicates that the quantity of these radicals remains below the detection level of the ESR-analysis. This could indicate that phenoxy radicals do not leave the active site of the P450 involved at all or at best in very low quantities. In addition, the reducing effects that agents like NADPH and protein thiol groups have on phenoxy radicals rather than the physical instability of the respective free radicals might also prevent detection by ESR. Thus, the findings substantiate the fact that formation of PAR-dependent phenoxy free radicals is possibly relevant in tissues rich in peroxidase-activity such as the kidneys whereas it is probably irrelevant in P450-dependent hepatoxicity.

3,5-Disubstitution by electron-donating groups and electron-withdrawing substituents seems to improve, respectively impair ratio between the analgesic activity and the safety of PAR as measured mostly *in vitro*. Although the PAR analogues

studied were shown to be substrates for P450s of the CYP1A subfamily, no reliable QSAR could be established for this P450-dependent oxidative biotransformation as this activity was only determined indirectly by measuring GSSG formation. Chemical as well as enzymatical nucleophilic attack of GSH on the NAPQI analogues is also important and could result per saldo in different correlations with oxidation potentials of PAR and PAR analogues.

The investigations on the potential chemoprotective compound ebselen provided an unexpected novel potential mechansims for the protection of essential protein thiol groups. As ebselen protected hepatocytes against cytotoxicity upon coincubation with toxic concentrations of PAR, several protective mechanisms were investigated. The most likely mechanisms is that ebselen is a proximate chemoprotector. Formation of an ebselen selenol in a reaction with GSH is highly indicated. Subsequently, the supposedly superior reductant properties of ebselen selenol over GSH with respect to NAPQI are suggested to be responsible for the fast reduction of NAPQI to PAR as no indication has been found for the formation of a nucleophilic substitution product between NAPQI and ebselen or its selenol. Although highly speculative, ebselen selenol might also function via a 'selenium-binding protein' by blocking of cysteine thiols by selenium thus forming thioselenols (R-CH_2-S-SeH). Thioselenols in turn, would reduce NAPQI to PAR. In addition, peroxidase-like activity of ebselen could add to the protection of ebselen against PAR-induced cytotoxicity.

Thus, from the investigations on derivatives of PAR described several perspectives can be delineated. The data obtained provide valuable knowledge on the analgesic and toxic properties of PAR and the underlying molecular mechanisms. A new guide to structural modification of PAR in order to obtain a safer analgesic substance should combine two leads. As mentioned in the review of Chapter 2, the analgesic lead prescribes an acetanilide combined with an -OH (or maybe an -NH_2) substituent at *para*-position. The 'toxicologically safe' lead prescribes substituents *ortho* to this group that are metabolically relatively inert, such as alkane substituents, and not liable to a rapid addition-elimination attack onto the oxidative phase I metabolites (NAPQI analogues) by GSH or other sulfhydryl nucleophiles, such as halogen atoms. Examples of potentially promising compounds of which some have been preliminary investigated are PAR-derivatives that are disubstituted with -OCH_3, -SCH_3, -OCOOH or -OC_2H_5 groups *ortho* to the -OH group. *In vitro* experiments already provided evidence for a favourable analgesic activity as well as decreased hepatotoxic activity for 3,5-diOCH_3-PAR and 3,5-diSCH_3,-PAR (Chapter 3). As this thesis was aimed at *in vitro* investigations with respect to hepatotoxicity only, the *in vivo* relevance of these findings as well as the potential toxicity for other organs remains to be established, however.

In perspective, the analytical method that was improved for GC-MS analysis after peralkylation of GSH- as well as protein thiol-conjugates of PAR analogues with varying molecular weights, is likely applicable to other compounds to be investigated for their potential thiol-depleting properties. Moreover, the newly developed concepts of assessing the analgesic and hazardous properties of PAR analogues could be applicable to other compounds with a PAR-like chemical structures that are known to

be patented but that have not yet been investigated as to their potential toxicity. Finally, the molecular toxicological findings as reported and reviewed in this thesis could result in a more rational elimination of the hazardous properties while developing safer PAR analogues and to a more rational approach in chemoprotection (antidotes as well as co-administration).

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SAMENVATTING, CONCLUSIES EN PERSPECTIEVEN

Algemene introductie en doel van het onderzoeksproject

Paracetamol (PAR, 4'-hydroxyaceetanilide) is de actieve stof in een aantal veel gebruikte, vrij verkrijgbare geneesmiddelen, soms met dezelfde naam. PAR heeft pijnstillende en koortswerende eigenschappen. Hoewel in het algemeen wordt aangenomen dat PAR veilig is bij voorgeschreven gebruik, veroorzaakt het ook ernstige schade aan lever en nieren bij overdosering. De afgelopen tientallen jaren werden vele pogingen beschreven om de veiligheid (uitgedrukt als therapeutische index) van PAR te vergroten. Moleculair farmacologisch onderzoek toonde aan dat de gewenste eigenschappen van PAR veroorzaakt worden door verminderde aanmaak van prostaglandines via prostaglandine synthase. De farmacologische eigenschappen van PAR zijn voor een belangrijk deel gebaseerd op aanwezigheid van een fenol groep. Waterstof abstractie van deze -OH groep (ook wel oxidatie genoemd) speelt vermoedelijk een rol bij de remming van prostaglandine synthase. Ironisch genoeg is, zoals in het vervolg verder belicht wordt, oxidatie van deze -OH groep tevens een belangrijke stap in de metabole activering (toxificatie) van PAR.

In de moleculaire toxicologie is reeds lang bekend dat PAR in de lever door zogenaamde fase II enzymatische reacties gekoppeld wordt aan gemodificeerd glucuronzuur (in het Engels afgekort als UDPGA) en gemodificeerd sulfaat (PAPS). Hierdoor ontstaan wateroplosbare produkten. Voor PAR kwantitatief minder belangrijk zijn omzettingen door prostaglandine synthase (PGES) en cytochroom P450 (P450) in zogenaamde fase I biotransformatie. Hierbij wordt PAR geoxideerd waarbij een reactieve groep ontstaat ten behoeve van daaropvolgend fase II metabolisme. Bij PGES resulteert deze reactie waarschijnlijk in zowel het vrij radicaal deeltje *N*-acetyl-*p*-benzosemichinonimine (NAPSQI) als het *N*-acetyl-*p*-benzochinonimine (NAPQI). Dit is gebaseerd op zogenaamd *in vitro* ('in de reageerbuis') onderzoek. P450-afhankelijke oxidatie resulteert in enige vorming van 3-hydroxyparacetamol (3-OH-PAR) maar vooral van het toxicologisch meer relevante *N*-acetyl-*p*-benzochinon imine (NAPQI). In een daaropvolgende fase II ontgiftigingsreactie wordt dit omzettingsprodukt NAPQI weer gekoppeld aan het tripeptide glutathion (GSH), een essentieel stofje in alle lichaamscellen, waarbij ook weer een wateroplosbaar produkt ontstaat. Bij hoge concentraties van PAR, met name in de parenchymcellen in de lever, als gevolg van een overdosering, leidt een toenemende vorming van NAPQI tot uitputting van de voorraad GSH in de deze cellen. Dit resulteert vervolgens weer in een zo goed als onomkeerbare binding van dit NAPQI aan enzymatische eiwitten maar ook in aantasting van het vermogen van cellen om essentiële posities (thiol groepen) in deze enzymen in een functionele (gereduceerde) staat te houden.

Bij het begin van het onderzoek dat beschreven staat in dit proefschrift waren, zoals gezegd, reeds vele pogingen ondernomen om de in potentie gevaarlijke eigenschappen van PAR te verminderen. De meeste pogingen waren gericht op het voorkomen van de door NAPQI veroorzaakte uitputting van GSH in de parenchymcellen van de lever. Veelal

werd de basisstructuur veranderd door enkelvoudige of dubbele zogenaamde substitutie door alkylgroepen (koolwaterstofgroepen) op diverse posities in de ring van PAR. Met name 3,5-dialkylering (dat is aan beide zijden naast de -OH groep) resulteerde in een duidelijke afname of zelfs voorkoming van levertoxiciteit in muizen, waarbij overigens de fase I metabole activering in stand bleef. Helaas werd wel enige algemene toxiciteit buiten de lever waargenomen bij toediening van mono- en dialkylderivaten (3- en de 3,5-posities), hetgeen overigens niet geheel onverwacht genoemd mag worden omdat deze structuur verwanten van PAR ontworpen waren om specifiek de levertoxiciteit te verminderen. De farmacologische consequenties van structurele veranderingen kregen tot nu toe minder aandacht. In dit proefschrift werd het doel verbreed. Er werd in eerste instantie onderzocht of structurele verandering van PAR gebruikt kon worden om de fase I biotransformatie te moduleren met behoud van analgetische (pijnstillende) eigenschappen. Voor fase I biotransformatie zou de nadruk liggen op P450.

De toename in oxidatie van de fenolische -OH groep bij hoge intracellulaire concentraties leidt tot meer vorming van een zeer reactief omzettingsprodukt. Vandaar dat gesteld kan worden dat de redox potentiaal van PAR en structuurverwanten waarschijnlijk net zo belangrijk is voor de in potentie gevaarlijke als wel voor de farmacologische eigenschappen. Om nu allerlei onderzoek gemakkelijker te maken, werd het uit de plant afkomstige enzym mierikswortel peroxidase gebruikt als model enzym met de potentie om een één-electron oxidatie (oftewel enkelvoudige watersof abstractie) van PAR te katalyseren. Hiervoor bezit dit enzym zowel eigenschappen van het farmacologisch relevante PGES als van het toxicologisch relevante P450. In feite is de door P450 gekatalyseerde twee-electron oxidatie van PAR tot NAPQI (of tweevoudige waterstof abstractie) geen klassieke monooxygenase reactie omdat er geen inbouw van zuurstof plaatsvindt. In plaats daarvan wordt een tweede waterstof onttrokken hetgeen per saldo resulteert in een zogenaamde dehydrogenering. In tegenstelling tot deze dehydrogenering is de ook door P450 gekatalyseerde vorming van 3-OH-PAR, zoals eerder gemeld, wel een klassieke monooxygenase reactie.

Tweevoudige substitutie van PAR met electronenstuwende alkyl groepen naast de -OH groep had zoals gezegd slechts een geringe invloed op fase I metabole activering (P450-afhankelijke oxidatie). Modulatie van de electronische eigenschappen van de -OH groep door introductie van electronenzuigende groepen maar ook van electronenstuwende groepen anders dan alkylgroepen, zou mogelijk kunnen resulteren in meer of minder oxidatie door P450. Echter, de volgens verwachting gevormde NAPQI verwanten zouden ook andere eigenschappen kunnen krijgen. Met name door wijziging van de electronegatieve eigenschappen van de plek waar GSH aanvalt zouden de fase II reacties kunnen veranderen. Aldus werd verwacht dat uitbreiding van het aantal modificaties van PAR niet alleen zou resulteren in 'mechanistisch gereedschap' voor onderzoek van fase I (P450-gekatalyseerde oxidatieve) bioactivatie maar ook voor fase II ontgiftiging (detoxificatie).

Overzicht van moleculair toxicologische en biochemische aspecten

In **Hoofdstuk 2** is een uitgebreid overzicht gepresenteerd van moleculair toxicologische en biochemische onderzoek naar het mechanisme van schade aan met name de lever en in mindere mate de nieren zoals veroorzaakt door PAR. Ook worden mogelijkheden voor chemische bescherming en van structurele aanpassingen om het evenwicht tussen gewenste werking en potentieel gevaar van PAR te verbeteren.

Wat betreft gevoeligheid voor het potentiële gevaar, de giftigheid (toxiciteit) van PAR, bestaan er markante verschillen tussen diverse diersoorten. Dit blijkt voor een groot deel te verklaren door biochemische en moleculair toxicologische verschillen. De ene diersoort, zoals de muis, heeft veel enzymen die zorgen voor metabole activering (de fase I enzymen) terwijl een andere, bijvoorbeeld de rat, weer meer ontgiftigingsenzymen bezit.

Schade ontstaat zeer waarschijnlijk doordat koppeling van NAPQI met de zogenaamde thiol groepen van GSH en eiwitten in de lever dichtbij de plek van vorming, via een zogenaamde 1,4-Michael additie leidt tot zo goed als onomkeerbare thioether bindingen. Met name eiwitten in het zogenaamde endoplasmatisch reticulum worden gebonden. Een mogelijke verklaring voor het verschijnsel dat bindingen van NAPQI ook buiten de lever gevonden worden is de vorming van een relatief instabiele zogenaamde *ipso*-koppeling tussen NAPQI en GSH. Middels dit instabiele produkt zou NAPQI naar andere organellen in de levercel en zelfs naar diverse plekken buiten de lever getransporteerd kunnen worden. Hier zou NAPQI weer vrijkomen om vervolgens ter plekke met thiol groepen van eiwitten te reageren. Aldus kunnen eiwitten in het cytoplasma, in de mitochondriën, in het plasma membraan en zelfs in de celkern van met name de levercel beschadigd worden. Vorming van de eiwit-PAR bindingen speelt zeer waarschijnlijk een zeer belangrijk rol in Stadium I van acute levernecrose. De produkten hiervan zijn aangetoond in bloedplasma van patiënten met PAR-vergiftiging (PAR-intoxicatie). De belangrijkste veranderde eiwitten, waarvan de functies nog niet zijn opgehelderd, worden aangeduid als de 55 tot 58 kDa 'PAR-bindende eiwitten'. Hun functie zou kunnen zijn het wegvangen van reactieve metabolieten of lichaamsvreemde stoffen.

Een toenemend aantal *in vitro* onderzoeken en epidemiologische rapporten wijzen op allerlei effecten op de celkern. Genoemd zijn onder andere verminderd herstel van beschadigd DNA en zelfs verandering van DNA. Naast zogenaamde necrose zou ook apoptose een rol kunnen spelen bij PAR-intoxicatie. De relevantie en mogelijke gevolgen van deze bevindingen voor patiënten met PAR-intoxicatie en gebruikers van PAR in toegestane doseringen zijn nog onduidelijk. Het wordt de laatste jaren wel steeds duidelijker dat er nog vele effecten volgen op de Stadium I effecten binnen de leverparenchymcel. In de eerste uren na PAR-intoxicatie spreiden deze zich uit over het hele leverweefsel. Voorbeelden van deze verschijnselen, betiteld als Stadium II effecten, zijn uitscheiding van groeifactoren, ontstekingsmediatoren en reactieve vormen van zuurstof. Sommige van deze mediatoren leiden tot weefselherstel, anderen tot weefselbeschadiging. Tot nu toe is onduidelijk of er een relatie bestaat tussen de waargenomen effecten op de celkern en deze Stadium II effecten. De resultaten kunnen

echter interessante ideeën opleveren voor mogelijke klinische behandeling van acuut leverfalen.

In het verleden zijn met name in proefdieren veel stoffen onderzocht op mogelijke vermindering van leverbeschadiging zoals veroorzaakt door PAR. Een goed onderzocht en klinisch veel gebruikt middel is *N*-acetylcysteine. Helaas zijn de meeste andere middelen van weinig waarde voor de kliniek omdat ze vóór PAR (chemische preventie) toegediend zijn. Middelen die tegelijk met PAR (chemische bescherming) toegediend zijn bieden mogelijk meer perspectief. Daarom wordt het idee aangedragen om gevoelige moleculaire doelen in de cel te beschermen door stimulering van de 'PAR-bindende eiwitten' b.v. door selenium bevattende verbindingen. Bovendien is de meeste initiële schade (Stadium I) vaak reeds opgetreden bij aankomst in een ziekenhuis. Zoals hierboven gemeld zou interventie in Stadium II dus een mogelijk onderwerp van onderzoek kunnen zijn voor behandeling in de kliniek van leverschade bij PAR-intoxicatie.

Tot slot wordt beschreven dat structurele verandering van PAR onderzocht is voor verbetering van pijnstillende eigenschappen en veiligheid van PAR. Een geheel nieuwe lijn voor structurele modificatie van PAR zou twee aspecten moeten combineren. Voor de pijnstillende werking is een anilide nodig met een substituent (-OH of $-NH_2$) in zogenaamde *para*-positie. Voor de veiligheid mogen de groepen naast de -OH (of $-NH_2$) niet gemakkelijk leiden tot een reactie met GSH in een fase II biotransformatie reactie die volgt op de vorming van een NAPQI derivaat. Bovendien mogen deze groepen ook weer niet volledig inert te zijn voor biotransformatie omdat dat verwijdering van de *N*-acetyl groep in de hand werkt (hetgeen kan leiden tot toxische aminofenolen). Substitutie naast -OH door alkylgroepen maar dan via een ether of thioether binding zou beide eisen (pijnstillende werking en veiligheid) kunnen inwilligen.

In zijn algemeenheid kan geconcludeerd worden dat er weinig klinisch bruikbare resultaten geboekt zijn ondanks de enorme hoeveelheid onderzoek naar chemisch voorkomen van schade, naar chemische bescherming en naar diverse structuurverwanten van PAR. Echter, de voordelen van de nog steeds toenemende kennis aangaande de moleculaire mechanismen van PAR-intoxicatie zijn evident. Op mechanistische kennis gebaseerde ontwikkeling van chemisch beschermende middelen maar ook van veiligere structurele derivaten van PAR gaat waarschijnlijk gewoon door. Bovendien wordt in dit overzicht beargumenteerd dat kennis van de moleculair toxicologische aspecten van het 'modeltoxine' PAR belangrijk is voor het begrijpen van de mechanismen van toxiciteit van vele klinisch relevante en klinisch niet-relevante chemische stoffen. Inzicht in deze moleculaire en biochemische mechanismen kan behulpzaam zijn bij het ontwikkelen van verbeterde methodes voor vroege behandeling van intoxicaties maar ook van verfijning van methoden die gebruikt worden in de humaan toxicologische risicobeoordeling van chemische stoffen.

Experimenteel werk

In **Hoofdstuk 3** is een nieuwe serie van 3,5-digesubstitueerde derivaten van PAR onderzocht met betrekking tot fysisch-chemische (oxidatie potentiaal), farmacologische (remming van cyclooxygenase, een activiteit van PGES) en

toxicologische aspecten (celtoxiciteit) om zodoende mogelijke structuur-activiteitsrelaties (SAR) op te sporen. Zoals in de algemene introductie hierboven gezegd, speelt waterstofabstractie van de -OH groep van PAR waarschijnlijk een rol in de remming van PGES. Vandaar dat de redox potentiaal van de -OH groep een belangrijke parameter te zijn bij pogingen om de pijnstillende werking van PAR te verbeteren. 3,5-Substituenten met electronenstuwende werking (R = $-CH_3$, $-OCH_3$, $-SCH_3$) bleken zoals verwacht, de oxidatie potentiaal duidelijk te verlagen in vergelijking met PAR. Ook werd de cyclooxygenase remmende activiteit van PAR vergroot en de celtoxiciteit in vers geïsoleerde leverparenchymcellen (uit de rat) geremd. Electronenzuigende groepen daarentegen (R = -F, -Cl, -Br of -I) verhoogden de oxidatiepotentiaal en verminderden de cyclooxygenase remmende activiteit van PAR. Paralel hieraan bleek dat de pijnstillende werking van de gemaakte derivaten bij muizen in vergelijking met PAR verminderd was. De meeste electronenzuigende groepen bleken de celtoxiciteit ten opzichte van PAR licht te verminderen hoewel die vermindering voor R = -I in dit test systeem substantieel was.

Er kan voorzichtig geconcludeerd worden dat betere *in vitro* remming van cyclooxygenase door derivaten van PAR met electronenstuwende groepen naast -OH correleert met een lagere oxidatiepotentiaal en verminderde celtoxiciteit. Deze verbindingen zouden dus wel eens tot veiligere pijnstillers kunnen zijn dan PAR met betrekking tot levertoxiciteit bij hoge inname. Het tegenovergestelde kan gesteld worden voor derivaten met electronenzuigende groepen naast -OH, met uitzondering wellicht van de celtoxiciteit.

In **Hoofdstuk 4** worden alternatieve zuiveringsmethoden beschreven voor de P450's CYP1A1 en CYP2E1 en voor het enzym P450 reductase, allen uit rattenlever. Hierbij worden de voordelen van grote schaal chromatografie onder invloed van de zwaartekracht gecombineerd met het gemak van programmeerbare 'HPLC'-apparatuur. Deze apparatuur maakt de toepassing van stapsgewijze gradiëntchromatografie mogelijk. Door gebruik te maken van een nieuw soort ionenwisselingschromatografie kolommen ('High Performance HiLoad') voor gebruik in eiwitzuiveringssystemen ('FPLC') bleken belangrijke verbeteringen mogelijk in vergelijking met de klassieke zuivering die meestal begint met hydrofobe interactie chromatografie.

Een proefzuivering van CYP1A1 resulteerde na 'solubilisatie' van de eiwitten met Emulgen 911 via slechts een ionenwisselingschromatografie stap ('Q-Sepharose') in ogenschijnlijk zuiver CYP1A1 (volgens analyse met 'SDS-PAGE' en 'Western blotting'). In een opzuivering op grote schaal van CYP2E1 werden eiwitten met Emulgen 911 'gesolubiliseerd' uit microsomen (endoplasmatisch reticulum) van ratten die met pyrazool waren voorbehandeld. Eiwitten met anion karakter werden eerst verwijderd met 'Q-Sepharose'. Vervolgens werd CYP2E1 (een eiwit met kation karakter) verder opgezuiverd met 'S-Sepharose'. Tot slot werd voor het verwijderen van Emulgen 911 met hydroxyapatiet ook onconventionele stapsgewijze chromatografie toegepast. Hierdoor konden behalve Emulgen 911 ook nog de nodige eiwitten verwijderd worden. Hoewel de opbrengst nog tegenviel bleek er uiteindelijk slechts één

onzuiverheid van ongeveer 45 kDa te zijn overgebleven. Verder bleek paralel met CYP2E1 ook het P450 reductase geïsoleerd te kunnen worden met 'Q-Sepharose' en 'ADP-Sepharose'.

In **Hoofdstuk 5** werd het gedrag van PAR en 3,5-digesubstituteerde structuurverwanten in fase I biotransformatie reacties onderzocht. Het exacte mechanisme van de door microsomaal P450 gekatalyseerde vorming van NAPQI uit PAR is nog niet duidelijk. Het is de vraag of het een radicaal mechanisme betreft, met vorming van bijvoorbeeld NAPSQI structuurverwanten, of niet. Behalve in systemen met P450 uit rattenlever werd de potentiële vorming van vrije radicaal deeltjes tijdens de oxidatie van PAR en derivaten daarom onderzocht in een simpel model systeem, het mierikswortelperoxidase dat bekend staat om de vorming van vrije radicalen. Radicalen werden wel gedetecteerd in experimenten met de structuurverwanten en mierikswortelperoxidase, echter niet bij incubatie met P450-systemen. Uit de waargenomen resonantie signalen ('ESR') werd afgeleid dat er fenoxy radicalen (NAPSQI en structuurverwanten) gevormd werden. Vrije radicaal deeltjes werden noch in microsomale systemen, noch in incubaties met gezuiverd P450 waargenomen. Sterker nog, toevoeging van onwerkzaam gemaakte (gekookte) microsomale systemen of NADPH, een cofactor, aan radicalen die reeds door mierikswortelperoxidase gevormd waren leidde tot neutralisatie van deze reactieve deeltjes.

Tot slot werd de abstractie van een atoom waterstof van de diverse structuurverwanten, hetgeen leidt tot de vrije radicaal deeltjes, gesimuleerd met een computermodel. Deze simulaties toonden aan dat de vorming van fenoxy radicalen het meest gunstig is en minder energie koste dan de abstractie van een waterstof van een stikstofatoom. Dit laatste zou leiden tot andere radicalen met andere resonantiesignalen dan die welke werden waargenomen in de incubaties. Het feit dat er in experimenten met P450 geen radicalen werden waargenomen geeft aan dat de concentratie van radicalen onder de detectiegrens bleef. Dit kan erop duiden dat de radicalen het actieve centrum van P450 niet of nauwelijks verlaten en/of dat de reagentia en/of eiwitten die in dit soort incubaties nodig zijn de radicalen wegvangen waardoor detectie verhinderd wordt.

In **Hoofdstuk 6** is de binding van PAR en 3,5-digesubstitueerde structuurverwanten aan microsomaal P450 uit rattenlever en de oxidatie door hetzelfde P450 tot bijvoorbeeld NAPQI derivaten bestudeerd. Alle structuurverwanten die bestudeerd zijn vertoonden binding aan P450, de mate waarin dat gebeurde correleerde met de lipofiliteit van de verbindingen. Ondanks het geringe aantal verbinding kon een voorlopige 'QSAR' (kwantitatieve structuuractiviteitsrelatie) opgesteld worden. Verder bleek via een andere analytische methode dan die in hoofdstuk 5, dat alle structuurverwanten van PAR wel degelijk geoxideerd worden door P450. Alleen is in deze meetmethode niet gekeken naar het exacte mechanisme van de oxidatie.

Om toch meer te weten te komen over de vorming van chinonimines (verwant aan NAPQI) in plaats van semichinonimines (verwant aan NAPSQI), werden een aantal

NAPQI-derivaten langs chemische weg bereid als referentiestof om middels UV analyse de enzymatische vorming te kunnen bekijken. Uit deze experimenten bleek dat zowel het mierikswortelperoxidase als het microsomaal P450 de structuurverwanten van PAR kan oxideren tot NAPQI verwanten. Ook werd een analytische methode aangepast om conjugaten (koppelingsprodukten) van GSH met deze NAPQI verwanten te kunnen detecteren. Deze NAPQI verwanten bleken in aanwezigheid van GSH uiteindelijk ook conjugaten te vormen. Een extra interessante bevinding was dat van de PAR derivaat met R = $-CH_3$ de *N*-acetyl groep afgesplitst was waarbij dus een *para*-aminophenol ontstaan kan zijn. Tot slot bleek dat alle bestudeerde structuurverwanten minstens even goede substraten waren voor het bestudeerde P450 waren als PAR zelf. Een 'QSAR' kon echter niet afgeleid worden hetgeen veroorzaakt zou kunnen worden door het feit dat hier, in tegenstelling tot eenvoudige binding aan P450, het eindpunt van meerdere reacties bekeken wordt, ieder met een eigen 'QSAR'.

In **Hoofdstuk 7** werden de 3,5-digehalogeneerde derivaten van PAR nader bestudeerd in incubaties met parenchymcellen die vers geïsoleerd waren uit rattenlever. In overeenstemming met de resultaten in hoofdstuk 3, bleken de gehalogeneerde structuurverwanten (R = -F, -Cl en -Br) net als PAR celschade te veroorzaken. Deze werd voorafgegaan door een snelle, concentratie- en tijdafhankelijke uitputting van GSH. Analyses toonden aan dat er wederom conjugaten met GSH maar ook met eiwitten gevormd waren. Koppeling vond plaats op regiospecifieke plekken in de ring van de PAR derivaten. Ook bleek dat met name een specifiek P450, namelijk het CYP1A1, een rol speelde bij de toxiciteit in deze parenchymcellen. Tot slot bleek uit de analyses dat er met name bij R = -Cl meervoudige GSH-conjugaten gevormd waren. Bovendien werden alle drie de bestudeerde derivaten ook ontgift (gedetoxificeerd) door koppeling met glucuronide waarbij een positieve correlatie gevonden werd met de grootte van de substituent.

In **Hoofdstuk 8** werden mogelijkheden onderzocht om in te grijpen in het proces van beschadiging van vers geïsoleerde levercellen uit de rat door PAR, middels chemische bescherming, dus zonder structurele modificatie. Het mechanisme van bescherming van ebseleen, een ontstekingsremmer, tegen celtoxiciteit door PAR werd in detail onderzocht. Toevoeging van ebseleen aan de cellen 1 uur vóór of tegelijkertijd met PAR beschermde o.a. tegen algemene celschade en uitputting van GSH. Bij toediening 1 uur na PAR werd geen bescherming waargenomen. Bovendien werden bij gelijktijdige toediening minder conjugaten van PAR en GSH gevormd terwijl ebseleen geen invloed had op de andere ontgiftigingsroutes, nl. sulfatering en glucuronidering. Ebseleen had ook geen invloed op P450 activiteit in het algemeen en reageerde ook niet direct met PAR.

Daarom werd het ebselen selenol, een ring-geopend produkt dat gevormd kan worden in aanwezigheid van GSH en mogelijk ook relevant is onder fysiologische omstandigheden, gemaakt en bestudeerd. Bij mengen van ebselen selenol en NAPQI bleek dat er PAR en ebselen diselenide (een koppelingsprodukt van twee ebselen selenol moleculen) gevormd werd. Er werd geen koppeling tussen NAPQI en ebselen selenol

waargenomen. Wellicht kan ebselen selenol het NAPQI beter reduceren dan GSH. Ook zou ebselen selenol, doordat het lipofieler is dan GSH, hogere concentraties kunnen bereiken in het endoplasmatisch reticulum om aldaar het NAPQI direct te ontgiften. Hierdoor zou voorkomen worden dat NAPQI reageert met essentiële onderdelen van de cel verder weg van de plek van vorming (het P450 in het endoplasmatisch reticulum).

Eindconclusies en slotbeschouwing

Het doel van dit onderzoek was tweeledig. Ten eerste zouden de moleculaire mechanismen van P450-afhankelijke oxidatieve omzetting van PAR in relatie tot levertoxiciteit bestudeerd worden en ten tweede de mogelijkheden voor bescherming daartegen door chemische bescherming en door structurele modificatie, zo mogelijk met behoud van farmacologische werking.

Alhoewel op dit moment nog geen allesomvattende mechanistische uitleg voorhanden is voor de waargenomen verschillen in analgetische eigenschappen en toxiciteit lijkt het er op dat dit onderzoek naar structurele modificatie van PAR redelijk succesvol is geweest. Zowel de omvang als de electronische eigenschappen van de substituenten naast de -OH groep zijn belangrijk voor binding in de actieve centra van fase I en fase II biotransformatie enzymen. De electronische eigenschappen zijn waarschijnlijk zeer belangrijk voor uiteindelijke omzetting door deze enzymen. In tegenstelling tot electronenzuigende groepen lijken electronenstuwende groepen daarbij ook nog de analgetische werking te versterken. Dit houdt klaarblijkelijk verband met de voorgestelde één-electron oxidatie van de -OH groep voor het mechanisme van de analgetische werking van PAR. Ook toxicologisch relevante enzymen blijken de -OH groep van de diverse structuur verwanten van PAR te kunnen oxideren. Hierbij konden diverse bevindingen bovendien voorspeld worden met computer modellen. Eén-electron oxidatie van de onderzochte verwanten van PAR kon niet bevestigd worden in incubaties met cytochroom P450, echter wel met een enzym model voor diverse fysiologische peroxidases, nl. het mierikswortelperoxidase. Dit zou kunnen betekenen dat dit mechanisme wellicht van betekenis is in weefsels die rijk zijn aan peroxidases maar niet of weinig relevant is voor P450-afhankelijke levertoxiciteit. Het lijkt er samenvattend op dat 3,5-disubstitutie door electronenstuwende en -zuigende groepen de ratio tussen analgetische activiteit en veiligheid van PAR verbetert respectievelijk verslechtert.

Het onderzoek naar de potentieel chemisch beschermende verbinding ebseleen leverde een onverwacht nieuw mogelijk mechanisme op voor de bescherming van essentiële thiol groepen in eiwitten. Ebseleen werkt waarschijnlijk als een 'precursor' (voorloper) van het mogelijk langs chemische weg beschermende stofje ebseleen selenol dat gevormd kan worden door reactie van ebseleen en GSH.

Aldus kunnen vanuit dit onderzoek diverse perspectieven gedestilleerd worden. De gegevens leveren waardevolle informatie op over de analgetische en toxicologische eigenschappen van PAR en de mechanismen die eraan ten grondslag liggen. Er kan mogelijk zelfs een leidraad voor nieuw onderzoek naar structurele modificatie uit gedestilleerd worden. Die leidraad combineert twee voorwaarden, namelijk dat voor analgetische werking een aceetanilide nodig is met een -OH (of wellicht -NH_2) groep in

tegenovergestelde positie en dat voor de veiligheid van deze stof de plekken naast deze groep enigszins 'beschermd' moeten worden door bijvoorbeeld alkylachtige groepen. Deze laatsten dienen echter ook weer niet volledig resistent te zijn tegen biotransformatie omdat anders te veel *N*-deacetylering kan optreden met vorming van toxische *p*-aminofenolen. Voorbeelden van dit soort beschermende groepen zijn $-OCH_3$, $-SCH_3$, -OCOOH of $-OC_2H_5$. Er zijn reeds voorzichtige aanwijzingen gevonden dat dit soort verbindingen beter scoren met betrekking tot analgetische werking en levertoxiciteit. Omdat het onderhavige proefschrift zich met name richtte op *in vitro* onderzoek dient de *in vivo* relevantie van deze resultaten, net name voor andere organen dan de lever, verder onderzocht te worden.

Het dient tot slot opgemerkt te worden dat de verbeterde methode voor analyse van GSH- en eiwitbinding door structuurverwanten van PAR ook bruikbaar zou kunnen zijn voor diverse andere chemische stoffen die mogelijk reageren met thiol-groepen. Ook kunnen de nieuw ontwikkelde concepten om de analgetische en in potentie gevaarlijke eigenschappen van structuur verwanten van PAR te onderzoeken wellicht gebruikt worden voor onderzoek aan PAR verwante stoffen, die reeds gepatenteerd zijn maar nog niet onderzocht met betrekking tot mogelijk toxiciteit. Tot slot kunnen de in dit proefschrift beschreven resultaten op moleculair toxicologisch terrein resulteren in meer rationele benaderingen bij pogingen om iets te veranderen aan de potentieel gevaarlijke eigenschappen van aan PAR verwante stoffen. Tevens zouden de uitkomsten kunnen leiden tot een meer rationele benadering van de chemische bescherming tegen PAR-intoxicatie (gelijktijdig formuleren) en bij gebleken PAR-intoxicatie (met behulp van een tegengif).

CURRICULUM VITAE

The author was born on the 16th of August 1963 in Margraten, The Netherlands. He followed secondary education (Atheneum) at 'Sophianum' in Vaals and Gulpen and graduated in 1981. Next, he started to study biology at the Wageningen Agricultural University. He successively passed the 'propaedeutic' (1982), the 'kandidaats' (1986) and the 'doctoraal' examination (1988). The latter entitled him as an 'ingenieur' ('ir') in the agricultural sciences. This graduation included cell biology (prof. dr W.B. van Muiswinkel), toxicology (ir B. van Ommen, dr P. J. van Bladeren and prof. dr J.H. Koeman) and biochemistry (ing. W.J. van Berkel and prof. dr C. Veeger) as main subjects and a practical period in molecular toxicology and biochemistry at the University of Maryland, USA (dr R.N. Armstrong).

Subsequently (1998-1990), the author fulfilled his duty as a conscientious objector military service in the Department of Biological Toxicology at the TNO-CIVO Toxicology and Nutrition Institute in Zeist. He was appointed as scientific research assistant in the Molecular Toxicology and Toxicokinetics section under supervision of ir B. van Ommen and dr P. J. van Bladeren.

From 1990 to 1994, he was appointed as a PhD student (AIO) in the Molecular Toxicology section of the Department of Pharmacochemistry (Faculty of Chemistry) at the Vrije Universiteit in Amsterdam, now also a part of the Leiden Amsterdam Center for Drug Research (LACDR). Under supervision of prof dr N.P.E. Vermeulen, dr J.M. te Koppele and dr J.N.M. Commandeur, the work was performed that is described in this thesis. In the meantime, some postgraduate toxicology courses were followed in order to become a board certified toxicologist.

Subsequently (1994-1996), the author worked as a standardization consultant for the development of Dutch (NEN), European (EN) and international standards (ISO), subsequently in the sections 'Environment' and 'Agriculture and Foods' at the Dutch Standardization Institute in Delft. Amongst others, he was appointed project leader for the automated interactive database 'Domino' containing analytical standards and environmental legislation.

From 1996 on, the author has been appointed as occupational toxicologist and scientist risk assessor, subsequently in the Departments of Occupational Toxicology and Toxicological Risk Assessment at the TNO Nutrition and Food Research Institute in Zeist. He is currently involved in the registration and notification and in international review activities for the competent authorities (national and in the European Union) as well as for private companies. This concerns hazard and risk assessment of plant protection products, biocides, (veterinary) medicines, novel foods and industrial chemicals with special emphasis on molecular toxicological and toxicokinetic aspects.

NAWOORD

Nu dit proefschrift klaar is, past een korte terugblik. Dit boekwerkje is niet alleen mijn werk. Het resultaat is er uiteindelijk dank zij velen die mij gedurende al die jaren geholpen en gesteund hebben. Met een aantal van hen heb ik tranen mogen delen, van verdriet, maar gelukkig ook van blijdschap. Ik zal nog heel vaak aan jullie denken.

Dank je wel!

Jos