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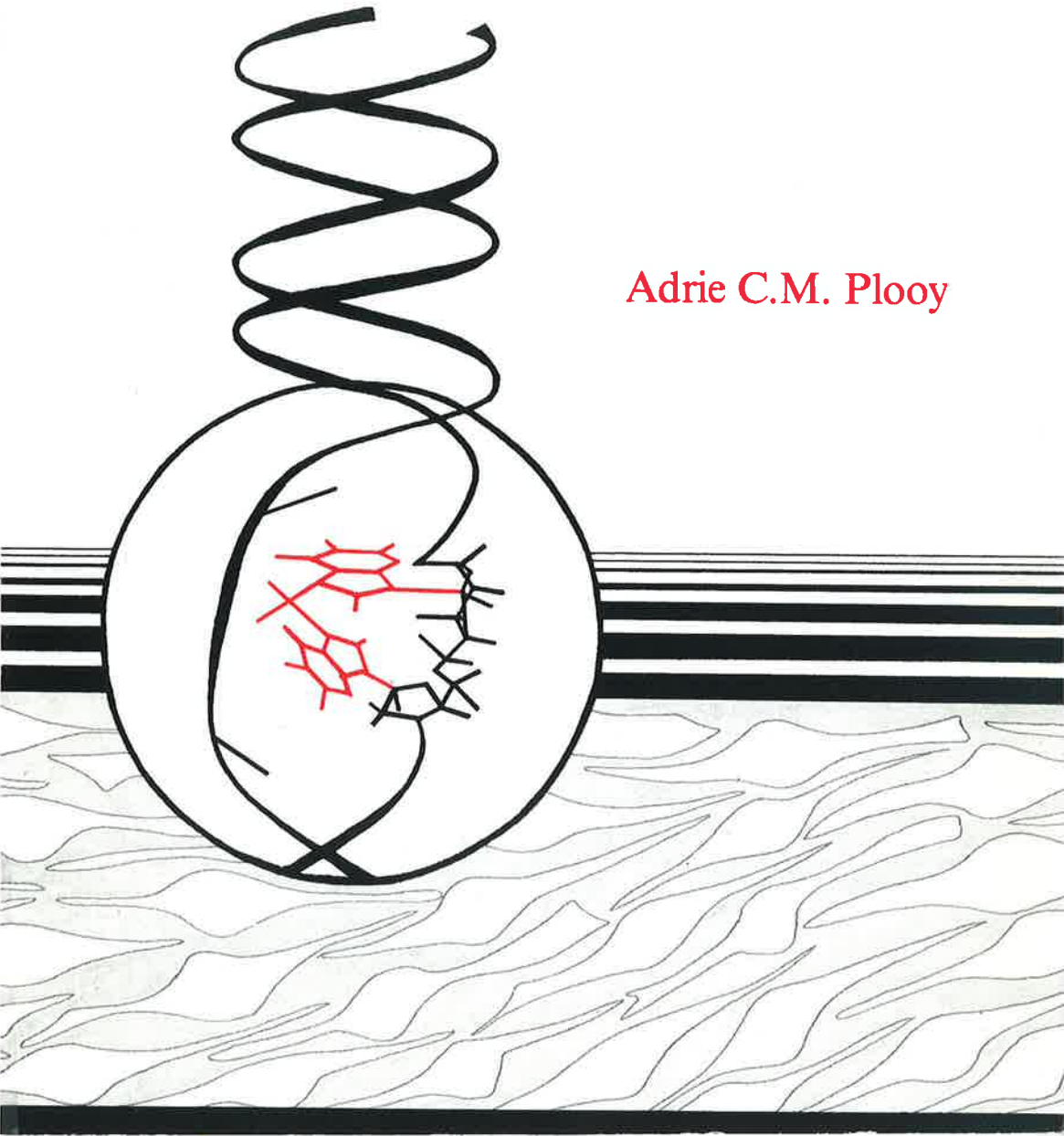
EFFECTS OF CISPLATIN AND RELATED *CIS*- AND *TRANS*-PLATINUM COMPOUNDS ON CULTURED MAMMALIAN CELLS:

genotoxic properties and their molecular background



Adrie C.M. Plooy

EFFECTS OF CISPLATIN AND RELATED *CIS*- AND *TRANS*-PLATINUM COMPOUNDS ON CULTURED MAMMALIAN CELLS:

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STELLINGEN

Behorende bij het proefschrift: Effects of cisplatin and related *cis*- and *trans*-platinum compounds on cultured mammalian cells: genotoxic properties and their molecular background.

1. De konklusie van Eastman, dat dichloro(ethylenediamine)platina-(II) intrastrand cross-links in DNA vormt tussen adenine en guanine, gescheiden door een derde base, lijkt niet geheel gerechtvaardigd.
   Eastman, A. (1983) Biochemistry, *22*, 3927-3933.

2. De reaktieprodukten van cisplatin met DNA behandeld *in vitro* en *in vivo* zijn volgens Johnson en Malfoy *et al.* niet identiek, deze konklusie lijkt een gevolg te zijn van de gebruikte detectiemethode.
   Johnson, N.P. (1982) Biochim. Biophys. Res. Comm., *104*, 1394-1400.
   Malfoy, B., Hartman, B., Macquet, J.P., Leng, M. (1981) Cancer Res., *41*, 4127-4131.

3. De betrouwbaarheid van mutageniteits-testen om de carcinogeniteit van een verbinding te voorspellen, is direkt gerelateerd aan het percentage carcinogenen in de onderzochte stoffen.
   Ashby, J., Styles, J.A. (1978) Nature, *271*, 452-455.
   ICPEMC comm. 3 final report (1983) Mut. Res., *114*, 179-216.
   ICPEMC comm. 2 final report (1982) Mut. Res., *99*, 73-91.

4. Er is een wezenlijk verschil tussen genotoxische en niet-genotoxische carcinogenen. Dit verschil is echter niet automatisch gelijk aan het verschil tussen gevaarlijk en ongevaarlijk.
   Ashby, J. (1983) Mut. Res., *115*, 177-213.
   IARC working group report (1981) Cancer Res., *42*, 5236-5239.
   ICPEMC publ. no 9 (1984) Mut. Res., *133*, 1-49.
   Jansen, J.D., Clemmesen, J., Sunderarm, K. (1980) Mut. Res., *76*, 85-112.

5. Genetic counseling draagt ertoe bij dat de geboorte van kinderen met aangeboren afwijkingen wordt voorkomen, wat niet alleen het welzijn van ouders, maar ook het financiële welzijn van de gezondheidszorg ten goede komt.
Gezondheidsraad: advies inzake genetic counseling.
Matsunaga, E. (1983) Mut. Res., 114, 449-457.

6. Bij de behandeling van een intoxicatie dient men erop bedacht te zijn, dat, na een aanvankelijk geslaagde therapie, reïntoxicatie vanuit tijdelijke opslag in een lichaamsdepot kan optreden.
Wolthuis, O.L., Berends, F., Meeter, E. (1981) Fund. Appl. Toxicol., 1, 183-192.
Van Helden, H.P.M., Benschop, H.P., Wolthuis, O.L. (1984) J. Pharm. Pharmacol., 36, 305-308.

7. In een studie naar de sekretie van humane interferonen door gist, gaan Hitzeman en medewerkers voorbij aan het feit, dat gedeeltelijke processing van interferon ook een veranderde antivirale aktiviteit tot gevolg kan hebben.
Hitzeman, R.A., Leung, D.W., Perry, L.J., Kohr, W.J., Levine, H.L., Goeddel, D.V. (1983) Science, 219, 620-625.

8. Semi-wetenschappelijke publikaties over nieuwe antitumorverbindingen kunnen valse hoop wekken bij patienten en hun familieleden en lijken meer te zijn geschreven voor de subsidiegevers.

9. Kritiekverwerking is niet hetzelfde als tekstverwerking.

10. Wat vandaag modern is, is morgen ouderwets, maar wat eergisteren ouderwets was, is overmorgen modern.

11. Het kost veel geld om er 'poor' uit te zien.

12. De schadelijkste schades geven de schadelijke nachtschaden (Solanaceae).

13. Crossen is link.

Adrie Plooy

Leiden, 17 oktober 1984.

EFFECTS OF CISPLATIN AND RELATED *CIS*- AND *TRANS*-PLATINUM COMPOUNDS ON CULTURED MAMMALIAN CELLS:

genotoxic properties and their molecular background

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.A.H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN TE VERDEDIGEN OP WOENSDAG 17 OKTOBER 1984 TE KLOKKE 14.15 UUR

door

Catrijna Adriana Maria Plooy

geboren te Schiedam in 1954

1984

Offsetdrukkerij Kanters B.V.,
Alblasserdam

Promotores: Prof. dr. ir. P. van de Putte
Prof. dr. J. Reedijk

Co-promotor: Dr. ir. P.H.M. Lohman

Referent: Dr. ir. A.A. van Zeeland

Overige leden: Prof. dr. F.J. Cleton
Prof. dr. P.H. van Knippenberg
Prof. dr. A.P. Zuur

The present investigation had been carried out at the Medical Biological Laboratory TNO, Rijswijk, The Netherlands, with financial support of the Queen Wilhelmina Fund (Dutch Organisation for the Fight against Cancer), project KWF-MBL 79-1.

aan mijn ouders

en Dennis

Cover design by Peter C.G. Glas
DNA helix: taken from Caradonna
and Lippard in: Platinum coordi-
nation complexes in cancer che-
motherapy, Hacker et al., (eds.),
1984, pp 18, with permission.

CONTENTS

PREFACE

All living organisms are composed of cells, in which a more or less complex organisation is present. Multicellular organisms originate from one cell through repeated replication (cell division) and differentiation. In each cell division the complete information (stored in DNA) which determines all features and properties of a cell, has to be converged to both daughter cells. It is very important, therefore, that the genetic information of a cell and its descendants is maintained during the replication and, later on, during its functioning in the whole organism. Cells are exposed to a constant barrage of agents as well as to spontaneous influences that cause alterations in the structure of DNA which may alter its coding properties or its function. Hence, it is not surprising that an elaborate array of mechanisms for the repair of damaged DNA has evolved. There seems to be a strong correlation between the ability of an agent to produce changes (mutations) in the DNA and its ability to cause cancer. Therefore, investigators have suspected that damage to DNA plays a causal role in carcinogenesis. The discovery that a deficiency in the repair of DNA photoproducts is correlated with the high incidence of sunlight-induced cancer in the human hereditary disease known as xeroderma pigmentosum (XP), supports the idea that DNA damage plays a role in carcinogenesis. A number of other hereditary diseases conferring predisposition to cancer also appear to involve deficiencies in DNA repair processes (Friedberg et al., 1979; Setlow, 1978).

The way most antitumor agents - compounds which preferentially kill tumor cells - exert their action has not yet been elucidated. Their action is attributed to their binding to macromolecules in the cells, in particular to the introduction of damage in the DNA. It is not known which type of DNA damage is responsible for the antitumor properties of certain drugs, nor is it known for which reason tumor cells are particularly susceptible to this damage: whether it is their high replicative activity, or a (partial) DNA repair deficiency or something else.

The purpose of this project was to study the mechanism of action of platinum coordination compounds - in use in chemotherapy of particular forms of cancer - at the cellular and molecular level. Because this project was part of a multidisciplinary research cooperation, the introduction deals rather extensively with various aspects of this work: reviews are given of the chemical, clinical and biological properties of platinum compounds, the properties of mammalian cells in culture, the various types of DNA repair and of the methods involved in research on cultured mammalian cells. Then, an overview is given of what was known of the mechanism of action of platinum compounds at the onset of this study. After an outline of this thesis five chapters are presented, in which the experimental work is described and discussed. Finally, conclusions based on the complete investigation and final remarks are given.

1. INTRODUCTION

1.1. INTRODUCTION TO PLATINUM COMPOUNDS AS ANTITUMOR DRUGS

The medical use of metal complexes had a peak during the first decade of this century. The decline since then is not due to the failure of metal complexes as useful drugs, but to the successes of organic chemistry and biochemistry, marked by the overwhelming triumph of the sulphonamides in the 1930's followed by the discovery of the antibiotics. The reintroduction of the metal complexes in medicine took until 1965, when Rosenberg and coworkers discovered the possible antitumor activity of square planar platinum coordination compounds (see review by Marx, 1976) (Fig. 1.1.).

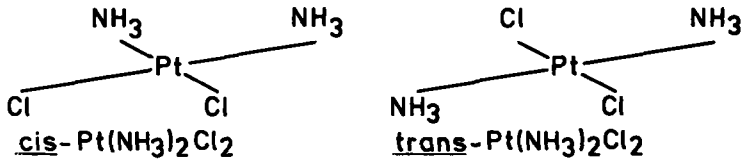

Fig. 1.1. Structure of cis- and trans-diamminedichloroplatinum(II).

Discovery

The motivation for the investigation on simple platinum coordination compounds as antitumor drugs came from a study on the effects of an electric field on cells growing in culture (Rosenberg et al., 1965; Rosenberg et al., 1967). The electric current caused a chemical reaction between ammonium chloride and the platinum electrodes, and the reaction products gave rise to filamentous growth of the bacterium Escherichia coli. One of the compounds causing the biological effect was cis-diamminedichloroplatinum(II) (cisplatin), a compound with a

chemical structure already known since 1845 (Basolo and Pearson, 1962; see Fig. 1.1.). By virtue of the observed effects in bacteria (Rosenberg *et al.*, 1967; Renshaw and Thompson, 1967), the conclusion was drawn that cisplatin might also inhibit cell division in rapidly growing tumor cells. The first tumor system studied was the solid Sarcoma 180 in mice. Cisplatin and a number of derivatives inhibited the development of this tumor (Rosenberg *et al*, 1969; Rosenberg and van Camp, 1970). Remarkably, the stereo-isomer of cisplatin, *trans*-diamminedichloroplatinum(II), was inactive. Subsequently, cisplatin was tested in a wide variety of animal tumor systems and found to be active against most of them (see Roberts, 1979). Toxicological data in dog, monkey and mouse were obtained to evaluate the dose to be used in clinical trials (Penta *et al.*, 1979; see 'Clinical trials').

Table 1.1.a. Results of the treatment of testicular cancer

period	type of therapy	remission (%)	duration of remission
before 1960	single drug *e.g.* adriamycin	10	weeks
1960-1970	chloorambucil methotrexate actinomycin	30	weeks to months
1970-1975	vinblastin bleomycin	40	months to years
1975-	cisplatin alone	70	years and total curation
	cisplatin + vinblastin + bleomycin	90	curation

Cisplatin was introduced in phase I clinical trials in 1971 (Wiltshaw and Carr, 1974; Williams and Carter, 1978) and activity against testicular and ovarian cancer was noted, as well as dose-limiting side effects (kidney toxicity, ototoxicity , nausea, vomiting, myelosuppression) (Helson et al., 1978; Kelsen et al., 1982; Goldstein and Mayor, 1983). In Phase II and III clinical trials, activity against head and neck, bladder, lung and bone cancer was observed as well (see review Burchenal, 1978; Rosenberg, 1980). No activity could be observed against leukemias and colorectal tumors (Rozencweig et al., 1977). Further decrease of toxicity and better response rates were obtained when cisplatin was used in combination with other antineoplastic drugs (Prestayko et al., 1979; see Table 1.1.a.). Cisplatin is now commonly used in the treatment of testicular cancer (see Table 1.1.a.; Prestayko et al., 1980; Hacker et al., 1984).

Structure-activity relationships

A large number of platinum compounds have been studied in an effort to find compounds with a broader spectrum of clinical applicability than cisplatin, and with lower toxicity (Braddock et al., 1975; Burchenal, 1978; Cleare et al., 1978; Wilkinson et al., 1978; Craciunescu et al., 1982). The vast majority of platinum complexes that have been examined has the general formula: PtA_2X_2, where X is an anionic reactive ligand, typically chloride, and A an amine (or A_2 a chelating diamine) ligand or a heterocyclic ligand (Tobe and Khokhar, 1977; Prestayko et al., 1979; 1980; Roberts, 1981; Brown et al., 1982; Lippard, 1982). Some properties appeared to be essential to obtain anti-cancer activity:

- The complex should be neutral in charge;
- There should be a pair of cis leaving groups (X), the reactivity of which influences the toxicity and antitumor activity of the compound. The reactivity of the leaving groups should not be too strong, otherwise the compound will react with other molecules before it reaches its target. The reactivity of the leaving groups should not be too weak either, because then almost no reaction will occur. This could explain why anions such as chloro

Table 1.1.b. Platinum compounds used in this study

name	abbreviation	reference
Pt(II)-compounds [1]		
cis-diamminedichloroplatinum(II)	cII, cisplatin	Chapter 2, 3, 4, 5, 6
trans-diamminedichloroplatinum(II)	tII	Chapter 2, 3
cis-dichloro(1,2-diaminocyclohexane)platinum(II)	$Pt(DACH)Cl_2$	Chapter 2
cis-dichlorobis(aminecyclohexane)platinum(II)	$Pt(ACH)_2Cl_2$	Chapter 2
cis-malonato(2,2-dimethyl-1,3-diaminopropane)-platinum(II)	Pt(DMDAP)mal	Chapter 2
cis-dichloro(1,2-diaminoethane)platinum(II)	$Pt(en)_2Cl_2$	Chapter 2
cis-dichlorobis(pyridine)platinum(II)	$Pt(py)_2Cl_2$	Chapter 2
Pt(IV)-compounds [1]		
cis-tetrachlorodiammineplatinum(IV)	cIV	Chapter 2, 3
trans-tetrachlorodiammineplatinum(IV)	tIV	Chapter 2, 3
cis-tetrachloro(2,2-dimethyl-1,3-diamminopropane)-platinum(IV)	$Pt(DMDAP)Cl_4$	Chapter 2

1) All platinum compounds have been synthesized by van Kralingen, 1979.

Table 1.1.c. Possibly successful second generation platinum compounds

1,1-bis(aminoethyl)cyclohexaneplatinum(II)sulfate	TNO-6	de Jong et al., 1983
cis-dichloro-trans-dihydroxo-cis-bis(isopropylamine)-platinum(IV)	CHIP	Bocian et al., 1983
cis-dichlorobis(cyclopentylamine)platinum(II)	cis-PAD	Connors et al., 1972
malonato(1,2-diaminecyclohexane)platinum(II)	AHM	Soloway et al., 1980
diammine(1,1-cyclobutanedicarboxylato)platinum(II)	CBCDA	Tobe and Khokhar, 1977
diamminehydroxymalonatoplatinum(II)	-	Pera et al., 1982
dichloro(2,2-dimethyl-1,3-diaminopropane)-platinum(II)	$Pt(DMDAP)Cl_2$	van Kralingen, 1979

or chloroacetate often form very active compounds, whereas strongly bound anions (NO_2^- or SCN^-) or weakly bound anions (NO_3^-) yield inactive and/or very toxic compounds. In some cases, however, cis leaving groups which are relatively stable in the test tube (i.e. malonate) were found to give rise to active compounds in vivo (Cleare et al., 1978), probably due to biological activation;

- The A ligands also play an important role. They should be relatively inert and neutral. Variation of the structure of the amine has given complex results and no clear activity pattern has emerged (Tobe and Khokhar, 1977). However, it seems a prerequisite for antitumor activity to have at least one proton in the amine group attached to platinum (van Kralingen, 1979). Some platinum compounds with a specific cyclic (A) ligand (e.g. cyclohexylamine) seem to be highly specific only for certain tumors (Cleare and Hoeschele, 1973a, 1973b; Dus and Kuduk-Jaworska, 1982).

For the study described in this Ph.D. thesis, 10 compounds have been synthesized (van Kralingen, 1979). The compounds (Table 1.1.b.) are chemically related, they differ in platinum valence (Pt (II) and Pt (IV)), stereoconfiguration (cis or trans) and in non-leaving (A) group (cyclic and noncyclic, different sizes). Trans-platinum compounds are inactive as antitumor drugs; they were included in this study in order to determine a possible difference in the mode of action between inactive and active platinum compounds in the cells.

Two of the newly synthesized compounds ($Pt(DMDAP)Cl_4$ and Pt-(DMDAP)malonate, see Table 1.1.b.) used in this study and one related compound ($Pt(DMDAP)Cl_2$), together with some other compounds (see Table 1.1.c) have been found to be active against animal tumors, while showing less toxic effects and/or higher antitumor activity than cisplatin. Therefore, these compounds might be an alternative choice as antitumor drugs. $Pt(DMDAP)Cl_4$, Pt(DMDAP)malonate and $Pt(DMDAP)Cl_2$ were investigated further in animal tests (see below), the first two compounds turned out to be less suitable for clinical trials than cisplatin, while $Pt(DMDAP)Cl_2$ is still under investigation and may enter

clinical trials in the future. Three of the listed analogs (Table 1.1.c) have entered Phase I or Phase II clinical trials (TNO-6, CBCDA and CHIP). Recently, TNO-6 has turned out to be too nephrotoxic and is not studied further (Vermorken et al., 1983); CHIP and CBCDA are still under investigation and may enter Phase III and IV if found promising (Hacker et al., 1984).

Clinical trials

Toxicological data (rat, mouse, dog, monkey) are used at present to establish a starting dose of new cancer therapeutic agents for clinical trials and to provide information about possible acute and cumulative toxic effects. Usually, one-third of the low toxic dose (LTD) in the most sensitive animal species, expressed in mg per m^2 body sur-

Table 1.1.d. Phases of clinical testing

Phase I[1)]	clinical pharmacological study to determine the maximal or optimal tolerated dose of a drug and to elucidate the clinical toxicological pattern in cancer patients.
Phase II	study to determine the antitumor activity of a drug on each particular tumor out of a set of signal tumors. Signal tumors are: brain, breast, cervix, colon, oesophagus, lung, myeloma, ovary, pancreas and head and neck; testicular tumors were included in initial cisplatin testing.
Phase III	study to compare the effect of a drug with drugs of known activity, these trials usually involve large numbers of patients.
Phase IV	validation of the research findings and introduction of the drug in actual clinical practice.

1) Starting dose is derived from toxilogical data in animals.

face, is used as a starting dose in patients (Penta et al., 1979). The first clinical trial (Phase I; see Table 1.1.d.) is a pharmacological study in which the maximal tolerated dose as well as the toxicological pattern is determined (Carter, 1978). Thereafter the drug enters Phase II, an efficacious screening of antitumor activity against several signal tumors (Carter, 1978; Table 1.1.d). Subsequently, when positive results are obtained, the drug enters Phase III and IV clinical trials.

Biological target of platinum compounds

When cisplatin dissolves in water, the chloride ions are relatively rapidly displaced by water molecules and the resulting aquated molecules can react further with biological macromolecules (Johnson et al., 1980a; Marcelis and Reedijk, 1983):

$$Pt(NH_3)_2Cl_2 \overset{H_2O}{\rightleftharpoons} Pt(NH_3)_2Cl(H_2O)^+ + Cl^- \overset{H_2O}{\rightleftharpoons} Pt(NH_3)_2(H_2O)_2^{2+} + 2Cl^-$$

$$Pt(NH_3)_2Cl(H_2O)^+ \rightleftharpoons Pt(NH_3)_2Cl(OH) + H^+$$

$$Pt(NH_3)_2(H_2O)_2^{2+} \rightleftharpoons Pt(NH_3)_2(OH)(H_2O)^+ + H^+$$

$$Pt(NH_3)_2(OH)(H_2O)^+ \rightleftharpoons Pt(NH_3)_2(OH)_2 + H^+$$

The time needed for the displacement of the chloride ions by water and for the reaction of the aquated forms with macromolecules is different for each step and for each compound (Rahn et al., 1980).

In the body fluid, with its high chloride concentration, cisplatin remains in the little active dichloro-form, but after entering the cell it is hydrolysed owing to the low Cl^- concentration inside the cell. Then, reaction of the aquated complexes with cellular macromolecules will follow.

Although platinum compounds can react very well with proteins (Kohl et al., 1980; Filipski et al., 1983; Haddad et al.; 1983) and RNA (Morris and Gale, 1973; Miller et al., 1983), the site of action resulting in cytotoxicity and tumor destruction is now generally believed to be DNA rather than other macromolecules (Roberts and Thom-

son, 1979). The following observations demonstrate effects of the platinum compounds attributable to interaction with DNA:

- induction of filamentous growth of E.coli (Rosenberg et al., 1967);
- induction of lysis in lysogenic bacteria (Reslova, 1971; van Kralingen, 1979);
- induction of mutants in a number of prokaryotic (Beck and Brubaker, 1975; Beck and Fisch, 1980; Brouwer et al., 1981; Mattern et al., 1982) and eukaryotic cell systems (Bradley et al., 1979; Lecointe et al., 1979; Turnbull et al., 1979; Wiencke et al., 1979; Johnson et al., 1980b; Hsie et al., 1981; Singh and Gupta, 1983). In the present study (Chapter 2) the mutagenicity of ten platinum compounds in CHO cells is reported.
- inhibition of DNA synthesis (Howle and Gale, 1970; Shimizu and Rosenberg, 1973; Pascoe and Roberts, 1974; Rahn et al., 1980; Salles et al., 1983). The ten Pt-compounds tested in this study all inhibit semi-conservative DNA synthesis in mammalian cells, be it to a different extent (Chapter 2).
- DNA repair deficient human cell lines are more susceptible to cisplatin than are normal cell lines (Fraval et al., 1978; Chapter 6).

1.2. PROPERTIES OF MAMMALIAN CELLS IN CULTURE

1.2.1. Introduction

Cell culture as an experimental procedure for studying biological effects outside the intact animal has been described for the first time by Harrison (1907). He showed that nerve fibres would grow out from fragments of frog spinal cord isolated in clotted lymph. Thereafter the 'organ culture' technique was initiated, in which pieces of tissues were cultivated (Thompson et al., 1914; Carrel, 1912). In this approach the small tissue fragments were maintained in a state as close as possible to their state in the intact animal. Subsequently, methods were developed to transplant cultivated animal tissues back into the animal ('transplantation cultures', Murphy, 1913; Greene 1941).

Simultaneously, research was done on 'single' cell cultures. For this purpose it was necessary to separate the cells of a piece of tissue, providing cell cultures which were rapidly growing and dividing and thus resembled the growth of protozoa or microorganisms (Carrel and Baker, 1926).

Cell cultures can be obtained by separating the cells of a piece of tissue e.g. by treatment with trypsin. In this way, primary cell lines can be obtained from trypsinized embryo, newborn or adult animal and human tissues. In particular, cell lines from rodent tissue are easily prepared. They are all diploid and grow through 20 - 30 cell doublings (cell divisions, passages); then, the growth rate gradually diminishes. The cells become senescent (old) after 50 or more divisions and finally will stop dividing and die. However, at a low frequency cells may undergo genetic changes of unknown nature, releasing them from senescence and enabling them to continue dividing: an established cell line is obtained. It is relatively easy to derive established cell lines from tissue of fetal or newborn rodents, but difficult to obtain them from normal human tissues. Cell cultures can be maintained in suspension, in roller bottles or in flasks by stirring. Also, cultures can be kept in monolayer on flasks or Petri dishes. Animal cells in suspension culture display the same growth pattern as do microorganisms. When cells are taken from a stationary - non-dividing - culture, and seeded at low density in medium, there is at first a 'lag phase' of some hours to some days before growth commences. Then growth (cell multiplication) proceeds steadily ('logarithmic phase') until the maximum population density is reached and the cells enter 'stationary phase'. In monolayer cultures, normal cells will show a constantly decreasing growth rate from the outset and will stop dividing when the surface of the culture flask or dish is covered with cells (confluent state). This phenomenon is called contact inhibition.

In early studies, cells of a cell culture were only able to grow when they were plated (seeded in a flask or on a dish) at high densities. Puck and Marcus (1955), however, were able to grow cells in a dish at low densities (e.g. 50-250 cells per 6-cm dish), through modification of the culture conditions and adaptation of the cell culture

technique in such a way that the cells were able to form distinct colonies, (sometimes also called clones), i.e. a population of 50 or more cells which are all descendants from one single cell. This technique made it possible to screen large numbers of cells for genetic as well as toxicologic and physiologic effects (e.g. cell survival and mutation induction, see 1.4.). The growth period required to form colonies and the plating efficiency, i.e. the percentage of plated cells that form colonies, will differ for various cell lines. Some cell lines need a so-called 'feeder layer' in order to be able to form colonies. This feeder layer consists of X-ray irradiated cells, which are unable to multiply but exhibit active metabolism. The test cells are thus provided with the required growth metabolites until they become self-sustaining.

At an early stage in the development of animal tissue culturing, investigations on the factors required for the growth and division of the cells under artificial circumstances were initiated (Carrel and Baker, 1926), which resulted in the development of media which are still in use. Cells in culture are usually maintained in liquid medium consisting of a mixture of inorganic salts, vitamins, amino acids and an energy source (often glucose). Buffers such as bicarbonate may be used to stabilize the pH at 7.1-7.6. The cells are covered with medium and kept at 37°C. Some cell lines require 5-10% CO_2 for optimal growth. Most cultured cells require the addition of blood serum to the medium. The serum provides as yet unidentified factors (Kraemer, 1983), which influence the growth of the cells as well as their reaction to certain chemicals. Serum is derived from various sources; (fetal and newborn) bovine calf serum and horse serum are most commonly used. Each of these will have its own effect on the cells. Even different serum batches from the same animal species, usually provided industrially, will have different constituents. Hence uncontrolled factors are introduced in the experiments.

Besides many advantages, the use of cells in culture for testing the effects of physical and chemical agents has also several disadvantages when compared with whole animal testing, namely:

- Especially, established cell lines will become genetically different

from the original cells in the animal. This renders the extrapolation of test results obtained from cells in culture to the whole animal rather risky.

- Cells in culture will partly loose their differentiated state and are not controlled anymore by factors which are operational in the whole animal, even though they still exhibit contact inhibition.
- Metabolic activation in cells in culture is different from that in the animal. This is very important in (geno)toxicity testing. Chemicals which require metabolic activation will not be scored as (geno)toxic when a cell line without metabolic activation is used (Styles et al., 1982). The addition of liver extract can provide the cells with metabolic enzyme systems (see review by Paul, 1965).

1.2.2. Characteristics of the various cell lines used

Rodent cells

Among the rodent species the hamster is most often used to obtain cell cultures, especially Chinese hamster V79 and Chinese hamster ovary (CHO) cells (Siminovitch, 1976). In the present study, CHO cells have been used for several assays (see 1.4.).

CHO cells are descendants of an established cell line, characterized by rapid growth. The doubling time of the cells is 8-12 hr and they are able to start growing from a small inoculum. The growth will stop when the cells cover the entire surface of the dish or flask. The plating efficiency is 75-95%, which permits reliable (geno)toxicity testing without the use of a feeder layer. The cells have a stable karyotype with an average chromosome number of 22 $\pm$ 1. CHO cells can be stored in liquid nitrogen and subsequently grown up easily, allowing the maintenance of large stocks of cells with identical and reproducible characteristics.

Human cells

The culture of human cells is usually started from primary cell lines, but established human cell lines exist as well (e.g. HeLa cells). Some cells may not start growing from a small inoculum or only

in such a way that irregular or small clones are obtained. A feeder layer is then required in cloning assays. The cells will stop dividing when they reach confluency. Most human cell lines are skin fibroblasts usually isolated from a 3-4 mm punch biopsy of the inner surface of the upper arm or from foreskins. Beside human cell lines isolated from healthy persons (normal cell lines), cells can also be isolated from patients with heritable diseases. Of special interest for the present study are the cells from patients thought to have a defect in the repair of DNA damage.

Xeroderma pigmentosum (XP)

XP is an autosomal recessive disease. The patient shows sensitivity to sunlight, photophobia, early onset of freckling, and subsequent neoplastic changes on sun-exposed surfaces of the skin. The frequency of the disease in the human population is 1:250,000 (Kraemer, 1983). Cultured XP cells have a normal karyotype without excessive spontaneous chromosomal abnormalities and generally grow normally when they are not exposed to DNA-damaging agents. The cells are extremely sensitive to ultraviolet irradiation (UV) (Cleaver, 1968; Zelle, 1980) and to several DNA-damaging chemicals. They are thought to be deficient in excision repair of UV-induced pyrimidine dimers (Friedberg et al., 1979; Lehmann, 1982; see Chapter 6). XP is the first mammalian syndrome described to have defective DNA repair. The study of these cells has greatly contributed to the understanding of DNA repair processes in higher organisms.

Fanconi's anemia (FA)

FA is an autosomal recessive disorder characterized by anemia, developmental defects, and a high incidence of neoplasia. The frequency of this disease in the human population is very low, only 200 patients have been discovered so far (Kraemer, 1983). The cultured cells show a high frequency of spontaneous chromosomal abnormalities; they are hypersensitive to agents that can cause cross-links in DNA and are, therefore, thought to be deficient in cross-link repair (Fujiwara et al., 1977; see section 1.3.2.; Chapter 6). The cells grow normally, but senescence starts early (above 15 cell doublings).

Mucolipidosis II

Beside the human cell lines defective in DNA repair, another mutant cell line has been used in the present study (Chapter 6). This cell line was obtained from mucolipidosis II patients which suffer from an enzyme deficiency, resulting in intralysosomal accumulation of (harmful) compounds. The patients show severe clinical and radiological symptoms and psychomotoric retardation. They usually die between 2 and 8 years of age, because of cardiorespiratory failure (Halley, 1980). Cultured cells derived from biopsies of these patients, show extreme intralysosomal storage, due to a deficiency in one of the lysosomal enzymes. This deficiency result in the accumulation of toxic metabolites in the cells. In the mucolipidosis cell lines used in our study (RD 172 and RD 164), the enzyme arylsulfatase A is deficient. The cells react normally to UV (Zelle, 1980), but are very sensitive to the platinum compounds. This sensitivity is not due to defects in the DNA repair system, but is probably caused by accumulation of this compound or its metabolites in the cells (Chapter 6).

1.3. REPAIR OF DNA DAMAGE

In all DNA-containing species, DNA damage is induced spontaneously or by DNA-damaging agents, each of which introduces specific DNA damages (see Table 1.3.1.). DNA repair systems have evolved in all organisms studied so far which usually prevent deleterious effects of the DNA damage (see for reviews Hanawalt, 1977; Hanawalt *et al.*, 1979; Lehman and Karran, 1981; Roberts, 1982; Haseltine, 1983; Kenyon, 1983). The repair processes induced by several agents are most profoundly studied in prokaryotes, especially the bacterium *Escherichia coli*. Detailed study of the bacterial repair processes became possible through the construction of mutants that are deficient in specific DNA repair enzymes (see review by Witkin, 1976).

1.3.1. *DNA repair in Escherichia coli*

The research on DNA repair mechanisms in *E.coli* has been mainly concentrated on the classical model, the UV-induced pyrimidine dimer.

Table 1.3.1. DNA damage caused by various agents

Breaks		
- single strand	X-and γ-rays, neutrons	Painter and Young 1972; Lehmann, 1978
- double strand	X- and γ-rays, neutrons	v.d.Schans et al., 1982
Missing bases	spontaneously	Lindahl, 1979
Base alterations		
- incorrect base	spontaneously	Hanawalt et al., 1978
- pyrimidine dimers	UV	Hanawalt et al., 1979
- N- and O-alkylations	alkylating agents	Lawley, 1979; Demple and Linn, 1980; Livneh et al., 1979 Demple and Karran, 1983
- bulky adducts	4-nitroquinoline, benz(a)pyrene, Pt-compounds	Hanawalt et al., 1979 Philips, 1983
Cross-links		
- DNA interstrand	Pt-compounds, mitomycine C, nitrogen mustard, sulfur mustard, psoralen	Fujiwara et al., 1977; Cole et al., 1976
- DNA intrastrand	Pt-compounds, UV	Brouwer et al., 1981 Haseltine, 1983
- DNA-protein	Pt-compounds, UV, cyclohexyl-nitrosourea, ionizing radiation	Kohn and Ewig, 1979 Cleaver, 1984

This DNA intrastrand cross-link on adjacent pyrimidines (thymidines and cytosines) is formed by the absorption of UV light. It represents a block to replication; in the absence of repair and of a 'tolerance' mechanism - a mechanism to circumvent the damage - one dimer in the entire genome of E.coli may already be lethal (Howard-Flanders and Boyce, 1966). Different repair pathways to handle the dimer have been discovered, namely: photoreactivation (Sutherland 1978a, 1978b), excision repair (see below), post-replication repair (see below) and SOS repair (Bridges and Mottershead, 1978; Little and Mount, 1982; Kenyon, 1983). Some of these systems together with proofreading function (Glass, 1982), mismatch repair (Glickman and Radman, 1980) or repair via the suicide enzyme (adaptive response, Samson and Grains, 1977; Karran et al., 1979), have been found to be active on other types of DNA damage as well. The enzymes involved in the first three processes are thought to be constitutively present in the cell (at a low level, which is enhanced after induction of damage; Brandsma, 1983), whereas the SOS repair and some of the other repair systems seem to occur only when damage is inflicted (Kenyon, 1983; Brandsma, 1983). Some of the repair systems are discussed below in more detail.

Excision repair

Excision repair, which is the best known and characterized repair process, can repair most forms of base damage. In this process damaged bases are recognized and excised by enzymes, whereafter a replacement region (patch) is synthesized with the opposite strand as template (see review by Grossman et al., 1975). The new stretch of nucleotides is joined to the preexisting DNA by ligase (Hanawalt et al., 1979; Pettijohn and Hanawalt, 1964; see Fig 1.3.1.a and 1.1.1.b) and intact, repaired, DNA is obtained.

The recognition of the damaged site and the first enzymatic (incision) steps require very specific enzymes (see review by Lindahl, 1979). Two pathways for excision repair appear to occur depending on the chemical form of the damage: base-excision repair (sometimes called short patch or X-ray-like repair) and nucleotide-excision repair (long patch or UV-like repair). In the base-excision repair process the damage, in the form of nucleobases, is excised by rupture of the

glycosidic bond (by a glycosylase) between the damaged base and the sugar. The resulting apurinic or apyrimidinic (AP) sites (Fig. 1.3.1.a) can be filled in directly by base-insertase to give intact DNA or they are recognized by AP-specific endonucleases (Haseltine, 1983), which introduce single-strand nicks in the DNA. Then exonuclease, polymerase and ligase restore the DNA helix. The patch size of base-excision repair is small and the repair process usually does not generate errors (Hanawalt et al., 1979). Base-excision repair will predominantly restore a variety of small base-alterations, like N7-alkylguanine and N3-alkylguanine (Livneh et al., 1979; Demple and Linn, 1980). Nucleotide-excision repair (Rupp et al., 1982; Haseltine and Gordon, 1982; Haseltine, 1983; Sancar and Rupp, 1983) is the predominant pathway for the removal of UV-induced dimers in E.coli and other types of (bulky) adducts. Briefly, the distortion of the double helix or the lesion itself is recognized by an endonuclease, which makes a single strand nick in the phosphodiester backbone at one side of the damage followed by removal of the damage and some adjacent bases by an endonuclease (Setlow and Carrier, 1974; see Fig. 1.3.1.b.). Otherwise, two nicks can be made simultaneously at both sides of the

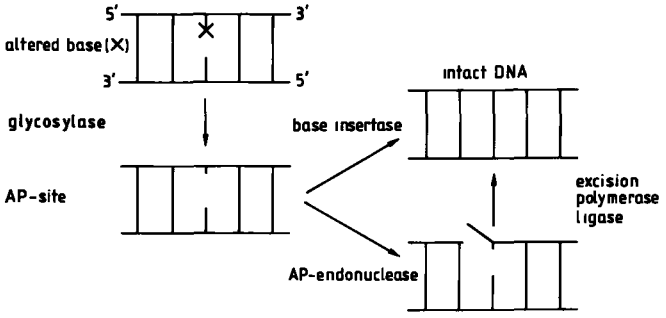

Fig. 1.3.1.a. Model of base-excision repair. The altered base is removed by a glycosylase. The obtained apurinic/apyrimidinic (AP) site can be filled in directly or can be recognized by an AP-endonuclease, resulting in a DNA break, which is processed further as in the original model of excision repair.

damage (Haseltine and Gordon, 1982), thereby excising an oligonucleotide fragment. Polymerase I will fill in the single strand region, which process can generate repair patches up to several hundred nucleotides, (Rothman, 1978; Cooper and Hanawalt, 1972; Hanawalt et al., 1979). The process is completed by ligase, through linkage of the re-

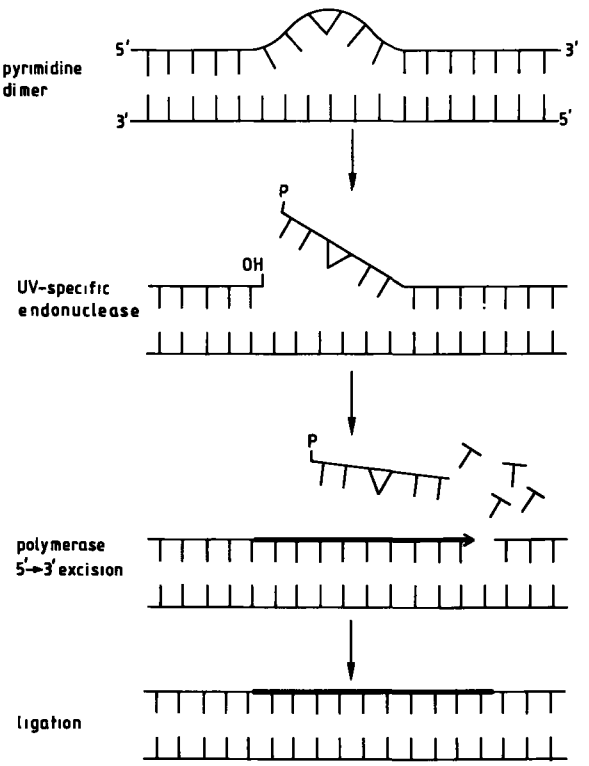

Fig. 1.3.1.b. Original model of excision repair of UV-induced pyrimidine dimers (Setlow and Carrier, 1964).

pair patch to the parental DNA. Excision repair is considered a highly accurate process; wrong bases leading to mutations are thought to be inserted only rarely (Cleaver, 1984).

Post-replication repair

DNA lesions which are not repaired before the DNA replicase reaches the site of the damage, are thought to slow-down the DNA replication machinery. Depending on the nature of the lesions, the extent of inhibition of DNA synthesis will be different. Sometimes DNA synthesis is reinitiated at a point beyond the lesion (Hanawalt et al., 1978), resulting in discontinuous daughter-strands (Roberts, 1981). It is also possible that the lesion is bypassed (through branch migration) (Roberts, 1981). For the ligation of DNA daughter strands and for branch migration, a model has been proposed called post-replication repair (Roberts, 1981; see Fig. 1.3.1.c), of which the details are not yet understood. Post-replication repair is thought to lead to strands of DNA still containing the primary lesions (Howard-Flanders,

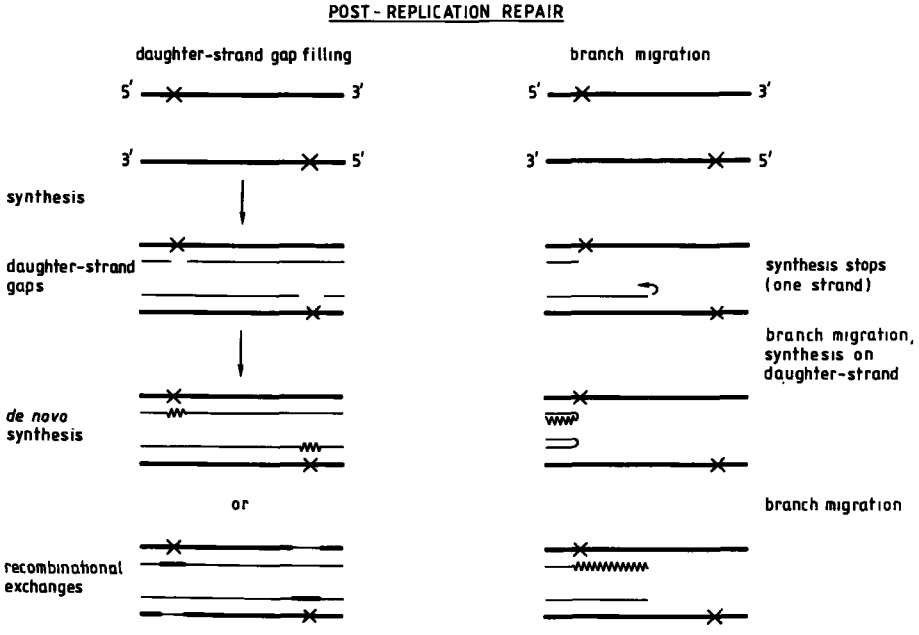

Fig. 1.3.1.c. Model of post-replication repair, which is supposed to be divided in two pathways, namely: daughter-strand gap filling and branch migration. The first pathway is suggested to generate errors only when the gaps are filled in de novo. Branch migration is thought to be an error-free pathway.

1968), although the amount of damage per cell will be diluted when the cells go through many cell doublings. The ultimate result of post-replication repair is the survival of the cell. Depending on the contribution of the different pathways of the post-replication repair acting on double stranded DNA matrices, the process can be more or less error-prone (Ganesan et al., 1982).

DNA interstrand cross-link repair

Interstrand DNA cross-links - covalent links between opposite DNA strands - can be formed by compounds that have two reactive sites. These types of lesions are very important in relation to the working mechanism of the platinum compounds and the repair of these lesions is relevant for all studies reported in this thesis.

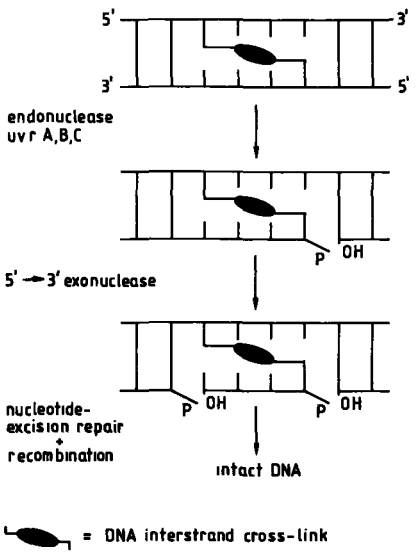

Fig. 1.3.1.d. Model for excision repair of psoralen-induced cross-links in bacteria. The DNA strand is broken at one side of the cross-link followed by a second nick at the other side of the cross-link in the same strand or both nicks are made simultaneously. In order to restore the damage, the unhooked cross-link is processed further by nucleotide-excision repair and recombinational events are necessary to obtain intact DNA.

Cole et al. (1976) proposed a model for excision repair of psoralen-induced cross-links in bacteria (see Fig. 1.3.1.d). Nicks are made in one DNA strand at either side of the cross-link. The intact DNA structure is then restored by means of recombinational events (Cole et al., 1976), which are not known yet. It is supposed by Haseltine (1983), that both strand incisions could possibly be made simultaneously through the nucleotide-excision repair pathway. Because the repair of DNA interstrand cross-links requires recombinational events, this type of DNA damage might be very effective in inducing mutations (Cole et al., 1976).

1.3.2. DNA repair systems in mammalian cells

In contrast to E.coli, which is haploid, the genome of mammalian cells consists of a diploid number of chromosomes and is organized in a much more complex way. Each chromosome is thought to consist of one uninterrupted DNA molecule (Natarajan et al., 1982) which is packed onto histone structures to form chromatin. The basic units of chromatin are the 'nucleosomes', each containing approximately 200 base-pairs. The 'core' of a nucleosome consists of 140 base-pairs wound around an octamer of 4 (x 2) different histone molecules. The remaining 60 base-pairs together with histone H_1 form the 'linker' region between two consecutive units (Hand, 1978; Kornberg and Klug, 1981). The nucleosome structure of the eukaryotic chromosome contains secondary and tertiary foldings of the DNA and the extent of the folding depends on the cell cycle and on the function of each particular region. These structures play a dominant role in the interactions between DNA and chemical or physical agents. The sites and rates of (excision) repair depend on the accessibility of these structures for enzymes. Damage in linker DNA might be repaired more rapidly than that in core regions. DNA-damaging agents may either produce randomly distributed lesions (e.g. lesions induced by X-rays and UV), or lesions predominantly located in the linker regions of the DNA (e.g. bulky adducts) (Cleaver, 1978), so that the rate of repair will depend on the agent causing the damage. The section of DNA being repaired is thought to acquire a relatively open (free of histones) structure,

subsequently, the repaired DNA is repacked into nucleosomes (Hand, 1978).

In mammalian cells the repair mechanisms appear to differ to some extent from those originally discovered and defined in bacteria (see reviews: Nichols and Murphy, 1977; Cleaver, 1978; Hanawalt et al., 1979; Lehmann and Karran, 1981; Meneghini et al., 1981; Roberts, 1981; Natarajan et al., 1982; Hanawalt, 1983). The activity of DNA repair mechanisms in eukaryotic cells seems to be dependent on the state of the cell cycle, the type of cells, the organ and the species involved (Hart and Setlow, 1974; Holliday et al., 1977; Nichols and Murphy, 1977; Holliday, 1978; Kidson, 1978; Aaron et al., 1980; Kato et al., 1980; Purchase, 1980; Styles et al., 1982; Vijg et al., 1984).

Excision repair

Excision repair seems to be the prevailing pathway of repair in mammalian cells. The early steps of these pathways involve numerous enzymes which recognize and attack damaged sites of the eukaryotic chromosome. These steps are not understood very clearly. Later steps of the excision repair in mammalian cells, involving exonucleases, polymerases and ligases, are understood better (Hanawalt, 1977; Cleaver, 1978; Hanawalt et al., 1979; Lehmann and Karran, 1981; Ganesan et al., 1982; Lehmann, 1982; Roberts, 1982; Hanawalt, 1983).

As described for E.coli, also in mammalian cells excision repair pathways with distinct early steps can be recognized (base-excision and nucleotide-excision repair). The patch size obtained after excision repair in mammalian cells generally is smaller than in E.coli. Nucleotide-excision repair might result in patches from 35 (Edenberg and Hanawalt, 1972) to 100 nucleotides (Clarkson, 1978) and base-excision repair might involve patches of 3 to 5 nucleotides (Painter and Young, 1972).

Post-replication repair

Little is known about the enzymes involved in post-replication repair and the details of this process in mammalian cells. The process is thought to have two pathways, as in bacteria (Roberts, 1981). Al-

though recombination (involved in daughter strand gap filling; see section 1.3.1.) does not seem to occur very often (Cleaver, 1978), post-replication repair is probably of fundamental biological importance in mammalian cells. A defect in the post-replication repair process (XP variants) correlates with multiple malignancies and early death (see review Lehmann and Karran, 1981; Lehmann, 1982; Meneghini et al., 1981).

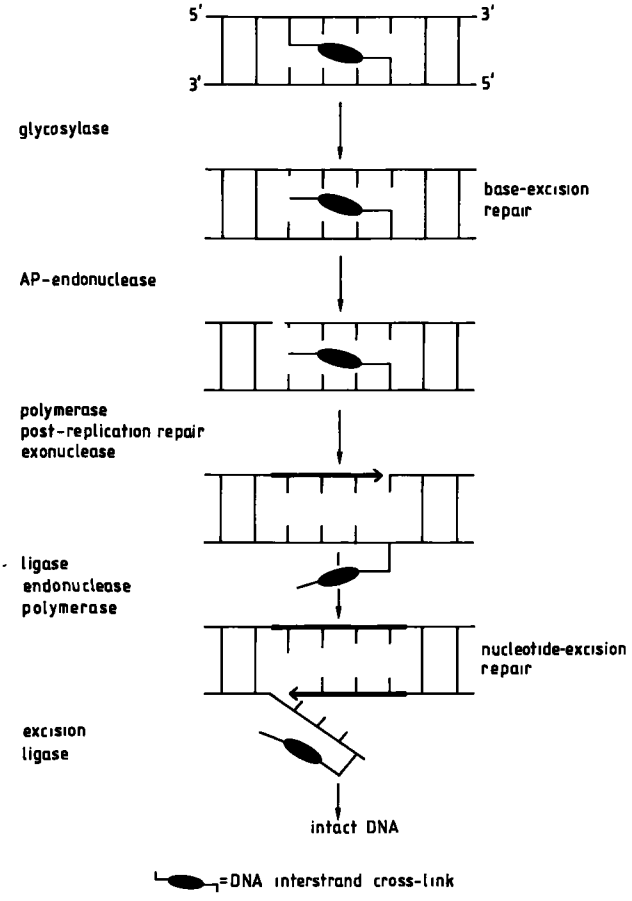

Fig. 1.3.2.a. Model of DNA interstrand cross-link repair in mammalian cells. The first arm of the cross-link is unhooked via the base-excision repair pathway and the second arm of the cross-link is removed by nucleotide excision repair. Post-replication repair is needed to restore the DNA helix.

DNA interstrand cross-link repair

DNA interstrand cross-links are predominantly formed in the linker region of the DNA of eukaryotic cells (Cech and Pardue, 1977) by bifunctional agents (e.g. platinum compounds). The model proposed by Cole (et al., 1976) for the repair of cross-links in E.coli, probably will not be applicable to mammalian cells, as it involves recombinational steps (Cleaver, 1971, 1978). From comparative studies in normal, xeroderma pigmentosum and Fanconi's anemia cells, a model for eukaryotic cross-link repair has emerged, which suggests that the opposite ends of DNA interstrand cross-links are not equivalent (Fujiwara et al., 1977). The initial step of cross-link repair might involve base-excision (i.e. first arm unhooked by glycosylase action) and the second step might require nucleotide-excision repair (Fig. 1.3.2.a; Fujiwara et al., 1977; see Chapter 6). The intermediates in cross-link repair may be particularly effective in stimulating sister chromatid exchanges (see section 1.4.5. and Chapter 3) during DNA replication (Perry and Evans, 1976).

Repair of other DNA cross-links

Two other types of cross-links can be formed in cells by various agents, namely: DNA intrastrand cross-links and DNA-protein cross-links. The former are cross-links between neighboring nucleotides or nucleotides separated by one or more bases in one DNA strand. These cross-links are thought to disturb the DNA configuration. They might be repaired conform the (nucleotide-) excision repair pathway, in the same way as pyrimidine dimers (Haseltine, 1983; Cleaver, 1984). If this assumption is true, only a very small proportion of the DNA intrastrand cross-links will result in mutations during their repair. The small amount of mutations might be induced by persistent DNA intrastrand cross-links by the time the DNA replication machinery has to pass them. DNA intrastrand cross-links which link bases over a long distance might require another type of (excision) repair.

DNA-protein cross-link repair has not been studied very extensively. Because protein, covalently bound to the DNA, is a bulky molecule, these cross-links are thought to be removed in the same way as other bulky DNA-adducts are, i.e. via excision repair (Cleaver, 1984).

Inducible repair

It has been suggested that in eukaryotic cells recovery of the normal rate of DNA synthesis, after DNA damage has been inflicted, could be an inducible process (Bianchi, 1982) resembling the SOS mechanism described for bacteria (Kenyon, 1983). Split-dose experiments have revealed evidence for such an inducible system; after the first dose a second dose will inhibit DNA elongation clearly less than does a single dose of the same magnitude (D'Ambrosio and Setlow, 1976). Pretreatment of host cells with radiation or chemicals enhance the survival of UV-irradiated viruses, which viruses have a higher mutation rate (Sarasin and Benoit, 1980). The study on inducible repair is still in its infancy. It is unknown whether some kind of error-prone post-replication repair is involved in it.

1.3.3. Model DNA-damaging agents

DNA-damaging agents usually induce a variety of DNA damages, but in most cases predominant lesions are formed. The spectrum of lesions induced by the model compounds (UV, X- and γ-rays and mitomycin C) used in this study, is discussed here.

Ultraviolet irradiation (UV)

The predominant lesions induced by UV are the pyrimidine dimers, but several minor photo-products, such as (6-4) products, cytosine hydrates, chain breaks, DNA-protein cross-links and DNA interstrand cross-links are formed as well. Most research on the effects of UV has been done on the pyrimidine dimers and has been discussed already (see above), the other photo-products have not been studied extensively so far and their biological effects are not well known. Very recently, however, the (6-4) products were found to be possibly responsible for mutagenic effects (Haseltine, 1983). UV has only been used in this study as a reference DNA-damaging agent (Chapter 6), in order to compare the repair of platinum-DNA adducts with the relatively well understood response of cells to UV-irradiation.

X- and γ-rays

X- or γ-rays produce a heterogeneous mixture of damages in DNA, including single- and double-strand (ss and ds) breaks, sugar damage, base loss (alkali labile lesions) and base damage such as saturation of the 5-6 double-bond of pyrimidines. X- and γ-rays have been used in alkaline elution experiments in our study (Chapter 3, 4 and 6).

Mitomycin C

Mitomycins, generally nonreactive in the neutral oxidized state, behave as bifunctional 'alkylating' agents, after activation with NADPH-generating systems (Iyer and Szybalski, 1964). Mitomycin C is able to bind amino-groups of adenines or cytosines, but it predominantly links together two O^6 atoms of guanines (Lown, 1979; Iyer and Szybalski, 1964). The way in which O^6-guanine cross-links are repaired is not known. O^6-guanine monoadducts seem more difficult to be repaired than N7-guanine monoadducts, therefore, O^6-guanine interstrand cross-links might be more harmful for the cell than are N7-guanine interstrand cross-links (Sognier and Hittelman, 1983). Furthermore, the O^6-adducts have influences on the hydrogen bonding of the bases, leading to mispaired bases, e.g. guanine based to thymidine or cytosine (Loveless, 1969). Mitomycin C is used here as a model DNA interstrand cross-linking agent (Chapter 6).

1.4. METHODS TO DETECT DNA DAMAGE AND ITS EFFECTS IN MAMMALIAN CELLS

1.4.1. Introduction

When cells are treated with DNA-damaging agents, the DNA-repair machinery will be activated, resulting in various responses which are outlined for mammalian cells in Fig. 1.4.1.(taken from ICPEMC committee 1 final report, 1983, with permission). These responses can be investigated at several levels. Firstly, it is possible to study the induction and the disappearance of the DNA lesion itself (upper part

of Fig. 1.4.1.). Secondly, we can study the presence or absence of DNA repair processes (e.g., by measuring unscheduled DNA synthesis, by studying the time-course of DNA synthesis inhibition; middle part of Fig. 1.4.1.). Thirdly, we can look to the ultimate (phenotypic) effects in cells (last line of Fig. 1.4.1.) by measuring biological endpoints, such as cell death, mutation induction, chromosomal damage (see review by Baan et al., 1984).

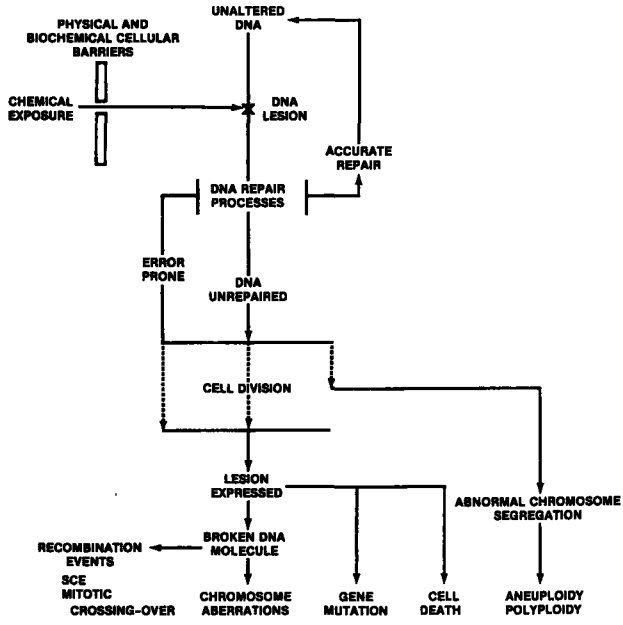

Fig. 1.4.1. Pathways leading to the induction of genetic end points.

1.4.2. Measurements of the induction of DNA lesions

Detection and quantification of the original DNA damage allow the study of the persistence and the biological significance of a particular type of damage. The occurrence of some types of DNA damage can be detected with the aid of physical-biochemical methods. Other types of DNA damage, such as adducts and pyrimidine dimers, can be determined

after they have been isolated by chromatography (e.g., high performance liquid chromatography; HPLC). Otherwise, identified adducts can be measured by immunochemical techniques.

Physical-biochemical methods

A change in the size of the DNA within the cells caused by e.g. X-rays (decrease) and cross-linking agents (increase), respectively, can be detected by physical-biochemical methods. The induction of strand breaks (either single or double strand breaks) will reduce the molecular size of the nuclear DNA, which can be measured by sedimentation of the DNA in alkaline or neutral sucrose gradients (van der Schans et al., 1969; Chapter 2). These methods are rather insensitive and have been replaced recently by more sensitive methods, viz elution through membrane filters under alkaline (Kohn et al., 1976; Chapter 3) or neutral (Bradley and Kohn, 1979; Chapter 2) conditions. In the filter elution procedures, the rate of elution of DNA through a small-pore size filter is related to the length of the single- or double stranded DNA (the smaller the DNA, the faster the elution). Therefore, the amount of DNA eluted can be considered as a function of the size of the DNA (see appendix to Chapter 3).

When cross-links (either DNA interstrand or DNA-protein cross-links) are induced in cells, the elution rate of the single-stranded DNA (alkaline elution) will be reduced. The decrease in elution rate is related to the number of DNA interstrand and DNA-protein cross-links (Chapter 3). The two types of cross-links can be distinguished by the fact that DNA-protein cross-links are sensitive to proteinase digestion (Fornace and Little, 1979). The alkaline elution procedure is a indirect method to detect cross-links, especially DNA-protein cross-links (Kohn, et al., 1976; Kohn and Ewig, 1979; Chapter 3).

Isolation, characterization and quantification of DNA damage by chromatography

During the past years considerable progress has been made with regard to the precise characterization of DNA components structurally modified by chemical carcinogens and mutagens. Until recently, radiochromatography has been the method of choice for the analysis of che-

mically modified DNA (Baird, 1979). In this method, DNA is modified with radioactively labeled carcinogens and degraded to nucleosides or nucleotides. The degradation products are separated by means of e.g., HPLC, whereafter the radioactive DNA-adducts can be isolated and characterized. The sensitivity of the radio-chromatographic method is limited by the specific radioactivity of the respective carcinogens and by the relatively large amounts of DNA required for analysis. Furthermore, its application is hampered by the limited availability of radiolabeled carcinogens (Müller and Rajewsky, 1981). These shortcomings may be circumvented by the use of immunochemical techniques to detect the presence of well-known DNA modifications in a degraded DNA sample after separation. Since immunochemical techniques can be highly specific and sensitive, DNA-degradation and/or separation of the DNA-adducts from the bulk of unmodified DNA particles might not always be necessary.

Immunochemical techniques

Antibodies are characterized by an exceptional capability to specifically recognize subtle alterations of molecular structures (Müller and Rajewsky, 1978). High-affinity antibodies specifically directed against DNA components structurally modified by carcinogens, in combination with sensitive immunoassay procedures, have recently become available to detect low levels of DNA damage (Müller et al., 1982). The sensitivity of the immunochemical techniques largely depends on the affinity of the antibody for its antigen or hapten. Antibodies have been raised in rabbits against several immunogens, i.e. specific haptens - DNA nucleotide or nucleoside damages prepared in vitro - which have been coupled to immunogenic proteins (e.g. bovine serum albumin) (Poirier, 1981; Müller et al., 1982; van der Laken et al., 1982; Baan et al.,1982). The antibodies raised in this way sometimes exhibit a higher affinity for single carcinogen-modified nucleosides or nucleotides than for the corresponding products contained in single or double stranded DNA (Baan et al., 1982). The antibodies are used to carry out quantitative and qualitative assays (Farr, 1958; van der Laken et al., 1982).

In the so called 'solid phase assays' antibody binding to antigen (coated onto a solid plastic surface) is monitored by the binding of a second antibody. This second antibody carries a covalently bound enzyme (e.g. alkaline phosphatase) which is able to cleave a chromogenic or fluorogenic group from its substrate (Shalev et al., 1980). This enzyme linked immunosorbent assay (ELISA; Engvall and Perlmann, 1972) can easily be used in large scale experiments (= direct ELISA). Especially when the ELISA is used in the competitive mode (see Chapter 5), only very small amounts of modified DNA (intact DNA or DNA degradation products) are needed to measure quantitatively the level of modification. In this procedure the digested DNA samples (containing unknown amounts of antigen) is incubated with a known amount of antibodies (preincubate). This preincubate is added to cups which are coated with in vitro prepared antigens (see Chapter 5). The unreacted antibodies of the preincubate will bind to the antigens in the cups, the amount of cup-bound antibodies are determined by the same procedure as used with the direct ELISA.

When the competitive ELISA is used to detect low levels of Pt-DNA-adducts in HPLC fractions of enzymatically digested DNA from treated cells, a reproducible and sensitive test system is obtained, because the modified components are separated from the large excess of unmodified nucleotides (Fichtinger-Schepman et al., 1984). In this way it was possible to detect low levels of Pt-DNA-damage in cultured cells (see Chapter 5).

1.4.3. DNA repair measurements

In mammalian cells, replicative DNA synthesis occurs only in the S-phase of the cell cycle, i.e. it is 'scheduled'. Repair synthesis is not subject to this constraint and it can, therefore, be measured by a variety of techniques which distinguish 'unscheduled' from 'scheduled' DNA synthesis. The first evidence for unscheduled DNA synthesis (UDS) in mammalian somatic cells was provided by Rasmussen and Painter (1964), when they demonstrated that UV light induced the incorporation of labeled thymidine (TdR) into the DNA of non-dividing cultured mammalian cells.

A method to detect UDS is by autoradiography (Williams, 1977), which uses the ability of radioactive DNA precursors to blacken a photographic emulsion to detect the amount of radioactivity incorporated in the cells. To this end, the number (or area) of black silver grains in the photographic emulsion, which is layered on top of the cells, is counted. S-phase cells will show a very heavily blackened nucleus and can be easily distinguished. The autoradiographic method is thought to be reliable, because no S-phase inhibitors are needed. The UDS assay will only measure the type of excision repair yielding substantial repair patches. The level of UDS is determined by the rate of excision and polymerization and the size of the repair patches (Lonati-Galligani et al., 1983). Usually, agents that cause damage removed by short-patch excision repair will induce small amounts of UDS, as do the platinum compounds (Chapter 2 and 6). In that case UDS measurements will be insensitive. In general, a positive UDS answer indicates that the agent under study induces DNA damages, but it does not necessarily mean it is harmful, since probably not the repaired, but rather the unrepaired lesions are most noxious for the cell, and the latter will not give rise to UDS (Mirsalis, 1983). Nevertheless, UDS has been shown to be evoked by agents which have been identified as genotoxic or carcinogenic (ICPEMC, committee 4 final report, 1983; Lehmann and Stevens, 1980), but in the screening of unknown chemicals, the UDS test-system will not give a clearcut answer (Lohman et al., 1984).

1.4.4. DNA synthesis inhibition

An early response of cells to DNA damage is inhibition of DNA synthesis. DNA-damaging and non-DNA-damaging agents can be distinguished by the changes in the rates of DNA synthesis after treatment. With a DNA-damaging agent, the rate of DNA synthesis remains decreased or continues to decrease, whereas with a non-DNA-damaging agent the rate of DNA synthesis rapidly increases. We can explain this phenomenon as follows: a non-DNA-damaging agent may inhibit the DNA synthesis by interaction with cellular metabolism. With the removal of the agent the metabolic block is removed and recovery begins immediately, ra-

pidly bringing DNA synthesis back to the normal level. When a DNA-damaging agent is removed from the medium, however, the DNA damage remains and DNA synthesis remains inhibited until the damage has been repaired. The potential of a certain compound to act as a DNA-damaging agent can be estimated by measurement of the DNA synthesis within the first 1-3 hr after treatment of cells with this compound (Painter and Scheafer, 1969a, 1969b; Painter, 1977; Painter and Howard, 1978). This test has been suggested to be useful for the screening of genotoxic chemicals (Painter, 1977; Chapter 2 and 6).

1.4.5. Ultimate effects in the cells

Cell killing

Cell killing can be described as the loss of proliferative capacity of cells after they have been treated with an agent. Usually, cell killing is expressed as the percentage of surviving cells (number of cells that are able to form colonies relative to the number of colonies formed by the control cells) after a certain dose of an agent.

Many years ago, a direct relationship was noted between the number of microorganisms which were killed as a result of radiation expo-

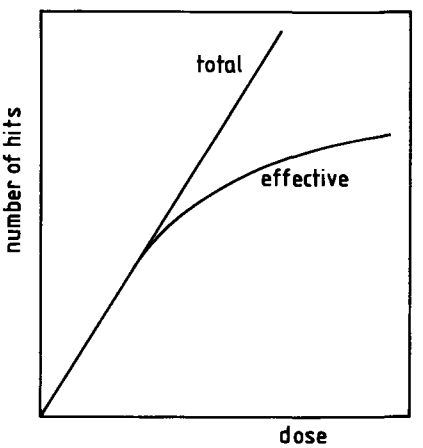

Fig. 1.4.5.a. Relation between the total and effective hits produced by an agent and the dose.

sure and the radiation dose they had received. In order to describe this relationship in mathematical terms, the target theory was formulated (Lea, 1955). The target theory stated that the production - by ionizing radiation - of damage in a particular molecule or substrate (target) is responsible for the measured effect (cell killing). The induction of an effective event in the target is called a hit. In the simplest form of the target theory, one hit is sufficient to kill the cell (simple target theory). The theory assumes that each event occurs at random in an irradiated system. The relation between the dose and the number of hits is given in Fig. 1.4.5.a. At higher dose the number of hits occurring in targets that have already been hit, will increase, resulting in a decreased number of effective hits. Therefore the relationship between the dose and the number of effective hits is not linear. The number of effective hits will correspond to the number of cells which are killed by the radiation exposure. Cell killing in relation to dose is usually expressed in survival curves, in which the surviving fraction of the irradiated cells is plotted against the dose. By doing so the number of surviving cells decreases in an exponential way. The numerical relationship of the simple target theory can be given by $S = e^{-kD}$, where S is the fraction of surviving cells, D is the radiation dose and k is the 'proportionality' constant.

It is assumed that the number of hits which reaches a target follows the Poisson distribution (since each event occurs at random), and therefore k is equal to $1/D_0$ (Casarett, 1968), where D_0 is the dose that gives an average of one hit per target. The fraction surviving a dose of D_0 will be e^{-1}, which is approximately 0.37. Therefore, when there has been an average of one hit per target, 37% of the original number of cells will survive. The D_0 value corresponds to the dose at which 37% of the cells will survive.

In the multi-target theory it is assumed that cells contain more than one target which can be hit and in order to inactivate the entire cell, each of the targets must receive a hit. When the probability of a target being hit is one minus the probability of not being hit and the probability of all targets being hit is the probability of one target being hit raised to the power n, a mathematical expression can

be made: $S = 1-(1-e^{-kD})^n$ (which can be simplified, for large values of D, to $S = ne^{-kD}$). When such a relationship is plotted in a semilogarithmic way (see Fig. 1.4.5.b.), the result is a straight line with a shoulder, the extrapolation of the straight line to the vertical axis is n (the number of targets). The D_0 value can be defined as the dose increase which results in the survival of 37% of the cells in the linear part of the curve (Fig. 1.4.5.b.). The dose at which 37% of the

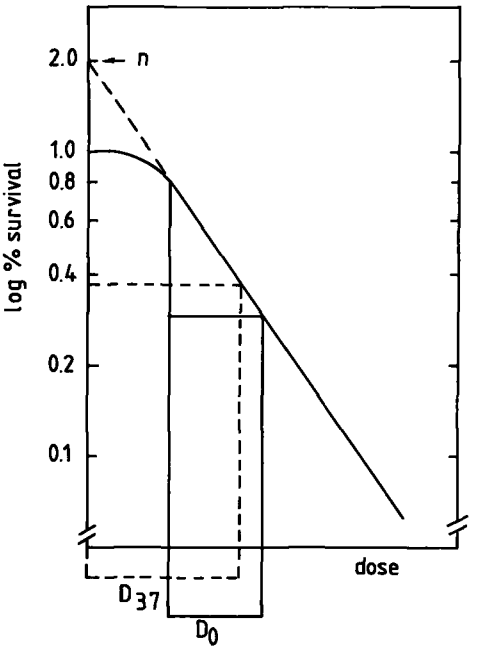

Fig. 1.4.5.b. Semi-logarithmic plot of the relationship derived for the multi-target theory.

cells survive - including the shoulder - is called the D_{37} value. D_0 and D_{37} values therefore, represent different criteria to describe the cytotoxicity of a compound (Chapter 2 and 6).

Multi-hit, or multi-hit/multi-target theories have been proposed as well, but will not be described here.

The radiation effect on mammalian cells in culture can be studied in the same way as in microorganisms (see Elkind and Whitmore, 1967). The first survival curves of cultured mammalian cells exposed to radiation were given by Puck and co-workers (1956). When the relationship between the surviving fraction of the cells was plotted semilogarithmically against the dose, a straight line with a shoulder was obtained. The survival curve obtained in this way, was thought to correspond to that obtained by the multi-target theory of microorganisms. The target was supposed to be DNA and the number of targets was 2, which was thought to correspond to the diploid number of chromosomes: i.e. both chromosomes of a set must be inactivated to result in cell death (Puck et al., 1956). However, Elkind (Elkind et al., 1961; Elkind and Whitmore, 1967) assumed that the shoulder in the survival curve is due to the repair of sublethal damage and that the linear part of the curve would fit the single hit-to-kill (simple target theory) theory.

The theories on the survival curves of cells after radiation exposure can be applied on the data obtained after treatment of cells with various DNA-damaging agents (Barendsen, 1962; Elkind and Whitmore, 1967). From the shape of the survival curves the mechanism by which inactivation of the cells occurs (single- or multi-hit, single- or multi-target, presence or absence of sublethal damage repair) may be derived.

Some factors will influence the cell killing properties of an agent. Firstly, the cell cycle phase will determine the sensitivity of the cells. Usually, G1 phase or early S phase cells are the most sensitive ones. Secondly, environmental factors will influence the sensitivity of the cells, i.e. CHO cells are more sensitive to γ-rays when they are irradiated in the presence, than in the absence of oxygen (van der Schans et al., 1982). Thirdly, cell killing might be affected when feeder layers are used. The influence of the metabolites of the feeder layer cells on the processing of the damage in the test cells cannot be excluded. It is recommended, therefore, to use the same cell type for feeder layer and test cells, to avoid for example the leakage of DNA repair enzymes from repair competent feeder cells

into the medium, resulting in the removal of DNA damage in repair deficient test cells.

The loss of proliferative activity of the cells is usually due to DNA damage. This is supported by the finding that treated cells which are allowed to repair their damage in non-dividing conditions, will have an increased survival when they are subsequently given the opportunity to divide. (Meneghini et al., 1981).

Gene mutation

Mutation induction can be studied in mammalian cells. For the detection of mutants generally two factors are of extreme importance: firstly, the mutation must be expressed in the cells and secondly, a selection procedure must be available.

For the expression of a recessive mutation in diploid cells, it is necessary that the mutation is situated in a (partly) haploid region of the genome. Fortunately, there is a haploid region available in mammalian cells. Namely, male cells contain only one X-chromosome and of the two X-chromosomes in female cells, one is inactivated (Lyonisation) (Howard-Flanders, 1981).

The selection of mutants requires a phenotypic change in the mutant cells, i.e. there must be a detectable change in morphology, in growth requirements or in some other observable property. In order to score the altered phenotype, often the resistance to a drug is investigated. A very suitable gene product for the selection of mutants is the hypoxanthine guanine phosphoribosyl transferase (HGPRT). In all mammalian cells the gene coding for this enzyme is located on the X-chromosome. HGPRT is essential for the re-utilization of the degradation products hypoxanthine and guanine by converting these purines into the corresponding nucleoside-5'-monophosphates, which then can be used for the synthesis of DNA. The enzyme is not essential for cell survival, because the cells can synthesize these bases de novo via an alternative pathway. Under certain, artificial conditions the presence of HGPRT can be harmful to the cells, as it enables these to incorporate toxic analogs of these bases. For instance, cells with a functional HGPRT gene product are killed by the supply of 6-thioguanine (a

toxic analog of guanine), whereas the cells with a non-functional HGPRT gene product will survive (Cole et al., 1983; Chapter 2 and 3).

The HGPRT gene is inactivated after the induction of a wide variety of mutations, such as base-pair substitutions, frameshifts, deletions and chromosome rearrangements (Bradley et al., 1981). Therefore, the HGPRT system can be used for the screening of mutation induction after treatment of cells with many chemical and physical agents.

Mutant selection involves the formation of colonies by the mutant cells. Because human cells often need the addition of feeder layers to form colonies, these cell lines are not very practical in mutation studies, although there are reports on mutation induction at the HGPRT gene in human cells (Grovosky and Little, 1983a, 1983b). The most widely used cell lines for mutation studies are V79 Chinese Hamster and Chinese Hamster Ovary (CHO) cells (Siminovitch, 1976). Especially the HGPRT/CHO system has been investigated extensively (Hsie et al., 1981; Hsie, 1981; Hsie et al., 1983).

Sister chromatid exchanges (SCE).

SCE, which can be considered as a special class of chromosomal aberrations, are the result of exchanges of homologous parts of sister chromatids in cells. The exchanged DNA regions are double strand (Wolf and Perry, 1975) rather than single strand (Bender et al., 1973).

To study the induction of these abnormalities, cells treated with an agent are allowed to go through two cell cycles in the presence of 5'-bromo-2'-deoxyuridine (BUdR) (Latt, 1973). During the first cell cycle the chromatids will build in BUdR in one DNA strand as a result of semi-conservative DNA replication; after the second round of replication, of each pair of chromatids, one will have incorporated BUdR in both DNA strands, its sister only in one. Then, strikingly clear demarcation of the differential incorporation of BUdR between the sister chromatids can be observed after staining the cells with Hoechst 32258 and Giemsa (Latt 1973; Perry and Wolf 1974; Korenberg and Freedlender, 1974) and exchanges will be observed easily.

SCE can occur spontaneously (Galloway, 1977) and SCE induction is depressed by caffeine, which might reflect the action of post-replica-

tion repair (Latt et al., 1981). The sites of SCE correspond to regions undergoing DNA synthesis (Latt et al., 1981). In general, for most substances tested the induction of SCE is linearly dependent on the dose (Carrano, 1983). The number of SCE is roughly proportional to the length of the metaphase chromosomes and the genome size in mammalian cells (Kato, 1977). Damages resulting in deformations of the DNA backbone are more likely to provoke SCE than others (e.g. damage induced by platinum compounds, Chapter 3).

The ratio of the number of SCE induced by a certain treatment and the frequency of induced mutations differs widely for various agents (Carrano et al., 1978; Bradley et al., 1979; Jostes, 1981; Jostes et al., 1981; Carrano, 1983), this suggests that the conversion of DNA damage to SCE or a mutation is lesion dependent. Even though the molecular mechanism of SCE has not been established, this should not preclude its use in quantifying exposure, because even at subtoxic doses of agents SCE are induced.

1.5. WHAT WAS KNOWN OF THE MECHANISM OF ACTION OF PLATINUM COMPOUNDS AT THE ONSET OF THIS STUDY

1.5.1. Sites of binding in DNA

The major target of platinum compounds in cells was considered to be DNA (Munchhausen, 1974; see section 1.1). Within the DNA, the nucleobases were found to be the reactive entities. Although in vitro the platinum compounds were able to bind to sugar, like D-mannitol (Eshaque et al., 1977) and to the phosphate group in cytidylic acid (Louie and Bau, 1977), these types of binding were not detected in vivo. The hydrated forms of cisplatin rather than the chloride form reacted with DNA, therefore, these forms were held responsible for the biological reactivity (Horacek and Drobnik, 1971; see section 1.1.). The binding to the nucleobases appeared to be very stable (Johnson et al., 1980a), but could be disrupted by cyanide (Stone et al., 1974) and thiourea (Zwelling et al., 1979c), albeit under non-physiological conditions.

Binding of cisplatin to DNA strongly perturbed the DNA structure,

resulting in partial unwinding of the DNA strands, in reduction of the apparent length of the DNA (Cohen and Bauer, 1979), in changes in the circular dichroism (CD) spectra (Ganguli and Theophanides, 1979) and in a decrease of the melting temperature (Hermann et al., 1979). The perturbations of DNA after treatment with the trans-stereoisomer were much less severe (see Roberts, 1979). From binding studies of the platinum compounds with nucleosides and nucleotides it had been concluded that binding occurred with the N7 of guanine (Theophanides, 1980; Marzilli et al., 1980), the N7 and N1 of adenine (Lock et al., 1976) and the N3 of cytosine (Louie and Bau, 1977). In mixtures of nucleotides or nucleosides and in DNA, cisplatin was preferentially bound to the N7 of guanine (Stone et al., 1974). Binding to adenine occurred to some extent and binding to cytosine took place only at high Pt/base ratios, but even under these conditions binding to thymine was never found. The stereo-configuration of the amine groups of the platinum compounds was shown to be retained during the binding to DNA.

1.5.2. Modes of binding to DNA and their possible importance

Although the trans-compounds had been found to react extensively with DNA, they were chemotherapeutically inactive. Therefore, the conformation of the platinum complexes was judged critical for their biological activity. Several different interactions with DNA had been detected after treatment of DNA and cells with platinum compounds. The studies to elucidate the working mechanism of the platinum compounds focussed mainly on the relevance of Pt-DNA-adduct and their repair. Especially those adducts which were exclusively induced by the cis-platinum compounds appeared relevant with respect to the antitumor action of the platinum compounds, although those adducts which invoked different repair systems in comparison to the trans-platinum-DNA-adducts were in view as well.

Binding of platinum compounds to DNA was found to occur either monofunctionally or bifunctionally. The latter was supposed to be performed through reaction of a monofunctional adduct with a second target site. The first binding step was thought to be fast, while the

second step appeared to be much slower (Marcelis *et al.*, 1980). It was thought unlikely that *cis*-platinum compounds exerted their tumoricidal action through monofunctional binding, because the *trans*-complexes which have no antitumor activity were more effective in forming monofunctional adducts (Pascoe and Roberts, 1974). Thus, the bifunctional mode of binding was more likely to be the basis of the antineoplastic action of the *cis*-compounds.

Several types of bifunctional adducts were shown to be formed by the platinum compounds. Firstly, the formation of a chelate within one guanine base by binding of the platinum compound at N7-O^6 had been found *in vitro* (Macquet and Theophanides, 1975; Dehand and Jordanov, 1976). The chelated base was thought to be a candidate for the antitumor action of the *cis*-compounds (Millard *et al.*, 1978; Theophanides, 1980), because O^6-guanine adducts had proven to be extremely mutagenic (Demple and Karran, 1983) and since the *trans*-compounds were unable to form this type of lesions (Mansy *et al*, 1973). However, this lesion had never been detected *in vivo*.

Secondly, Harder (1975) and Macquet and Theophanides (1975) have shown that the *cis*- and *trans*-platinum compounds were able to form cross-links between two bases in opposite strands of the double helix (DNA interstrand cross-links). These cross-links were formed between two guanines and have been detected *in vitro* (Roos, 1977), as well as *in vivo* (Zwelling *et al.*, 1978a, 1978b, 1979a, 1979b, 1979c). They represented only a small fraction of the total amount of platinum DNA adducts (Pascoe and Roberts, 1974). For a long time, DNA interstrand cross-links had been held responsible for the antitumor activity of the platinum compounds, because their presence correlated reasonably well with the observed toxicity in various systems (Zwelling *et al.*, 1978a, 1978b, 1979a, 1979b, 1979c).

Thirdly, *cis* and *trans*-platinum cross-links between DNA and protein molecules had been observed in eukaryotic cells (Zwelling *et al.* 1979a; Kohn and Ewig, 1979; Fornace, 1979). The *trans*-complexes were much more effective in inducing DNA-protein cross-links, predominantly between DNA and histones (Lippard and Hoeschele, 1979). The biological importance of this type of cross-links was not known.

Fourthly, the platinum compounds formed cross-links between two bases in the same strand of the DNA double helix (DNA intrastrand cross-links). Several types of DNA intrastrand cross-links are thought to be formed, namely:

- intrastrand cross-links between adjacent guanines, they have been found in vitro (Stone et al., 1976; Kelman and Buchbinder, 1978; Ganguli and Theophanides, 1979; Marzilli et al., 1980).
- intrastrand cross-links linking nucleobases more than one base apart (Macquet and Butour, 1978).

The role of intrastrand cross-links in the antitumor action of platinum compounds was not understood at that time.

Incubation of cells after treatment with platinum compounds had resulted in the release of platinum from the DNA of the cells. The platinum removal was not due to chemical instability, but had to be ascribed to an enzymatic repair process (Fraval and Roberts, 1979b; Rahn et al., 1980). The rate of loss inversely correlated with the sensitivity of the cells to the platinum drug. Cells growing in stationary phase (nondividing, displacing slower rates of repair) were more sensitive to cisplatin treatment than exponentially growing cells (Fraval and Roberts, 1979a; Roberts and Fraval, 1978). The repair of the platinum damage did not result in low molecular weight DNA and the damaged sites were not recognized by UV-endonuclease (Micrococcus luteus; Fraval et al., 1978). The repair mechanism that removed the major part of the platinum damage was thought to be excision repair, involving specific endonucleases which recognize the damage (Fraval and Roberts, 1978a; 1978b).

One explanation for differential cell killing between tumor cells and normal cells by cisplatin was that tumor cells were proliferating more frequently than did normal cells and therefore, displayed a higher rate of DNA synthesis. Another explanation was that the ultimate consequences of cisplatin's action upon any cell might be determined by the potential of that cell to repair the drug-inflicted damage (Zwelling and Kohn, 1979). For reviews on this matter, see Theophanides, 1980; Zwelling and Kohn, 1979; Roberts and Thomson, 1979; Prestayko et al., 1980).

1.6. OUTLINE OF PRESENT STUDY

The purpose of this project was to study the mechanism of action of platinum coordination compounds at the cellular and molecular level. In this approach special emphasis was given to studies on the relation between the structure of the platinum compounds, their biological effects, the nature of the DNA-adducts formed and the action of enzymatic processes specifically involved in the repair of these Pt-DNA-adducts.

Ten platinum coordination compounds, varying in charge of the central Pt-atom (II and IV), in configuration (cis or trans) and in the nature of the ligands, were involved in the first part of the investigation (Chapter 2). Their ultimate effects (cytotoxicity, mutagenicity) in Chinese hamster ovary (CHO) cells were compared to the inhibition of the DNA synthesis, induction of repair synthesis, induction of Pt-DNA-adducts (total and specific adducts), the induction of breaks (single strand and double strand) and alkaline labile sites (ALS) in the DNA of these cells. The results of this study were compared to the effects of these platinum compounds in other systems (van Kralingen, 1979; Brouwer et al., 1981; Mattern et al., 1982; Chapter 2). Four platinum compounds, differing in charge and configuration, were then studied in more detail as to the relation of the formation and repair of DNA cross-links (DNA-protein and DNA interstrand) to the

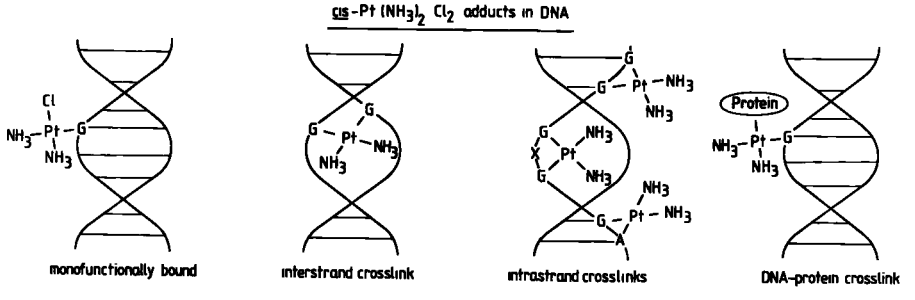

Fig. 1.6. Possible DNA-adducts formed after reaction of DNA with cis-diamminedichloroplatinum(II).

biological effects (cytotoxicity, mutagenicity, chromosomal damage) in CHO cells (Chapter 3). One platinum compound (cisplatin) was involved in a study on the effects of treatment temperature on the cytotoxicity, mutagenicity, platinum binding to DNA and on the induction of DNA interstrand cross-links (Chapter 4). Furthermore, the induction and repair of specific Pt-DNA-adducts (monofunctional adducts, DNA intrastrand cross-links) induced in CHO cells by cisplatin was investigated with recently developed highly sensitive immunochemical detection methods which allowed studies under relative mild conditions of treatment (Chapter 5; see Fig. 1.6.). Finally, cisplatin was used in experiments on normal and mutant human cells, in order to determine the contribution of different DNA repair and metabolic enzymes on the effects of this compound (cytotoxicity, repair synthesis and induction and repair of cross-links) (Chapter 6).

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2. COMPARATIVE STUDY ON THE EFFECTS OF TEN PLATINUM COORDINATION COMPOUNDS IN CHINESE HAMSTER OVARY CELLS.

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Summary

Ten platinum coordination compounds, differing in valence (II and IV), in ligands (both reactive and nonreactive groups) and in configuration (cis and trans) were used to perform a comparative study on the induction of DNA damage (single- and double-strand DNA breaks, alkali labile sites and platinum binding to DNA) and biological effects (cytotoxicity, mutagenicity, DNA synthesis inhibition, DNA repair synthesis) in CHO cells. Their antitumor activity in vivo, mutagenicity in Salmonella typhimurium and λ-prophage induction in Escherichia coli reported in the literature were compared to the effects found in CHO cells.

The cytotoxicity for CHO cells was found to differ considerable for the 10 compounds; it was inconsistent with the cytotoxicity in vivo, but did correspond to that in Salmonella typhimurium. The trans-compounds and $Pt(ACH)_2Cl_2$ were not cytotoxic in all systems. The mutagenicity in CHO cells (HGPRT-mutants) was determined in relation to survival and showed to be comparable to that in Salmonella typhimurium (Mattern et al., 1982) and coincide with the λ-prophage induction in E.coli (van Kralingen, 1979). In general, the mutagenicity in E.coli, as far as investigated (Brouwer et al., 1981), was only comparable to that of cII in CHO cells. The mutagenicity in CHO cells showed to coincide with the antitumor activity in vivo, except compound $Pt(ACH)-Cl_2$, which was non-mutagenic in CHO cells, but does have antitumor activity in one tumor system. All platinum compounds do react with DNA,

although the amount of Pt-DNA-adducts formed in the cells after treatment with equitoxic doses of the compounds was variable. The highly toxic compounds seemed to induce less Pt-DNA-damage at the D_{10} dose level, suggesting that the nature of the adducts rather than their total amount is responsible for the biological effects of the compounds. All platinum compounds did inhibit the semi-conservative DNA synthesis and induced marginal to very low amounts of DNA repair replication (UDS).

The compounds did not induce single- and double-strand DNA breaks and alkali labile sites in the DNA of the cells. It can be concluded that test systems as mutagenicity in CHO cells and Salmonella typhimurium and λ-prophage induction in E.coli seem to be predictable for the antitumor activity in vivo, although we found one exception. All data suggests that the nature of the formed Pt-DNA-adducts determines the action of the platinum compounds.

Introduction

Since the discovery of the antitumor properties of cis-diamminedichloroplatinum(II) (cII; cisplatin) (Rosenberg et al., 1967), much attention has been paid to the elucidation of the working mechanism of this and related platinum coordination compounds (Brown et al., 1982; Carter, 1984). Because cisplatin, although a good antitumor agent, is very cytotoxic, analogues called second generation platinum compounds have been synthesized (Litterst, 1984). These all have the general formula of PtA_2X_2, where X represents the reactive ligand and A is the non-reactive group. By varying the A and X group, it was hoped to obtain alternatives for cisplatin, in which low toxicity is combined with high antitumor activity (Burchenal 1978; Craciunescu et al., 1982; Hydes, 1984).

Platinum coordination compounds react in cells with cellular macromolecules (Roberts and Thomson, 1979). In particular the damage induced in the DNA of the cells is thought to be involved in the antitumor effects of the drug (see review Roberts, 1981; Lippard, 1982;

Macquet et al., 1984). In DNA the bases, preferentially guanine, are now accepted to be the target of cisplatin, leading to formation of monofunctional as well as bifunctional Pt-DNA-adducts. It is not known which DNA-adducts are responsible for the biological effects of cisplatin and related compounds, but the bifunctional Pt-DNA-adducts are considered the most likely candidates (Hacker et al, 1984).

The aim of this study was to determine the molecular and cellular effects of 10, carefully selected, platinum compounds (Pt(II) and Pt(IV), cis- and trans-compounds, variation in the A and X groups). Although the selected compounds all react with DNA in vitro, the nature and extent of their reactivity was expected to be different (van Kralingen, 1979). Furthermore, on the basis of antitumor tests in vivo it was found that cytostatic activities could only be observed among cis-Pt-compounds (van Kralingen, 1979). In this paper the molecular and cellular effects of the 10 compounds studied in Chinese hamster ovary (CHO) cells are reported. The tests performed were: de-

Table I Platinum compounds used

Pt-compounds:		A-ligand: N coordinated to Pt	abbreviation:
cis-Pt(II)	cis-diamminedichloroplatinum(II)	NH_3	cII
	cis-dichloro(1,2-diaminoethane)platinum(II)	RNH_2	$Pt(en)Cl_2$
	cis-malonato(2,2-dimethyl-1,3-diaminopropane) platinum(II)	RNH_2	Pt(DMDAP)mal
cis-Pt(IV)	cis-diamminetetrachloroplatinum(IV)	NH_3	cIV
	cis-tetrachloro(2,2-dimethyl-1,3-diaminopropane)platinum(IV)	RNH_2	$Pt(DMDAP)Cl_4$
cis-Pt(II) with ring ligands	cis-dichloro(1,2-diaminocyclohexane)platinum(II)	RNH_2	$Pt(DACH)Cl_2$
	cis-dichlorodi(aminocyclohexane)platinum(II)	RNH_2	$Pt(ACH)_2Cl_2$
	cis-dichlorobis(pyridine)platinum(II)	RN	$Pt(py)_2Cl_2$
trans-compounds	trans-diamminedichloroplatinum(II)	NH_3	tII
	trans-diamminetetrachloroplatinum(IV)	NH_3	tIV

Platinum compounds tested in this study. Indicated is the presence or absence of a proton at the N coordinated to Pt in the non-leaving (A) ligands. Abbreviations given are used in the text.

termination of cytotoxicity and mutagenicity, inhibition of semi-conservative DNA synthesis, induction of DNA repair synthesis, binding of platinum to DNA, induction of single- and double-strand DNA breaks and alkali labile sites.

Materials and Methods.

Compounds

The compounds used are listed in Table I. They were synthesized in the department of Chemistry (State University Leiden, The Netherlands). The solubility of the compounds decreased with increasing bulkyness of the (A) ligands. $Pt(ACH)_2Cl_2$, however, can only be dissolved under extreme conditions. Therefore, and to prevent the introduction of variation, stock solutions of each compound were prepared in dimethyl sulfoxide (DMSO), 0.5 - 1.0 hr before treatment. Dilutions were made in Ham's F10 medium supplemented with 7.5% NCS, final concentration DMSO < 1%.

Cell culture

Chinese hamster ovary (CHO) cells (10^6-10^7) were cultured in monolayers in flasks (75-cm^2 or 150-cm^2, Costar, Cambridge, Mass.) in Ham's F-10 medium (Flow laboratories, Cambridge, Mass.) without thymidine, supplemented with 15% new-born calf serum (NCS; Flow), 1 mM L-glutamine (BDH, Poole, UK), penicillin (100 U/ml, Gist-Brocades, NV, Delft, The Netherlands) and streptomycin (100 µg/ml, Gist-Brocades), at 37°C in a 5% CO_2 incubator (Heraeus, Hanau, Fed. Rep. Germany). The cells were subcultured by trypsinization every 2-3 days and checked periodically for possible mycoplasma contamination (Peden, 1975). All handlings were performed in a laminar down-flow system (Microflow Path Finder, Ltd, Fleet, UK) under yellow light (filtered T.L. light; Philips, Eindhoven, NL; wavelength > 525 nm).

Cell treatment

Cells were trypsinized in the culture flasks, counted and serially diluted in medium (15% NCS). Following this, the cells were given

radioactive DNA precursors or handled as described for the various tests. The cells were treated with platinum in monolayer cultures as follows: the medium was removed, platinum-containing medium (F10, 7.5% NCS, 37°C) was added and the cells were incubated in the dark for 1 hr at 37°C. Thereafter, the platinum-containing medium was removed and the cells were washed twice with PBS (phosphate buffered saline; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl).

Cell survival

Cells were seeded in 6-cm Petri dishes (Greiner) at a density of 500-10000 cells per plate in 4 ml medium. At 4 hr after seeding, they were incubated for 1 hr at 37°C with platinum-containing medium and handled as described earlier (Plooy et al., 1984a = Chapter 3). All survival experiments were performed at least in triplicate with 2 dishes for each dose applied. The B_0 and D_0 values (B_0 = dose at the intersection of the linear part of the curve with the line y = 100%; D_0 = dose increment in the linear part of the survival curve reducing survival to 37% of the original value) were calculated from the regression lines of the survival curve. The D_0 is given $\pm$ s.d. D_{10} values were derived from the regression lines at y = 10%.

Platinum determinations

For the determination of the amount of platinum bound to the DNA, 10^8 cells (10 times 10^7 cells in 14-cm petridishes, Greiner) were treated with the D_{10} dose of the platinum compounds. The cells were trypsinized and pelleted. The DNA of the cells was isolated according to the method of Kirby and Cooke (1967) and dissolved in 0.01 M phosphate buffer pH 7.4. The DNA concentration was measured spectrophotometrically (1 mg/ml has a A_{260nm} of 20) and the amount of platinum in the DNA sample was measured with a Perkin-Elmer atomic absorption spectrophotometer (model 4000), equipped with a HGA 500 graphite furnace and an AS 40 automatic sampler. K_2PtCl_6 solutions, supplemented with heat denatured salmon sperm DNA (500 µg/ml, Millipore corp., Freehold, N.J., USA) were used for calibration. The amount of platinum atoms bound per cell was calculated, assuming that there are 1.8 x 10^{10} nucleotides per cell.

Mutagenicity

The induction of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) mutants, resulting in 6-thioguanine resistency, was determined as described elsewhere (Plooy et al., 1984a = Chapter 3). Briefly, 1 to 5 times 10^6 cells were seeded in 150-cm^2 culture flasks, after 4 hr they were treated as described above. Subsequently, the cells were cultured for 10 days in F-10 medium without hypoxanthine (subcultured every 2 to 3 days) and mutant selection was carried out by plating 10^6 cells (in ten 9-cm Petri dishes) in medium without hypoxanthine, containing 5 µg 6-thioguanine per ml. The plating efficiency of these cells was determined simultaneously. After 7 days of culturing (37°C) the cells were stained and counted, the mutation induction was expressed per 10^6 surviving cells. Cell survival was determined simultaneously with each mutation induction experiment. The mutagenicity of the compounds was derived from a graph obtained by plotting the amounts of induced mutations as a function of log(%survival). From the regression lines fitted to the data, the amount of induced mutants per 10^6 surviving cells (± s.d.) at 10% survival was computed; this value was taken as a measure for the mutagenicity.

DNA synthesis inhibition

Cells (10^5) were seeded in a 3-cm Petri dish with the addition of 2 ml F-10 medium (15% NCS). After 16 hr, 1 ml of 0.04 µCi/ml [^{14}C]-thymidine (^{14}C-TdR; Amersham, UK; spec. act. 56.5 Ci/mol) in medium (15% NCS) was added and the cells were incubated for 8 hr. Thereafter the cells were washed twice with PBS, fresh medium was added and they were incubated for 16 hr. Subsequently, the cells were treated for 1 hr with platinum-containing medium, washed twice with PBS and incubated for 0, 1, 2, 3 and 24 hr in fresh medium followed by a 10-min pulse-labeling with [^{3}H]-thymidine (^{3}H-TdR; Amersham, UK; 1 µCi/ml; spec. act. 19 Ci/mmol) at 37°C. Then the cells were placed on ice, washed twice with ice-cold PBS, and 1 ml of (ice-cold) PBS was added. Finally, the cells were scraped off with a rubber policeman, suspended, transferred to a test tube and 2 ml of 10% trichloroacetic acid (TCA) in 0.01 M sodium pyrophosphate was added. After 0.5 hr at 0°C,

the cell suspension was filtered through a GFC (Whatman) filter and washed once with 10% ice-cold TCA and twice with 96% ethanol (ice-cold). The filters were dried in counting vials for 1.5 hr at 80°C and supplemented with 0.6 ml Soluene (Packard; 0.5M NH_4OH in toluene). Then they were shaken for 1 hr at 20°C and, after the addition of 14 ml of toluene (containing PPO and POPOP), counted in a liquid scintillation counter (Searle, Mark III). The ratio of $^3H/^{14}C$ was calculated and taken as a measure of the DNA synthesis. The values obtained for the treated cells relative to the untreated control value were plotted as a function of the dose of the drug.

Unscheduled DNA synthesis (UDS)

Two coverslips (1 x 1 cm) were aseptically placed in 6-cm Petri dishes. Afterwards 10^5 cells were allowed to adhere to the coverslips by incubation at 37°C in a 5% CO_2-incubator for 24 hr. The medium was replaced by fresh medium containing 15% NCS and 10 µCi ^{3}H-TdR per ml and the incubation was continued for 2 hr. Then, the radioactive medium was removed, the cells were washed twice (PBS), followed by a 1-hr treatment with the platinum compound (at 37°C). The cells were washed again (2 x PBS) and 3 ml ^{3}H-TdR containing medium (10 µCi/ml) was added. After 3 hr the radioactive medium was disgarded and the cells were washed twice with PBS and fixed with Bouin (750 ml picrinic acid (1.4 g/100 ml), 250 ml formaldehyde (35%) and 50 ml acetic acid) for 30 min. The coverslips were taken from the dishes, washed with deionized water, dried for 24 hr and mounted (with the cells at the free surface) on glass slides. They were dried for 24 hr, coated with Kodak NTB2 photographic emulsion at 33°C in the dark and stored at 4°C in light-proof boxes in the dark for 4 days. After the exposure time, the autoradiographs were developed at 16°C for 4 min in Kodak D 19, fixed for 10 min in Acidofix (Agfa-Gevaert) and stained with 0.02% toluidine blue in 0.1 M citric acid pH 5.0. Subsequently, another coverslip was mounted with malinol on top of the slides and dried. Autoradiographic slides were analysed with the use of a Zeiss microscope connected with an Artek model 880 automatic grain counter. Routinely, 25 nuclei per slide were counted.

Single-strand breaks (SSB) and alkali labile sites (ALS)

Cells (10^5) were seeded in a 6-cm petridish and prelabeled overnight with ^{3}H-TdR. Following treatment with the platinum compound for 1 hr, the cells were washed twice with PBS at 0°C, trypsinized (0.5 ml of 0.25% trypsine, 0.01 M EDTA) and suspended in the presence of 1 mg BSA (bovine serum albumine). From this cell suspension 50 µl was lysed for 1 hr at 20°C on top of an alkaline sucrose gradient (5-20% sucrose w/v, 2 M NaCl, 0.33 M NaOH, 0.01 M EDTA) between two layers of 75 µl lysis solution (1 M NaOH, 0.01 M EDTA) in the buckets of a SW60Ti rotor (Beckman). They were centrifuged for 210 min at 17000 rpm (Beckman L8-70 ultracentrifuge, 20°C). The gradients were fractionated (8 drops per fraction) on paperstrips. The DNA on the strips was fixed with 5% TCA (in 0.01 M sodium pyrophosphate) and subsequently washed twice in 96% ethanol, dried and the radioactivity counted in toluene containing PPO and POPOP. The number of breaks per unit length of DNA (10^9 dalton) was calculated from the sedimentation profiles according to Wade and Lohman (1980). Control value: 2.0 - 4.6 breaks/10^9 dalton DNA; γ-rays (100 Gy, at 0°C) induced 40.7 - 46.8 breaks/10^9 dalton DNA. For the determination of alkali labile sites, the cells were lysed on top of the alkaline sucrose gradient for 17 hr at 20°C ($\pm$ 0.5°C) and centrifuged for 155 min at 40000 rpm at 20 $\pm$ 1 °C. Then 16.6 - 21.7 breaks per 10^9 dalton DNA were detected in untreated cells, whereas ENNG (N-ethyl-N'-nitro-N-nitrosoguanidine, a positive control) induced 90.8 - 99.8 breaks/10^9 dalton DNA after a dose of 75 µM (for 1 hr, at 37°C).

Double strand DNA breaks (DSB)

Cells (10^5) were labelled as for the SSB determinations, treated with the platinum compound for 1 hr at 37°C and the number of DSB was measured by neutral filter elution according to the method described by van der Schans et al., 1983.

Results

Cytotoxicity of platinum compounds

The survival curves obtained after treatment of CHO cells with the various platinum compounds, show the normal exponential (see Chapter 1) dependence on dose, with a more or less pronounced shoulder

Table II Cytotoxicity and mutagenicity in CHO cells, antitumor activity reported in the literature and induction of DNA lesion by platinum compounds

compound:	adducts per cell ($\times 10^{-5}$)	B_0 (μM)	D_0 (μM)	induced mutations per 10^6 cells (D_{10} dose)	antitumor activity					
					L1210			ADJ/PC6		
					LD_0	ID_{50}	T/C	LD_{50}	ID_{90}	T.I.
cII	14	3.3	9.4 ± 0.6	122 ± 15	30	2.3	205	13.0	1.6	8.1
$Pt(en)Cl_2$	6	2.7	37.4 ± 3.8	96 ± 8	25	6.2	132	22.5	10	2.25
Pt(DMDAP)mal	86	29.8	93.3 ± 9.0	64 ± 6	25	6.3	149		nd	
cIV	4	4.7	9.3 ± 0.6	97 ± 13	43	1.2	152		nd	
$Pt(DMDAP)Cl_4$	25	2.1	7.6 ± 0.3	113 ± 11	6.3	1.6	186		nd	
$Pt(DACH)Cl_2$	30	9.8	41.8 ± 2.0	99 ± 11	18	0.13	177	14.5	20.5	6.9
$Pt(ACH)_2Cl_2$	152	0	468 ± 27.7	8 + 4		inactive		>3200	12	>267
$Pt(py)_2Cl_2$	33	16.2	38.5 ± 2.4	17 ± 6		inactive			inactive	
tII	60	240	250 ± 19.3	16 ± 3	167	67	116	27	>27	<10
tIV	100	28.8	115 ± 10	20 ± 3	216	54	121		inactive	

Platinum binding to DNA, cytotoxicity (B_0 and D_0), mutagenicity in CHO cells and antitumor activity reported in the literature. Pt-DNA-binding studies were performed as described in Methods, and calculated per cell (a nucleotide content of 1.8×10^{10} was assumed). The survival studies in CHO cells were performed at least 3 times with 2 dishes per dose applied, at 7 different doses down to about 1% survival. Both B_0 and D_0 values (μM) are calculated from regression lines of the obtained survival data, beyond the shoulder region (D_0 ± s.d.). The mutagenicity by treatment of the cells with the platinum compounds was determined by measuring the induction of 6-thioguanine resistancy (HGPRT mutants). The mutation studies were performed at least 3 times. The amount of mutants per 10^6 surviving cells (± s.d.), after dosages resulting in 10% survival (D_{10} dose), were calculated from the regression lines of curves in which the mutant frequencies had been plotted as function of the survival of the cells. Antitumor data for the ADJ/PC6 tumors in Balb/c mice were taken from Connors et al. (1972) and from test reports of Johnson Matthey research Centre (UK); the data for L1210 tumors in Swiss or CD_2F_1 mice were taken from Macquet and Butour (1983) and test reports from the National Institute of Health (USA). LD_{50}: dose needed to kill 50% of the animals; LD_0: maximal tolerable dose at which no animals are killed; ID_n: dose at which n% of the tumor is inhibited; T.I.: LD_{50}/ID_{90}, measure of the selectivity of the drug; T/C: ratio of tested over control survival as percentage; nd = not detected.

(data not shown). The survival curve obtained with $Pt(ACH)_2Cl_2$, however, was completely exponential. From the linear part of each graph in the plot of log(%survival) versus dose, the best fitting straight line was determined by regression analysis. The survival curves were characterized by the B_0 and D_0 values presented in Table II (B_0 = dose at the intersection of the regression line with the line y = 100 %; D_0 = dose increment reducing survival to 37% of the original value). The presence of a shoulder is considered to be indicative for the induction of some kind of potential lethal damage (see Chapter 1) that is repaired before progression through the cell cycle starts again, $Pt(ACH)_2Cl_2$, however, does not seem to induce such damage. Both, B_0 and D_0 values show large variations for the different compounds. Roughly speaking, a high value of B_0 coincides with a high D_0, with $Pt(ACH)_2Cl_2$ as the obvious exception. All cis-compounds are exceedingly more toxic for CHO cells than trans-compounds, except again the cis-compound $Pt(ACH)_2Cl_2$. Compounds with similar chemical ligands, but differing in the valence state of the Pt-atom (II or IV) are either equally cytotoxic to CHO cells (cII and cIV) or the Pt(IV)-compound is more toxic and less capable of repairing sub-lethal damage than the Pt(II)-compounds (Pt(DMDAP)mal versus $Pt(DMDAP)Cl_4$; tII versus tIV; see Table II).

The induction of mutations at the HGPRT-locus

The induction of mutations at the HGPRT gene in CHO cells treated with platinum compound was measured; cells mutated in this gene will become resistent to the base-analogue 6-thioguanine (Hsie, 1981). Table II shows the mutation induction in CHO cells at equitoxic dosages (D_{10} = 10% survival) for the various platinum compounds. Two cis-compounds, $Pt(ACH)_2Cl_2$ and $Pt(py)_2Cl_2$, and the 2 trans-compounds, tII and tIV, which are hardly toxic, are not significantly mutagenic either. The mutagenicity of the other cis-compounds, at the equitoxic dosages used, is of the same order of magnitude. The least mutagenic of these cis-compounds - Pt(DMDAP)mal - is approximately 10-fold less toxic than the strongest mutagenic cis-compound - cII. Consequently, cII induces 20 times more mutants than Pt(DMDAP)mal when compared at equal molar concentrations.

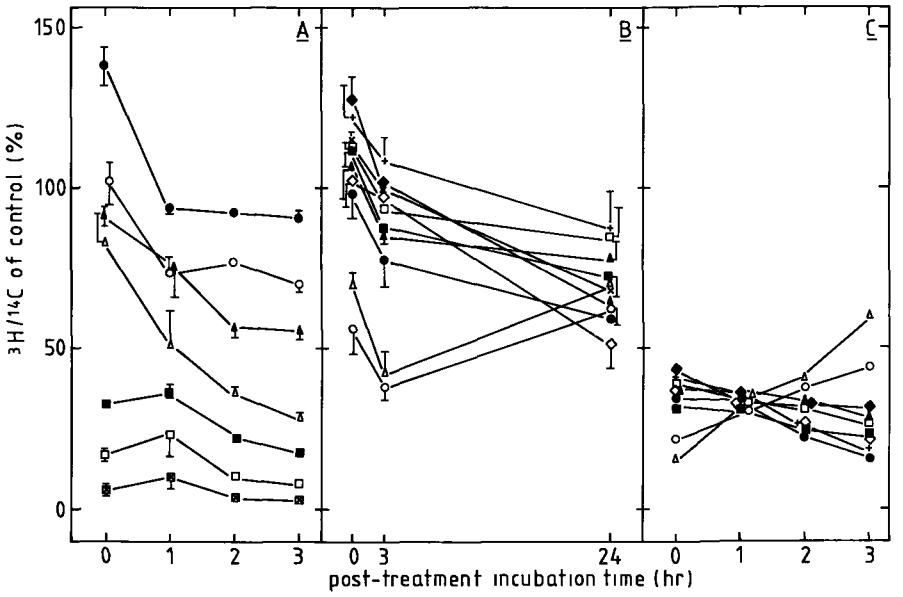

Figure 1: Inhibition of the DNA synthesis by the platinum compounds. In Fig. 1A the DNA synthesis inhibition after treatment of the cells with various doses of cII is given. ● 3.3 μM; ○ 17 μM; ▲ 33 μM; △ 83 μM; ■ 165 μM; □ 330 μM; ▣ 660 μM. The amount of $^{3}H/^{14}C$ (dpm/dpm) as percentage of that of the control (control = 100%) is plotted as function of the post-treatment incubation period of the cells, t = 0 represents the moment directly after the 1 hr treatment of the cells. The vertical bars represent s.d. of three individual dishes tested. In Fig. 1B, the DNA synthesis inhibition after cell treatment with platinum compounds resulting in 30-70% cell survival is plotted. The pulse-labelling was performed directly after the treatment of the cells (t = 0), or after a repair period of the cells (3 and 24 hr, respectively). The cells were treated with approximately equitoxic doses of the drugs, mean values of 2 independent experiments in which each point was in triplicate, are given ± s.d. In Fig. 1C, the DNA synthesis inhibition is plotted resulting in approximately the same initial (t = 0) inhibition.

symbol	abbrev.	dose (μM) (Fig. 1B)	%surv.	dose (μM) (Fig. 1C)
●	cII	8	65	165
▲	cIV	8	70	135
+	$Pt(DMDAP)Cl_4$	8	55	115
■	$Pt(en)Cl_2$	33	43	300
◆	$Pt(DACH)Cl_2$	37	54	260
□	$Pt(py)_2Cl_2$	50	32	130
◇	Pt(DMDAP)mal	64	30	500
△	tIV	125	50	350
○	tII	275	70	1000
×	$Pt(ACH)_2Cl_2$	625	30	1200

Inhibition of semi-conservative DNA synthesis

The presence of Pt-DNA-adducts in the cell may inhibit the DNA replication, which could result in blockage of the cell cycle. In Fig. 1A the DNA synthesis inhibition in cells treated with different dosages of cII are plotted, indicating that the inhibition of the DNA synthesis induced by cisplatin is dose-dependent. At very low dosages, an initial enhancement of the DNA synthesis by this compound (Fig. 1A and 1B) is observed. This can be explained by a (partial) synchronization of the cell population during the 1 hr treatment with cII. This synchronization becomes effective at the moment the drug is removed from the cells, resulting in a stimulation of the incorporation of DNA precursors, because more cells enter the S-phase (phase in which the DNA is replicated).

All 10 platinum compounds inhibit the semi-conservative DNA synthesis (Fig. 1B and 1C), although the kinetics of inhibition seem to be different for the two trans-compounds in comparison to the cis-compounds. After treatment with a low dose (Fig. 1B; 30-70% survival), the trans-compounds show a significantly stronger initial inhibition which increases during the first 3 hr. Thereafter, no further increase seems to occur, in contrast to the inhibition by the cis-compounds which continues to increase after that period. When treatments are compared which result in approximately the same inhibition 1 hr after the removal of the drug (Fig. 1C), again the trans-compounds show different kinetics. Their inhibition tends to decrease in the first 3 hr after treatment, whereas that of the cis-compounds tends to increase. It might, therefore, be suggested that the trans-induced lesions are more easily circumvented by the DNA replication machinery, or that they are repaired more quickly.

The induction and repair of DNA damage

- Unscheduled DNA synthesis

To get an indication of the repair of the Pt-DNA-adducts in CHO cells, unscheduled DNA synthesis (UDS) was measured. It appeared that all the platinum compounds induced only marginal amounts of UDS, even

although high, toxic dosages of the compounds were used (Table III). This is in agreement with findings described for cII in human cells (Plooy et al., 1984d = Chapter 6). Ultraviolet light (UV - 254 nm), in contrast to platinum compounds, induced a high level of UDS at sub-toxic dosages (Table II; see also Lohman et al., 1984). The amount of lesions present per cell after a D_{10} dose treatment with the platinum compounds (Table II) or after a D_{10} dose of UV (pyrimidine dimers - data taken from Zelle, 1980) is of the same order of magnitude. The

Table III *Unscheduled DNA synthesis in CHO cells after treatment with Pt-compounds or UV (254 nm)*

compound	UDS		adducts per cell ($\times 10^{-5}$)	D_{10} (μM)
	$m-m_0$	m/m_0		
cII	16 ± 5.7	3.3 ± 1.6	14	29.1 ± 1.7
$Pt(en)Cl_2$	21 ± 10	2.1 ± 0.5	6	90.3 ± 6.5
Pt(DMDAP)mal	11 ± 8.1	1.6 ± 0.4	86	245.8 ± 23.7
cIV	14 ± 5.5	3.0 ± 1.4	4	24.2 ± 1.5
$Pt(DMDAP)Cl_4$	22 ± 9.1	2.1 ± 0.5	25	19.7 ± 0.9
$Pt(DACH)Cl_2$	10 ± 6.4	3.5 ± 2.5	30	106.7 ± 5.2
$Pt(ACH)_2Cl_2$	5 ± 4.5	2.3 ± 1.6	152	1083.4 ± 64.2
$Pt(py)_2Cl_2$	17 ± 9.1	1.9 ± 0.2	33	105.3 ± 6.5
tII	8 ± 3.8	3.7 ± 2.6	60	827.7 ± 64.0
tIV	8.5 ± 5.0	3.8 ± 2.9	100	296.4 ± 25.6
UV (254 nm)	48 ± 5.4	7.0 ± 1.6	17[1)]	28.4[1)]

Unscheduled DNA synthesis at variable dosages and amount of platinum atoms bound to the DNA per cell after treatment of the cells with a D_{10} of the platinum compound or UV (254 nm). The UDS values are derived from treatment of the cells with 300-400 mM platinum. UDS experiments were performed by autoradiography and 25 nuclei were counted. The background grain countings were not identical in all experiments, therefore, the relative ($m-m_0$ = grains per nucleus treated minus grains per control nucleus) amount of UDS is given ± s.d. and the ratio of the values over the control (m/m_0) ± s.d. Formulas used $A - B \pm \sqrt{a^2 + b^2}$, where a and b are s.d.'s and $A/B\ (1 \pm \sqrt{a^2/A^2 + b^2/B^2})$. UDS data obtained after UV-irradiation were taken from Lohman et al. (1984), the amount of UV-induced pyrimidine dimers were taken from Zelle (1980) and survival experiments were performed by Plooy (unpublished). The amount of Pt-DNA-adducts per cell is calculated after D_{10} dose treatment, assuming a nucleotide content of 1.8×10^{10} per cell. 1) 28.4 sec UV 254 nm (dose rate 0.39 J/m²) = D_{10} dose, resulting in 17 ($\times 10^5$) pyrimidine dimers per cell.

amount of UDS per lesion after UV is, therefore, much higher than that after treatment with platinum compounds, if present at all. This suggests that, if excision repair is involved in the removal of platinum-induced damage, the repair patches obtained will either be very small or only a small fraction of the lesion is repaired that way.

- DNA breaks and alkali labile sites

Many genotoxic agents can induce DNA breaks (both single- and double strand) and DNA alkali labile sites (ALS). The induction and repair of such lesions can be measured with the help of alkaline sucrose gradient centrifugation and filter-elution methods (Wade and Lohman, 1980; van der Schans et al., 1983).

None of the platinum compounds used in this study, however, induced DNA breaks in the DNA of CHO cells. In time-course experiments with cells treated with cII, cIV, tII and tIV, upto 48 hr after the treatment, induction of double strand breaks (DSB) could not be demonstrated (with neutral filter elution); only between 48 and 96 hr, a few DSB were found in DNA from cells treated with cII or cIV (data not shown).

In an earlier paper, preliminary results indicating the induction of ALS by platinum compounds, especially tIV, were descibed (Plooy and Lohman, 1980). ALS are defined as DNA lesions that result in strand breaks when the DNA is kept in alkaline conditions. This conversion of ALS into breaks is strongly dependent on the temperature during the treatment with alkali. In this study we repeated the ALS induction experiments, using more stringent conditions and a carefully controlled temperature ($20 \pm 0.5\ ^{0}C$) during alkaline treatment. To check the reliability and reproducibility of these conditions, a positive control was induced in the experiments, i.e., the induction of ALS by N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG). The amount of ALS induced by ENNG was easily detectable (4 to 5 times the background); the results were comparable to those published by Abondandolo et al. (1982), therefore, the assay was suitable for detecting ALS. However, even extremely high dosages of tIV, the strongest inducer of ALS in the preliminary expe-

riments, did not induce a significant increase in the amount of breaks in comparison to the untreated control (3600 µM tIV, 21.3 - 27.0 breaks/10^9 dalton DNA; control: 16.6 - 21.7 breaks/ 10^9). We have to conclude that the platinum compounds used do not induce ALS.

- Covalent binding of Pt-compounds to the DNA of CHO cells

As shown in Table II, all cis-compounds, when used at D_{10} dosages, induce Pt-DNA-adducts in CHO cells. The amounts, however, show strong variation (about 40 x), with the highest values for Pt(DMDAP)-mal and $Pt(ACH)_2Cl_2$. For cis-compounds with similar ligands it holds that directly after treatment at equitoxic dose less Pt(IV) compounds are bound to DNA than Pt(II)-compounds. The two trans-compounds also appeared strong inducers. In contrast to the cis-compounds, here the Pt(II) compound induced less Pt-DNA-adducts than the Pt(IV) analogue. In general, the level of Pt-DNA-binding is correlated neither with the cytotoxicity, nor with the mutagenicity of the compounds, suggesting that it is not the quantity of Pt-DNA-adducts which is the main parameter for the biological effects of the compounds in the cells, but rather the nature of the induced Pt-DNA-adducts.

The presence of Pt-DNA-adducts, however, shows that both cis- and trans-compounds do react with the DNA in CHO cells and that, therefore, the lower toxicity and non-mutagenicity of trans-compounds and $Pt(ACH)_2Cl_2$ cannot be attributed to an absence of DNA damage.

Discussion

By comparison of the genotoxic, biological and cytostatic properties of various platinum coordination compounds it was hoped to get more insight in the working mechanism of the antitumor platinum compounds. The compounds used in this study (Table I) were carefully selected in order to study (i) the effect of the steric composition of the ligands around the Pt atom (cis- versus trans-compounds), (ii) the influence of the valence state of the Pt-atom (cII - cIV,

Pt(DMDAP)mal - $Pt(DMDAP)Cl_4$, tII - tIV) and (iii) the effect of particular (cyclic) ligands, suspected to play a role in the antitumor activity. Compounds with cyclic ligands were chosen because in animal studies, evidence was found that the absence of a proton at the amine coordinated to platinum (e.g. $Pt(py)_2Cl_2$; see Table I) would have drastical influence on the antitumor activity. In Table II the compounds are listed according to their chemical properties. In this Table the effects measured in CHO cells are summarized and compared to their antitumor activity as described in the literature for two experimental tumors. These data illustrate that the trans-compounds have no cytostatic action. Furthermore it shows that the cytostatic properties of Pt(II) and Pt(IV) compounds with common ligands are of the same order of magnitude and that indeed special ligands strongly influence the cytostatic properties. Among the compounds with cyclic ligands, $Pt(py)_2Cl_2$ which misses a proton at the N coordinated to Pt, is non-cytostatic; whereas the compounds with the saturated cyclic ligands are active, $Pt(DACH)Cl_2$, in both tumor systems and $Pt(ACH)_2Cl_2$ only against the ADJ/PC6 tumor.

When the cytostatic proporties of the ten platinum compounds were compared with their mutagenicity in the HGPRT system, it appears that, all cis-compounds, except $Pt(ACH)_2Cl_2$ (see below), are both mutagenic in CHO cells and cytostatic in vivo; the two trans-compounds are non-mutagenic in CHO cells as well as non-active in vivo. Moreover, both the spread in the mutagenicity at equitoxic dosages in CHO cells and the inhibitory properties in vivo (T.I. and T/C) of the various cis-compounds does not differ dramatically.

The induction of mutations in Salmonella typhimurium (Mattern et al., 1982) and the induction of prophage λ in Escherichia coli (van Kralingen, 1979) by the various platinum compounds (see Table IV) correlate very well with the mutagenicity in CHO cells. The effects in both systems were found to have a similar relationship with the cytostatic proporties in vivo as was detected in case of the mutagenicity in CHO cells (Table II and Table IV). The induction of mutations (base-pair substitutions) in the lacI gene in E.coli (Brouwer et al., 1981) seems to be comparable to the mutagenicity in CHO cells only for

cII, but not for the other compounds tested. The data obtained with the Ames-test, λ-prophage induction in E.coli and the mutagenicity at the lac gene in E. coli suggest that, at least in bacteria, some kind of SOS-type (error-prone) repair processes are involved in the mutagenicity of the cis-compounds. Nothing is known yet about possible error-prone repair pathways in CHO cells or cells of mice in vivo, although it may be of interest to look for such processes when methods become available to study the repair of individual Pt-DNA-lesions (Plooy et al., 1984b = Chapter 5).

The cytotoxicity of the compounds in CHO cells (D_0) does not correspond with the cytotoxicity, as far as measured, of the compounds in vivo (LD_{50}, LD_0 - Table II). However, it holds that: (i) cis-com-

Table IV Mutagenicity in CHO cells and Salmonella typhimurium and λ-prophage induction in Escherichia coli

compound	CHO cells	S.typhimurium uvr$^+$		E.coli
	HGPRT mutants	fr	bp	λ-prophage
cII	122 ± 15	13.0	28.1	+
$Pt(en)Cl_2$	96 ± 8	10.8	8.1	+
Pt(DMDAP)mal	64 ± 6	10.1	7.4	+
cIV	97 ± 13	10.2	10.1	+
$Pt(DMDAP)Cl_4$	113 ± 11	6.0	5.7	+
$Pt(DACH)Cl_2$	99 ± 11	14.7	20.1	+
$Pt(ACH)_2Cl_2$	8 ± 4	2.7	1.4	nd
$Pt(py)_2Cl_2$	17 ± 6	5.7	2.3	nd
tII	16 ± 3	4.0	7.0	-
tIV	20 ± 3	1.6	2.9	-

Comparison of the mutagenicity of ten platinum compounds in CHO cells and Salmonella typhimurium and λ-prophage induction in Escherichia coli. Mutagenicity in CHO cells is taken from Table II to facilitate the comparison. The λ-prophage induction data are taken from van Kralingen (1979). The mutagenicity in S.typhimurium determined as his$^+$ revertants (Ames-test) in two different strains are taken from Mattern et al. (1982). The ratio of the maximal values over the control values (n/n_0) are given for the doses at which the amount of cells is starting to decline (D_{max}). The abbreviations fr and bp mean: frameshift mutations and base-pair substitutions strain, respectively.

pounds are considerable more toxic than trans-compounds in CHO cells and in vivo (at least in the L1210 system) and (ii) the low toxicity of the compound $Pt(ACH)_2Cl_2$ in CHO cells is also found in vivo (Table II). This compound is rather insoluble in water, however, and this might have influenced the data. In general it should be stated that the cytotoxicity found in CHO cells is neither a good measure for the cytotoxicity of a Pt-compound in vivo nor for its cytostatic properties.

The possibility that the non-cytotoxic and non-mutagenic action of some of the platinum compounds could be attributed to the fact that they do not reach the DNA in cells or do not react with DNA, was investigated. The compounds did not induce lesions such as breaks or alkaline labile sites in the DNA, but, all were found to inhibit DNA synthesis (Fig. 1), suggesting that reaction with DNA in the cells occurs. The extent of the inhibition of the DNA synthesis seems to be dependent also on the nature of the Pt-DNA-lesions, since at equitoxic dosages the kinetics of the inhibition after treatment with trans-compounds was different from that observed with cis-compounds. The trans-induced lesions might be more easily circumvented by the DNA replication machinery or might be faster repaired. Two DNA adducts induced by the trans-compounds, the DNA-protein and DNA interstrand cross-links, are indeed repaired faster than the cis-induced ones (Plooy et al., 1984a = Chapter 3). Furthermore, all platinum compounds became covalently bound to the DNA of CHO cells (Table II). The level of Pt-DNA-binding of the various platinum compounds could not be correlated with their cytotoxic or mutagenic properties. At the D_{10} dose level, the highly cytotoxic compounds induced lower amounts of Pt-DNA-adducts than the less toxic ones, indicating that specific noxious lesions are induced by the former compounds.

Roberts and co-workers (Roberts et al., 1982) found in liquid holding experiments (repair of DNA lesions stimulated by stopping the progression of the cells through the cell cycle), that this treatment makes CHO cells less sensitive to treatment with cII. Evidently, repair of Pt-DNA-adducts takes place. However, when excison repair was measured in CHO cells (Table III), only a marginal amount of repair synthesis was found, even after high-dose treatment. These results are

in agreement with those obtained in human cells (Plooy et al., 1984d = Chapter 6). This does not mean that excision repair does not take place in these cells, Pt-DNA-lesions might be repaired by a mechanism which induces very small repair patches. Because small-patch repair is thought to be predominantly error-free, the major part of the Pt-DNA-damage will be removed without introducing errors, if this explanation is correct.

Since the total amount of Pt-DNA-lesions does not correspond with the cytotoxicity nor with the mutagenicity in CHO cells (Table II), some specific Pt-DNA-adducts induced by the compounds might be responsible for the observed biological effects. It is thought that the toxic, mutagenic and antitumor active cis-compounds induce other Pt-DNA-adducts than the trans-compounds, in particular bifunctional adducts. Not only the spectrum of the lesions induced may differ, also the repair of the adducts may be different (Plooy et al., 1984a = Chapter 3, 1984c = Chapter 5). Furthermore, particular Pt-ligands might invoke a typical binding to DNA resulting in lesions which are crucial for the antitumor activity.

The results obtained in this study with the compound $Pt(ACH)_2Cl_2$ could be an example of the specificity of induced Pt-DNA-adducts. This compound is non-toxic and non-mutagenic in CHO cells, but is a good inducer of Pt-DNA-adducts and a strong antitumor agent, be it only in the ADJ/PC6 system. $Pt(ACH)_2Cl_2$ is also the only compound that gives a shoulderness survival curve (B_0 = 0; Table II) in CHO cells, indicating there is no repair of certain potentially lethal damages at low dosages. Then, an irrepairable minor lesion induced by this compound in the DNA of mammalian cells should be responsible for the killing; it is tempting, though speculative, to assume that this lesion might also be the one that shows the effect in the ADJ/PC6 system. It remains to be explained, however, why this lesion does not have the same effects in the L1210 system.

In conclusion, it can be stated that the CHO in vitro system cannot be used to discriminate between the biological effects of cis-Pt(II) and Pt(IV) compounds. The system is capable of showing differential biological effects of platinum compounds with various ligands. The effect of the pyridine group ($Pt(py)_2Cl_2$), missing a hydrogen at

the N coordinated to Pt, can especially be demonstrated and the effects in CHO cells correspond well with those observed in vivo. As a test system for cytostatic properties of platinum compounds, only the mutagenicity studies in CHO cells seem to be advisable. Comparison with bacterial test systems does suggest that also in mammalian cells error-prone repair systems may be of importance for the action of the platinum compounds. However, these repair processes will most likely not affect all type of induced DNA lesions, but rather specific ones.

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3. INDUCTION AND REPAIR OF DNA CROSS-LINKS IN CHINESE HAMSTER OVARY CELLS TREATED WITH VARIOUS PLATINUM COORDINATION COMPOUNDS IN RELATION TO PLATINUM BINDING TO DNA, CYTOTOXICITY, MUTAGENICITY AND ANTITUMOR ACTIVITY.

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Summary

Several effects of four diamminechloroplatinum compounds (II and IV) in Chinese hamster ovary (CHO) cells were studied. The two cis-compounds (cis-diamminedichloroplatinum(II); cII and cis-diamminetetrachloroplatinum(IV); cIV) are known to possess antitumor activity, whereas the two trans-stereoisomers (trans-diamminedichloroplatinum-(II); tII and trans-diamminetetrachloroplatinum(IV); tIV) are inactive.

When the effects of the cis- and trans-Pt-compounds were compared after treatments that resulted in the binding of equal amounts of platinum to the DNA of the cells, the following differences were found: a) the cis-platinum adducts gave a much higher cytotoxicity; b) only the cis-platinum-DNA complexes were strongly mutagenic (forward mutations at the hypoxanthine-guanine phosphoribosyltransferase locus); c) the cis-platinum adducts induced more sister chromatid exchanges; d) the cis-compounds initially induced fewer DNA-protein cross-links (factors 5 to 8), but these cis-platinum cross-links were much more persistent; e) for both cis-complexes, the amount of DNA interstrand cross-links passed through a maximum between 6 and 12 hr after treatment, and the cross-links were repaired slowly. One trans-compound (trans-diamminetetrachloroplatinum(IV); tIV) resembled the cis-complexes with respect to the overall kinetics of formation and

disappearence of this type of lesion, but the repair went faster. For the other trans-compound (trans-diamminedichloroplatinum(II); tII), the highest number of cross-links was detected directly after the treatment of the cells, and they were rapidly eliminated.

Neither the number of platinum-DNA lesions as such nor the initial amount of DNA interstrand cross-links could be related to the (geno)toxic effects of the compounds. However, as the slow repair of the cis-platinum-induced interstrand and DNA-protein cross-links leads to a certain persistency of these adducts, the unrepaired lesions might be responsible for cytotoxicity, mutagenicity and antitumor activity. This indicates discriminating properties of the repair systems for certain cis- or trans-platinum-DNA adducts. The sister chromatid exchange (SCE) induction seems to be related to the persistent DNA interstrand cross-links.

Introduction

CII has been found to be superior to other available antitumor drugs for the treatment of some types of cancer (Prestayko et al., 1980, part II: Clinical Studies). The stereoisomer of this coordination complex, tII, does not show comparable cytostatic properties. In clinical chemotherapy, cII shows severe side effects (Prestayko et al., 1980); this has stimulated the search for more selective congeners. Specific attention has been paid to the elucidation of the working mechanism of cII at the molecular level (Prestayko et al., 1980; Roberts and Thomson, 1979). For review of the literature on cII and related platinum compounds see the studies of Roberts and Thomson, 1979 and Prestayko et al., 1980 and their cited literature.

Upon entering the cell, the platinum compounds will become hydrolysed (Johnson et al., 1980), and then they can react with DNA as well as with RNA and proteins. DNA, however, is believed to be the principal site of action (Munchhausen and Rahn, 1975). Within DNA, the main targets are the nucleobases, especially guanine (Marzilli, 1977). Only at high exposure levels are adducts of adenine and cytosine

formed with platinum but, so far, no complexes with thymine have been detected (Munchhausen, 1974).

Two types of platinum DNA binding have been found: a) monofunctional; and b) bifunctional. The monofunctional binding is unlikely to be responsible for the observed cytostatic effects, since the trans-compounds are at least as effective as the cis-compounds in forming monofunctional platinum-DNA adducts (Munchhausen and Rahn, 1974). Bifunctional binding results in chelation and various kinds of cross-linking, presumably by a 2-step reaction of which the last step is rate limiting (Marcelis, 1982). The formation in vitro of a chelate with one guanine base by binding of cII at the $N7\text{-}O^6$ positions has been reported (Dehand and Jordanov, 1976), but there are no reports of chelation in vivo.

In mammalian cells, the platinum compounds can form DNA-protein cross-links (Zwelling et al., 1979a), of which the biological importance is not yet known. Intrastrand cross-links are the major type of bifunctional lesions induced by cII in DNA in vitro (Fichtinger-Schepman et al., 1982). They have been detected between 2 adjacent guanines (Fichtinger-Schepman et al., 1982), 2 adjacent adenines, adjacent adenine and cytosine (Roos et al, 1974), and between 2 guanines separated by one (Brouwer et al., 1981; Marcelis, 1982), or more bases (Alix et al., 1981). Also in Escherichia coli, intrastrand cross-links have been found; there, they can lead to base-pair substitution (Brouwer et al., 1981). In mammalian cells, DNA intrastrand cross-links are, therefore, likely to be induced. These lesions might be responsible for antitumor activity, since they are not formed, at least not in vitro, by the non cytostatic trans-compounds (Fichtinger-Schepman et al., 1982; Royer-Pokora et al., 1981). Interstrand DNA cross-links are formed by both cis- and trans-compounds, in vivo as well as in vitro (Zwelling et al., 1979a). In mammalian cells, the cis-compounds show a delayed formation of these cross-links, while the trans-DNA interstrand cross-links are almost immediately formed (Zwelling et al., 1978; 1979). For some other cytostatic agents, e.g., for cyclophosphamides (Fleer and Brendel, 1982), the formation of interstrand cross-links is closely related to the cytotoxicity but, in

the case of the platinum compounds, a clear relationship has not been demonstrated (Pera et al., 1981; Zwelling et al., 1979a).

In the present study, an attempt is made to relate the effects of different platinum compounds to the lesions they form in CHO cells. In particular, the relation between the formation and repair of DNA interstrand and DNA-protein cross-links and the induced genotoxic and cytotoxic effects is investigated. For this purpose, 4 platinum compounds (cII, cIV, tII and tIV), which are known to react differently with DNA in vitro (Van Kralingen, 1979) and in Salmonella typhimurium (Cochiarella et al., 1980; Mattern et al., 1982), have been studied. Previously, we reported (Bonatti et al., 1983; Plooy et al., 1982; Plooy and Lohman, 1980) some other effects of these platinum compounds in CHO cells: a) induction of micronuclei and unscheduled DNA synthesis; b) the inhibition of DNA synthesis and c) the absence of induction of single-strand breaks.

Material and methods

Compounds

The compounds used were: cII, cIV, tII, tIV. The platinum compounds were synthesized and kindly provided by the group of Professor Dr. J.Reedijk (State University Leiden, Netherlands). Solutions of the compounds were freshly prepared in dimethylsulphoxide. Dilutions were made in Ham's F-10 medium (Flow laboratories, Cambridge, MA) supplemented with 7.5% newborn calf serum (Flow), with final concentration of dimethylsulfoxide < 1%. Treatment of the cells was performed in the dark for 1 hr at 37° in a humidified atmosphere containing 5% CO_2.

Cell cultures

Monolayers of CHO cells were grown in flasks (75 sq cm; Costar, Cambridge, MA) in Ham's F-10 medium, supplemented with 15% newborn calf serum, 1 mM L-glutamine (BDH, Poole, United Kingdom), penicillin (100 units/ml; Gist-Brocades NV, Delft, Netherlands) and streptomycin (100 µg/ml; Gist-Brocades NV) at 37° in a 5% CO_2 incubator (Heraeus,

Hanau, Federal Republic of Germany). The cultures were handled in a laminar downflow system (Microflow Pathfinder Ltd, Fleet, United Kingdom) under yellow light (filtered T.L.light wavelength > 525 nm; Philips, Eindhoven, Netherlands) and checked periodically for possible Mycoplasma contaminations (Peden, 1975).

Cell survival

Cells were trypsinized, counted in a haemocytometer (AI 134, Analysis Instruments, AB, Stockholm, Sweden), serially diluted, and subcultured in 6-cm Petri dishes (Greiner GmbH and Co KG, Nürtingen, Federal Republic of Germany) at a density of 500 to 10,000 cells/plate. At 4 hr after seeding of the cells, the medium was replaced by medium containing 7.5% newborn calf serum and platinum compound at the desired concentration. After 1 hr at 37°, the platinum-containing medium was removed, and the cells were washed twice with PBS (phosphate buffered saline; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl), and fresh medium was added. After 7 days of culturing, the cells were stained with 1% methylene blue, and the colony-forming ability of the treated cells was determined. All survival experiments were performed at least 3 times with 2 dishes for each dose used.

Platinum determinations

DNA was isolated according to the method of Kirby and Cooke (1967) and dissolved in 0.01 M phosphate buffer, pH 7.4. The DNA concentration was measured spectrophotometrically (E=6600/M cm), and the amount of platinum in the DNA samples was determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 4000, equipped with a HGA 500 graphite furnace and an AS-40 automatic sampler. K_2PtCl_6 solutions, supplemented with heat denatured salmon sperm DNA (500 µg/ml, Millipore Corp., Freehold, NJ), were used for calibration.

Mutation experiments

Mutation experiments (mutations at the hypoxanthine-guanine phosphoribosyltransferase locus, resulting in 6-thioguanine resistance) with CHO cells were carried out as described by van Zeeland and

Simons (1976). Briefly, cells were subcultured in 150 sq cm culture flasks (1 to 5.10^6 cells Greiner). At 4 hr after seeding, cells were exposed for 1 hr to medium containing the platinum compound and were then washed with PBS. Subsequently, the cells were allowed to grow for 10 days in medium without hypoxanthine (cells were subcultured every 2 to 3 days during this period). Mutants resistant to 6-thioguanine (Sigma Chemical Co., St Louis, MO), at a concentration of 5 µg/ml were selected by plating 10^5 cells (ten 9-cm Petri dishes) in medium without hypoxanthine. At the same time, the plating efficiency of the cells was determined by plating 500 cells in normal medium. After 7 days of culturing, the colonies were stained and counted.

Induction of SCE

CHO cells (7.10^5) were seeded in 50 sq cm tissue culture flasks. At 4 hr after seeding, they were treated with the platinum compound for 1 hr, followed by a 24-hr incubation in the dark in medium containing 5-bromodeoxyuridine (5µg/ml; Sigma). At 2 hr before collecting of the cells, colchicine (final concentration 1 µg/ml; Sigma) was added. Cells were processed as air-dried preparations and were stained differentially with Hoechst 33258 (Riedel-de Haën AG, Hannover, Federal Republic of Germany) and Giemsa (Hopkins and Williams R66 solution, BDH, Poole, United Kingdom) (Natarajan, personal communication). In each preparation 25 to 50 nuclei (haploid chromosome number is 21) were scored for SCE; these were expressed per chromosome.

Measurements of DNA interstrand cross-links

The alkaline elution method of Kohn et al. (1976), as modified by van der Schans et al. (1982), was adapted to allow measurement of DNA cross-links, as follows. CHO cells (10^5) were seeded in 6-cm Petri dishes. After 6 hr, 0.1 ml [^{14}C]-thymidine (TdR; 0.25 µCi/ml; specific activity, 56.5 Ci/mol; Amersham, Buckinghamshire, United Kingdom) was added to the control cells, and 0.1 ml [^{3}H]-TdR (2.0 µCi/ml; specific activity 24 Ci/mmol; Amersham) was added to the cells to be treated. Incubation was continued for 16 hr at 37°. After this labeling, the cells were washed twice with PBS. The control cells were given fresh

medium and incubated further, while the [^{3}H]-TdR-labeled cells were treated for 1 hr with the platinum compound. Thereafter, cells were washed, given fresh medium and "post-incubated" for the desired time. Subsequently, the cells were placed on ice, medium was removed and 1 ml of ice-cold PBS was added. At 0.5 to 1 hr later, the cells were irradiated with 6 Gy of X-rays (Philips MG321, 300kV, 10mA, 1.5 mm copper filter, 4.5 Gy/min; Hamburg, Federal Republic of Germany) or with 9 Gy of ^{60}Co-γ-rays (Gammacell 200 dose rate 1.1 Gy/sec. Atomic Energy of Canada Ltd., Canada) at 0°. Control and treated cells were irradiated simultaneously. After irradiation, the cells were kept at 0° and scraped off from the Petri dishes with a rubber policeman. Control and treated cells were mixed. The combined cell suspension was layered on a polyvinyl chloride filter (Millipore Corp.) 25 mm diameter, 2-μm pore size) and alkaline elution was performed as described (Schans, et al., 1982), with the exception that eleven 1.5-hr fractions (of 3.3 ml) were collected instead of fifteen 1-hr fractions (of 2.4 ml). The procedure includes a treatment with proteinase K (no 24568 Merck, Darmstadt, Federal Republic of Germany) to remove proteins possibly cross-linked to DNA.

The ^{3}H and ^{14}C radioactivity eluted in the fractions or remaining on the filter at the end of the elution were determined by scintillation counting. The results were expressed as the relative amount of radioactivity still retained by the filter at the moment the fraction was collected. These percentages of DNA not eluted were plotted (on a logarithmic scale) as a function of elution volume. The amount of breaks (in arbitrary units) induced by the irradiation was calculated from the averaged slope over the first 5 fractions of the elution pattern of [^{14}C]-TdR-labeled irradiated control cells, from which the slopes of the unirradiated controls were subtracted (Fig. 1). From the ^{3}H elution pattern the amount of cross-links present in the DNA of the treated [^{3}H]-TdR-labeled cells was calculated according to the method described below. In this method, the value for $C_{o-m^{1}/_{2}}$ must be known; this value is obtained from the experimentally determined elution profiles, in the way schematically shown on Fig. 1 ($m^{1}/_{2}$ is defined as the molecular weight of single-strand DNA fragments passing through

the filter at the 50% elution point of control DNA; see "Appendix").

In correctly performed experiments, only a very small proportion of the DNA isolated from the untreated, unirradiated control cells should be eluted. For this reason, control experiments showing more than 10% elution over the first 9 fractions were discarded.

Calculation of the number of DNA interstrand cross-links

Although alkaline elution is a method to measure DNA single-strand breaks, it can be used to detect the presence of DNA interstrand cross-links by studying the effect of the induction of a known number of single-strand breaks by X- or γ-rays. When interstrand

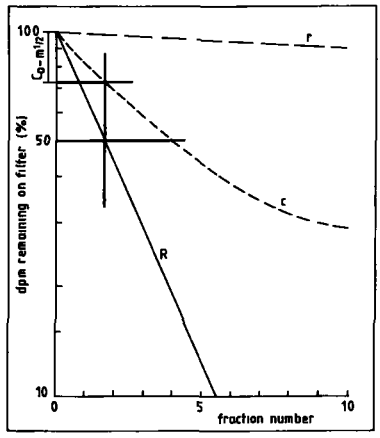

Figure 1: Model for the calculation of DNA cross-links from the elution pattern, obtained by the alkaline elution method. *Abscissa, fraction number; ordinate, the relative amount of* $[^3H]$- and ^{14}C-radioactivity remaining on the filter (logarithmic scale). r, ^{14}C elution pattern of the unirradiated, untreated control cells, ^{14}C-labeled; R, ^{14}C pattern of the irradiated, untreated control cells; ^{14}C-labeled; c, ^3H pattern of the irradiated, treated (cross-linked) cells, ^3H-labeled. In Curve R, the elution volume is determined at which 50% of the radioactivity (equals DNA) from the untreated, irradiated control cells has passed through the filter (the slope of this line is a measure for the single-strand breaks induced by the radiation). Then, $C_{o-m^1/_2}$ is read from Curve c, by estimation of the proportion of cross-linked DNA eluted at this volume. $C_{o-m^1/_2}$ is a measure for the number of cross-links (x) relative to the number of radiation-induced breaks (p), as is described in the "Appendix". The value for x/p is obtained with the help of Curve c in "Appendix", Fig. A-1, where $C_{o-m^1/_2}$ has been plotted as a function of x/p. In the accepted experiments, Curve r remained above 90% and no corrections of Curves R and c had to be applied. The $C_{o-m^1/_2}$ value of the cross-linked sample should not be too small, otherwise the determination of x/p becomes exceedingly inaccurate.

cross-links are present, interconnected single-stranded DNA fragments will not separate upon the alkaline denaturation, which results in a slower elution and, consequently, in a seemingly smaller number of single-strand breaks. Ewig and Kohn (1977; 1978; Kohn et al, 1976) have described a method to calculate the number of DNA interstrand cross-links from alkaline elution profiles, which leads to an underestimation of this number by at the most a factor of 2. We have used a more accurate method, which is explained in the "Appendix". This resulted in a curve (Fig. A-1, Curve c), which was used for the calculation of the amount of cross-links. From this curve, the ratio x/p corresponding to the experimentally determined value of $C_{o-m\frac{1}{2}}$ was read; x/p is the ratio between the number of interstrand cross-links (x) and the number of X- or γ-ray induced single strand breaks (p), both per unit length of DNA. Because p was derived from the elution pattern found for the irradiated control cells (see above), x could then be computed.

The alkaline elution method can be calibrated by extrapolation from results obtained with alkaline sucrose gradients (Van der Schans, personal communication). With our equipment, the irradiation induces 300 breaks/10^{12} daltons of DNA per Gy; this is in close agreement with values reported in the literature (Kohn et al., 1976).

The limitations of this method to calculate DNA interstrand cross-links are: a) that the number of cross-links should not surpass the amount of irradiation-induced breaks; and b) that corrections have to be made for the elution pattern of untreated, unirradiated control DNA from cells. These corrections are negligible, however, when only a small proportion of the control DNA is eluted, as was the case in our experiments that passed the <10% criterion mentioned above.

With the new method, the absolute number of cross-links are changed in comparison to the method by Ewig and Kohn (1977; 1978). In this particular case, however, qualitatively identical conclusions would have been drawn from our results when their method had been used.

In theory, single-strand breaks may be masked not only by interstrand cross-links but also by cross-links that connect 2 distant nucleotides within one DNA strand. It is not known whether such "long

distance" intrastrand cross-links are really formed to a significant extent. However, it appears highly improbable that these connections might reduce the elution of the DNA in the alkaline elution procedure, in view of the fact that the average distance between 2 X- or γ-ray-induced breaks amounts to 1 to 2.10^6 nucleotides, which is many orders of magnitude larger than the distance expected to be bridged by long-distance cross-links.

Calculation of the number of DNA-protein cross-links

DNA-protein cross-links can be measured with the alkaline elution method by omitting proteolytic digestion of the cell lysate with proteinase K. Then, the undegraded DNA-protein cross-links will, together with the DNA interstrand cross-links, reduce the elution rate of the labeled DNA on the filters. The calculation of the amount of both types of cross-links simultaneously from the elution patterns is identical to the calculation of the amount of DNA interstrand cross-links alone. After subtracting the amount of cross-links found after application of proteinase K (DNA interstrand cross-links), the amount of X- or γ-ray-induced breaks masked by DNA-protein cross-links remains. Kohn et al. (1976) and Kohn and Ewig (1979) have found a linear relationship between the amount of DNA-protein cross-links thus measured and the dose of several compounds used. Although the relation between the absolute amount of DNA-protein cross-links and the amount of masked radiation-induced single-strand breaks is unknown, this relationship has been considered to be 1:1. This permitted us to express the DNA-protein cross-links as Gy-equivalents or breaks per dalton of DNA (1 Gy induces 300 breaks/10^{12} dalton DNA; see above). If the relationship were different, the absolute amount of DNA-protein cross-links would change, but not the conclusions obtained in this study.

Results

The effects of the 4 platinum compounds on survival of CHO cells is shown in Fig. 2. In agreement with previous observations (Plooy and

Lohman, 1980; Plooy et al., 1982), the semi logarithmic survival graphs are straight lines with a slight shoulder. From the linear part of the graphs, the D_0 values (increase in dose resulting in a decrease in survival to 37% of the initial value) given in Table I were calculated. The 2 cis-compounds (cII and cIV) are highly cytotoxic, both with a D_0 of 8 μM, whereas the trans-compounds, tII and tIV, show a much lower toxicity (16 and 34-fold, respectively). These results compare reasonably well with earlier data obtained under somewhat different experimental conditions (Bonatti et al., 1983).

When the amount of platinum adducts formed (i.e., the amount of platinum bound to DNA) was determined as function of the treatment concentration, the differences between the 4 compounds were less pronounced. All 4 gave an incorporation of platinum that was proportional to dose (not shown). The amounts of platinum bound to DNA per μM of agent present during treatment differed less than one order of magnitude (see Table II under Pt-DNA adducts). According to this criterium, cII was the most active compound; cIV was distinctly less reactive (about 3.5-fold), and was clearly surpassed by tIV. These

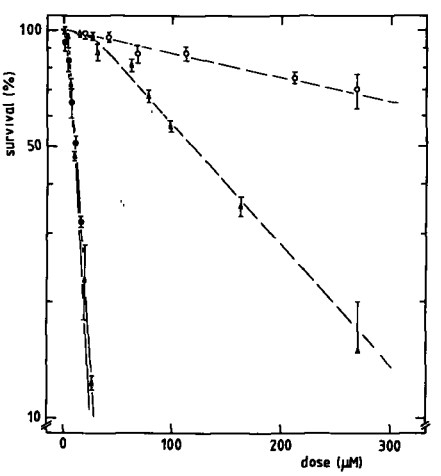

Figure 2: Survival of CHO cells as a function of the dose of the platinum compounds. Data represent the averages of at least 3 independent experiments, with duplicates for each dose; Bars, s.d. From the linear part of the graphs the D_0 values of the platinum compounds have been calculated. •, cII (D_0 = 8 μM); ▲, cIV (D_0 = 8 μM); o, tII (D_0 = 275 μM); Δ, tIV (D_0 = 125 μM).

results, in combination with the survival data, indicate that, on the average, lesions caused by the cis-compounds are more lethal than those induced by the trans-analogues, and that cIV lesions are more lethal than cII adducts, as is illustrated by Fig. 3A, where survival is plotted as a function of DNA-bound platinum.

Also, with regard to the ability to induce mutations at the hypoxanthine-guanine phosphoribosyltransferase locus of CHO cells, the cis-compounds appeared much more effective than the trans-isomers. When compared on the basis of the number of mutants induced per amount of Pt-DNA adduct present in the cell (Fig. 3B), the 2 cis-compounds were of almost equal potency, and about 40 times as active as the 2 trans-complexes. When the mutation induction was plotted as a function of survival (data not shown), cII and cIV were still more mutagenic than tII and tIV; in this comparison, under equitoxic conditions, cII appeared as mutagenic as cIV.

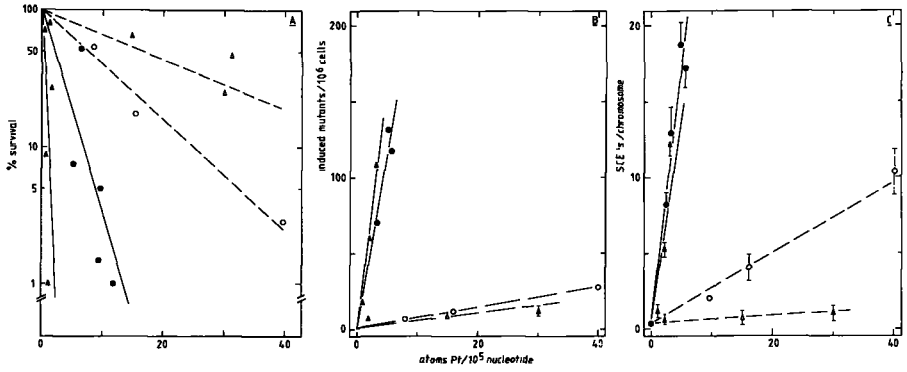

Figure 3: Amount of platinum bound to the DNA of CHO cells in relation to survival (A), mutagenicity (B) and SCE induction (C). Abscisca, amount of platinum bound to the DNA, measured by atomic absorption spectroscopy, is given as the number of platinum atoms per 10^5 nucleotides. (A), survival (plotted on a logarithmic scale) as a function of Pt-DNA binding; the results of 2 independent experiments have been computed. (B), Induction of hypoxanthine-guanine phosphoribosyltransferase mutants (per 10^6 surviving cells) as a function of Pt-DNA binding. The data from 4 separate mutation/survival experiments have been combined. The relation between survival and platinum binding was taken from (A). (C), SCE induced per chromosome as a function of Pt-DNA binding. Per dose, the SCE in 25 nuclei were counted (n=21). The results of 2 separate experiments are given (bars, s.d.). ●, cII; ▲, cIV; ○, tII; △, tIV.

The induction of SCE is another indicator for the occurrence of DNA damage. In CHO cells, these chromosomal abnormalities are readily induced by the 2 cis-compounds, and rather poorly by their trans-analogues. When expressed as a function of the number of Pt-DNA-adducts, as is done in Fig. 3C, cII and cIV are virtually indistinguishable, and about 9 times as effective as tII, while the SCE induction by tIV is almost negligible. Evidently, the lesions in DNA caused by the cis-compounds are much more harmful, also with respect to the induction of SCE.

The occurrence of interstrand cross-links in the DNA of CHO cells treated with any of the 4 agents was determined at different moments after the treatment to study both the induction and repair of these lesions. At the various moments after induction (after repair periods ranging between 0 and 48 hr), the number of DNA interstrand cross-links was, for all 4 compounds, approximately proportional to the concentration of the agent during treatment, up to a certain number of

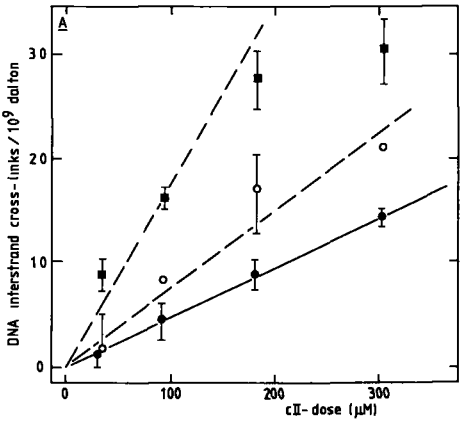

Figure 4: DNA interstrand cross-links induced by cII in CHO cells, measured by alkaline elution, after a 1-hr treatment at 37°. The amount of cross-links is given as function of the dose of cII, after various posttreatment incubation periods (at 37°). Data are mean values of 3 or more independent experiments; bars, s.d. (points without bars, values of less than 3 experiments). ●, directly after the removal of platinum compounds; ■, after 6-hr post-incubation; ○, after 24-hr post-incubation.

cross-links. Beyond this value, the induction leveled off. In Fig. 4, an illustration of this observation is given for 4 concentrations of cII, after 0, 6 or 24 hr of post-treatment incubation of the cells at 37°. An interesting phenomenon can be seen in this graph, namely, that during the first 6 hr of this incubation, a strong increase in the number of cross-links takes place. Apparently, additional cross-links are formed, although the agent has been removed, presumably because adducts with monofunctionally bound platinum, which still possess one Pt-Cl bond, become activated and react with a neighboring DNA strand. This observation is in agreement with the results of Roberts and Friedlos (1981).

A comparison of the 4 compounds with respect to the kinetics of formation or repair of cross-links is shown in Fig. 6A, where the number of DNA interstrand cross-links determined in cells treated with equitoxic dosages of cII, cIV, tII or tIV is plotted against the length of the post-treatment incubation. Striking differences are

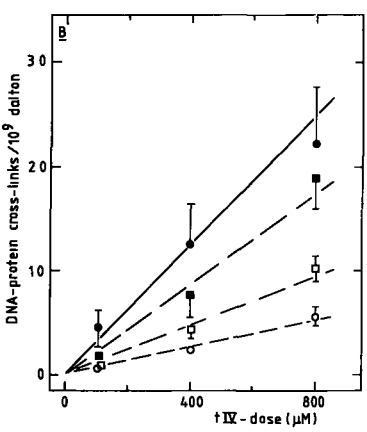

Figure 5: DNA-protein cross-links induced by tIV in CHO cells. The cells were treated for 1 hr at 37°; subsequently, the amount of cross-links (ordinate) was determined by alkaline elution. The DNA-protein cross-links present after different post-incubation periods (at 37°) have been plotted as a function of the concentration of the drug. Data are mean values of 3 independent experiments. Bars, s.d. ●, directly after treatment; ■, after 6-hr post-incubation; □, after 12-hr post-incubation.

seen, both in the number of cross-links and in the kinetics. The induction of DNA interstrand cross-links by tIV is extremely low, particularly when related to the total number of Pt-DNA adducts which, at equitoxic doses, is by far the highest for this compound (Fig. 3A). As for the kinetics, the amount of cross-links induced by both cis-compounds reaches a maximum 6 to 12 hr after the removal of the drug, probably because the continuing formation of cross-links overcompensates for the disappearance caused by repair reactions. Thereafter, repair exceeds formation. After 48 hr, some cis-induced DNA interstrand cross-links are still detectable in the DNA (Fig. 6A). Treatment of CHO cells with tII results in a maximum amount of DNA interstrand crosslinks directly after the treatment, followed by a fast repair process. The small amount of cross-links induced by tIV appears to show a slight increase during the first 6 hr after the removal of the drug. The essence of the results shown in Fig. 6A has

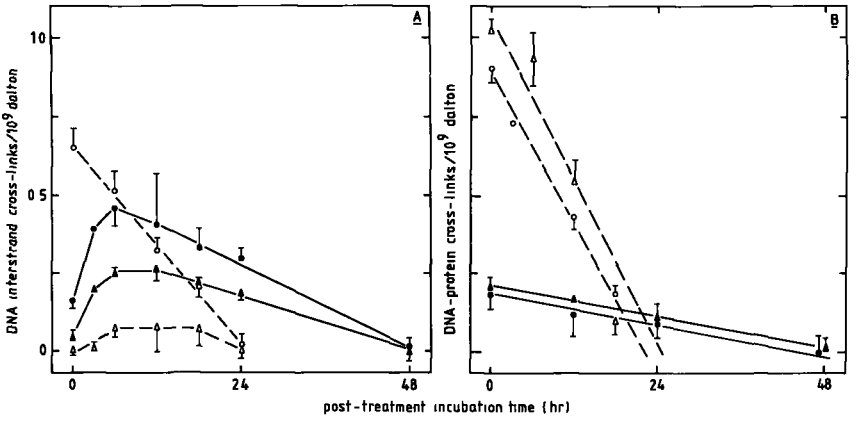

Figure 6: DNA interstrand cross-links (A) and DNA-protein cross-links (B), induced by cII, cIV, tII and tIV, as a function of the length of a post-treatment incubation period. Cells were treated with equicytotoxic doses of the platinum compounds and, after removal of the agent, incubated at 37° for various periods as indicated in the figure. Data of at least 3 independent experiments are given. Bars, s.d. (points without bars, data from less then 3 experiments). The plotted values for cII and cIV were interpolated from dose-response curves obtained after various post-treatment incubation periods (for cII, see Fig. 4). ●, cII, (30 μM); ▲, cIV, (30 μM); ○, tII, (1000 μM); △, tIV, (400 μM).

been summarized in Table I, where effects of the compounds are expressed per dose-increase equal to D_0. The conclusions from these data are: a) with regard to DNA interstrand cross-links, strong differences exist between tII and the 2 cis-compounds (and, possibly, tIV). Both cis-compounds show a delayed formation of these cross-links, whereas tII shows a strong immediate cross-link induction and not a trace of delayed formation; tIV appears to resemble the cis-compounds but is a much weaker inducer; b) the Pt(IV) complexes are less efficient cross-linkers than the corresponding Pt(II)-congeners; and c) cross-links induced by the cis-compounds are repaired more slowly than the trans-induced cross-links.

In DNA-protein cross-link studies, linear relationships were found between the dose of the drugs and the amount of DNA-protein cross-links, detected after various post-incubation times. Fig. 5 shows such dose-response curves for tIV, which (as tII) induces initially a large number of DNA-protein cross-links. In Fig. 6B and Table I, the data of the induction and repair of DNA-protein cross-links are given for all 4 platinum compounds when used at equitoxic

TABLE I Cytotoxicity and induction and repair of DNA interstrand and DNA-protein cross-links in CHO cells treated with platinum compounds, at a D_0 dose increment

Evaluation of the cytotoxicity and cross-linking data, calculated per single cell at a dose increase of D_0. Cytotoxicity (D_0) is derived from Fig. 2. The DNA interstrand and DNA-protein cross-links given at various times after treatment, are derived from Fig. 6, assuming a total DNA molecular weight per cell of 6×10^{12} (t=0, directly after the removal of the drug). Treatments were for 1 hr at 37°, post-treatment incubation was performed at 37°.

compound	symbol	D_0	DNA interstrand cross-links/cell				DNA-protein cross-links/cell		
		(μM)	0-hr	6-hr	24-hr	48-hr	0-hr	24-hr	48-hr
cis-$Pt(NH_3)_2Cl_2$	cII	8	448	1008	592	31.8	560	192	4.2
cis-$Pt(NH_3)_2Cl_4$	cIV	8	128	464	272	63.6	448	224	2.4
trans-$Pt(NH_3)_2Cl_2$	tII	275	1040	347	50	0	2277	66	0
trans-$Pt(NH_3)_2Cl_4$	tIV	125	150	244	19	0	2644	9.6	0

TABLE II Amount of DNA interstrand and DNA-protein cross-links and total Pt-DNA adducts per cell, per 1 μM drug, directly after the treatment of the cells (t=0)

The amounts of DNA cross-links and the total amounts of Pt-DNA adducts were derived from measurements directly after a 1-hr treatment of the cells (t=0) with approximately equitoxic dosages of the 4 platinum compounds, at 37°. From the results, the number of these lesions per cell, per 1 μM dose-increase, were calculated.

compound	DNA interstrand cross-links [a]			DNA-protein cross-links [a]			Pt-DNA-adducts [b]	
	per cell	%	per chromosome	per cell	%	per chromosome	per cell	per chromosome
cII	56	0.11	1.3	70	0,15	1.7	48,000	1,100
cIV	16	0.11	0.38	56	0.4	1.3	14,000	300
tII	3.8	0.05	0.09	8.3	0.12	0.2	7,000	200
tIV	1.2	0.004	0.03	21.2	0.06	0.5	34,000	800

a) DNA interstrand and DNA-protein cross-links were calculated per cell (DNA content of the cell, 6 x 10^{12}), and expressed as the percentage of the total Pt-DNA adducts per cell; in addition the number of these lesions per chromosome (2N = 42) is given. b) Data taken from Fig. 3C.

doses. All 4 platinum complexes show a maximal amount of these cross-links directly after the removal of the drug, but the number of DNA-protein cross-links induced by the trans-compounds is much higher than that caused by the cis-isomers. Furthermore, the tII- and tIV-induced DNA-protein cross-links are repaired much faster than those induced by cII and cIV. One-half of the initially detected cross-links have disappeared after about 3 to 6 hr in case of the trans-compounds; for the cis-isomers this process takes about 24 hr.

Table I summarizes the results of the time course experiments on induction and repair of cross-links, after mathematic conversion of the equitoxic doses used to D_0. As is indicated in the Table I, an estimate has been made of the cross-links still present per cell at 48 hr after the removal of the drug, as a consequence of a dose increment equal to D_0. Table II gives a survey of the amounts of cross-links and of total Pt-DNA adducts per cell and per chromosome directly after the treatment of the cells, and the fraction of cross-links in relation to

total adducts; in Table II, the 4 compounds are compared per equal increase of molar dose.

Discussion

Earlier we found some similarities in the action of the 4 platinum compounds (Plooy and Lohman, 1980; Plooy *et al.*, 1982; Chapter 2). All inhibit the DNA synthesis in CHO cells (although the recovery of the DNA synthesis is much faster after treatment of the cells with the *trans*- than with the *cis*-platinum compounds (see Chapter 2); they induce micronuclei (Bonatti *et al.*, 1983) and cause some repair synthesis (Plooy *et al.*, 1982; Chapter 2); with none of the platinum compounds used, treatments resulted in the induction of single strand breaks (Plooy and Lohman, 1980; Plooy *et al.*, 1982; Chapter 2). However, in the present study, differences in the action of the platinum compounds became apparent, now that other biological end points were measured. The 2 *cis*-platinum compounds (cII and cIV) are very cytotoxic and induce SCE as well as gene mutations, whereas the *trans*-compounds are much less active in this respect. The cytotoxicity seems to correlate with the other 2 measured biological end points.

The amount of Pt-DNA adducts cannot itself be held responsible for the differences between the *cis*- and *trans*-compounds, since, at equi-effective treatments, much more *trans*-complexes are bound to the DNA than *cis*-platinum compounds (Fig. 3A). Apparently, one or more very toxic and mutagenic adducts are exclusively or preferentially introduced by the *cis*-compounds. For a complete understanding of the mechanism of action of the platinum compounds, the nature of the various Pt-DNA adducts has to be identified, as well as the cellular responses to these adducts. Presently, only a part of the DNA lesions has been analyzed and their respective contributions to biological effects have not yet been established. Nevertheless, a few conclusions with regard to the relation between lesions and effects appear warranted.

Both, *cis*- and *trans*-platinum compounds can react with DNA either monofunctionally or bifunctionally. According to recent results,

initially, the majority of the adducts in the DNA of the cells consists of monofunctionally bound Pt (see Chapter 5). It is not expected that many differences will exist in the noxiousness or, with respect to repair, between monofunctional adducts formed by cis- and trans-platinum compounds. It is tempting, therefore, to attribute the much stronger cytotoxic and genotoxic effects of the cis-platinum compounds to the various bifunctional adducts, including DNA inter- and intrastrand cross-links and DNA-protein cross-links, particularly, since our results indicate such obvious differences, both in induction and repair of these lesions, between the cis- and trans-platinum compounds.

The formation of "short distance" DNA intrastrand cross-links, known to be formed in vitro (Fichtinger-Schepman et al., 1982) and in bacteria (Brouwer et al., 1981) by cis-platinum compounds, appears unlikely for the trans-compounds because of steric reasons (Royer-Pokora et al., 1981). This type of cross-link, which connect 2 nucleotides of one DNA strand that are adjacent or are separated by one nucleotide, have been found also in CHO cells after treatment with cII (see Chapter 5). Their biological importance in mammalian cells has not yet been established. The differences between cis- and trans-platinum compounds with regard to the induction, however, make these adducts to possible candidates for the genotoxic lesions. The steric factors that make the formation of "short-distance" intrastrand cross-links by trans-compounds rather unlikely, will not prevent the cross-linking of 2 more remote nucleotides of the same strand. There is no obvious reason to expect that the induction of these "long-distance" intrastrand cross-links should occur preferentially by either the cis- or trans-compounds and no reason, therefore, to attribute a particular biological significance to these (probably rare) lesions.

According to several authors, DNA interstrand cross-links induced by cII correlate with cytotoxicity. Strandberg et al. (1982), however, have reported that the DNA interstrand cross-links alone cannot be responsible for the cytotoxicity. In the present study, the DNA interstrand cross-links appear to be only minor reaction products when measured shortly after treatment (< 1 % of the total Pt-DNA adducts). However, the 2 cis-compounds and possibly the weakly inducing trans-

compound tIV show a delayed formation of these lesions, which has also been found by other authors (Roberts and Friedlos, 1982; Strandberg et al., 1982 and Zwelling et al., 1978). Eventually, the interstrand cross-links appear to be removed from the DNA. Fraval and Roberts (1979) demonstrated that this decrease in the amount of cross-links is not due to chemical instability of the cross-links or to the induction of breaks in the DNA, but has to be ascribed to the action of repair systems.

Our results indicate that the cellular repair systems are able to distinguish between DNA interstrand cross-links induced by cis- and trans-platinum compounds, respectively (Table I; Fig. 6). The trans-platinum-induced cross-links are repaired faster; this phenomenon may very well underlie the more rapid recovery of the DNA synthesis inhibition in CHO cells after treatment with a trans-platinum compound (see Chapter 2). Because of the delayed formation and slow repair, the cis-platinum induced cross-links show a rather persistent character. Persistent interstrand cross-links can be very noxious for the cells, especially during DNA replication. It is known (Fraval and Roberts, 1979) that cells in G_1 phase (the phase preceding the S phase, when DNA is replicated) are more sensitive for cII than asynchronously growing CHO cells (about 30% S phase) or than are cells half-way through the S phase. Moreover, DNA synthesis has been found to be required to express cII-induced cellular toxicity. These results may be considered to point to the interstrand cross-link as the biologically important DNA lesion. However, in our experiments, the DNA-protein cross-links also showed a persistent character and, therefore, cannot be ruled out as a (cyto)toxic lesion. A similar type of relation has been found for bifunctional alkylating agents (Murnane and Byfield, 1981); the irrepairable DNA interstrand as well as DNA-protein cross-links correlated with cytotoxicity.

In our results, the residual amount of persistent cross-links (DNA interstrand as well as DNA-protein) induced by the cis-platinum compounds in the cells correlates not only to cytotoxicity, but also to mutagenicity and SCE induction. In contrast to DNA-protein cross-links, the interstrand cross-links cause a mutilation of the genetic information in both DNA strands at (approximately) the same position.

To circumvent this double block on the template, recombination with homologous chromosomes appears to be necessary. Such a process is thought to be involved in the induction of SCE. It is tempting, therefore, to attribute great importance to the interstrand cross-links in relation to genotoxicity. This way of reasoning is not supported, however, by data of Bradley et al. (1982), who found that SCE are not the result of bifunctional platinum binding.

In summary, the persistent DNA interstrand cross-links, because of their damage to the DNA as a template, may account for the cytotoxicity, mutagenicity and presumably also for the antitumor activity of the cis-compounds. However, the contribution of DNA-protein and short-distance intrastrand cross-links cannot be excluded. Further research is required to establish, among others, the importance of monofunctionally Pt-DNA adducts and of intrastrand cross-links and their contribution to the observed effects in CHO cells.

Acknowledgements

The authors wish to thank Dr F.Berends, Dr A.M.J.Fichtinger-Schepman and Dr G.P.v.d.Schans for the critical reading of the manuscript and their helpful discussions, Dr. H.P.Liepman for the editorial assistance, and Dr A.T.M.Marcelis for the synthesis of the platinum compounds.

Appendix: Distribution of molecular weights in alkali-denatured DNA samples with randomly induced single-strand breaks and interstrand crosslinks.

Govert P. van der Schans, Adrie C.M. Plooy and Frits Berends

The elution of DNA single-strand molecules through membrane filters is assumed to depend on strand length. This assumption is supported by the observed dependence of DNA elution on the frequency

of single-strand breaks produced by X-rays (Ewig and Kohn, 1978; Kohn et al., 1976). The distribution of DNA over the fractions eluted from the filter is, therefore, determined by the molecular weight distribution in the DNA sample studied. The distribution in the DNA sample itself is determined by the distribution of the single-strand breaks and the cross-links over the original DNA molecules.

Let us consider a homogeneous population of double-stranded DNA molecules with molecular weight 2 M which contain, on the average, p breaks per single strand, randomly distributed. In the absence of cross-links, the weight fraction of the single-strand fragments with a molecular weight between m and m + dm is given by:

$$C_m = p^2.M^{-2}.m.e^{-mp/M}.dm \qquad (A)$$

provided M is large compared to m (i.e., p>>1) (van der schans et al., 1969). Next, we assume that the original DNA contained x randomly distributed cross-links/molecule. If we consider one cross-link to consist of 2 "links", there are x such links per original single strand with molecular weight M. This implies that in a population of single-strand fragments with molecular weight m, a fraction $e^{-mx/M}$ does not contain a link. Consequently, the weight fraction of fragments with a molecular weight between m and m + dm that contain one or more links, is:

$$X_m = p^2.M^{-2}.m.e^{-mp/M}(1-e^{-mx/M})dm \qquad (B)$$

We define $m_{\frac{1}{2}}$ as the molecular weight of single-strand DNA fragments that are passing through the filter at the point in the elution curve of non-cross-linked DNA, where 50% of the material has been eluted. For a random distribution of the breaks over the molecule, the number average molecular weight of the fragments (m_n) is equal to $m_{\frac{1}{2}}/1.68$ (Veatch and Okada, 1969). As $m_n = M/(p+1) \approx M/p$:

$$m_{\frac{1}{2}}p/M = 1.68 \qquad (C)$$

According to Equation B, the total weight fraction of fragments with a molecular weight (before cross-linking) between 0 and $m_{\frac{1}{2}}$, that contain at least one link, is given by:

$$X_{\frac{1}{2}} = \int_0^{m_{\frac{1}{2}}} p^2 . M^{-2} . m . e^{-mp/M} (1-e^{mx/M}) dm \qquad (D)$$

After resolution of this equation and substitution of Equation C, the next equation is obtained:

$$X_{\frac{1}{2}} = 0.50-(1+x/p)^{-2}+(1+x/p)^{-2}(0.50+0.31x/p)e^{-1.68x/p} \qquad (E)$$

In the experimental determination of crosslinks, we use $C_{0-m\frac{1}{2}}$, i.e., the percentage of total DNA already eluted at the point where non-cross-linked single-strand material with $m = m_{\frac{1}{2}}$ is expected to appear in the eluate. It is relevant, therefore, to ask what will be the elution behavior of the material in fraction $X_{\frac{1}{2}}$. Which part will acquire a molecular weight $> m_{\frac{1}{2}}$ by the cross-linking? This question can be easily answered for 2 extreme situations.

A. All cross-linked fragments have acquired a total molecular weight exceeding $m_{\frac{1}{2}}$ and are no longer eluted in the category $m < m_{\frac{1}{2}}$. In this case, Equation E directly gives the weight fraction that disappears from this category, so that the original 50% eluted with $m < m_{\frac{1}{2}}$ will be reduced to $0.50 - X_{\frac{1}{2}}$.

B. Only the fragments crosslinked to pieces with $m > m_{\frac{1}{2}}$ disappear from the category with $m < m_{\frac{1}{2}}$. As the cross-links have a random distribution and since, by definition, one-half of the material has a molecular weight $> m_{\frac{1}{2}}$, in this case, only 50% of the cross-links will be effective in shifting the fragments to the category with $m > m_{\frac{1}{2}}$. This implies that, in Equation E, x has to be substituted by the number of productive cross-links, i.e. by 0.5x, in order to obtain the weight fraction of material disappearing from the population with $m < m_{\frac{1}{2}}$.

In Fig. A-1, the weight fraction expected to be collected in the eluate at the point, when 50% would have been eluted in the absence of cross-links, has been plotted as a function of x/p, for both cases. It will be clear that the real curve will lie somewhere between these 2 extremes.

For a better approximation of the real curve, it has to be considered which fraction of the molecules with $m < m_{\frac{1}{2}}$ cross-linked to fragments of the same category will become larger than $m_{\frac{1}{2}}$. As a first approach, this group of fragments can be subdivided into a class of molecules with $m < \frac{1}{2}m_{\frac{1}{2}}$ (before cross-linking) and a class with $\frac{1}{2}m_{\frac{1}{2}} < m < m_{\frac{1}{2}}$. After integration of Equation A over these intervals, with the use of Equation C, we find that 20.5% of total DNA falls into the former category (a) and 29.5% into the latter (b). Since the number of links is proportional to the amount of DNA, the links will show the same distribution. In either class, one-half of the links will be connected to fragments with $m > m_{\frac{1}{2}}$ (according to the above situation B). Combination of two fragments from category b will result in material with $m > m_{\frac{1}{2}}$, whereas m will remain $< m_{\frac{1}{2}}$ when two molecules of category a are cross-linked. Finally, when a fragment of category a is

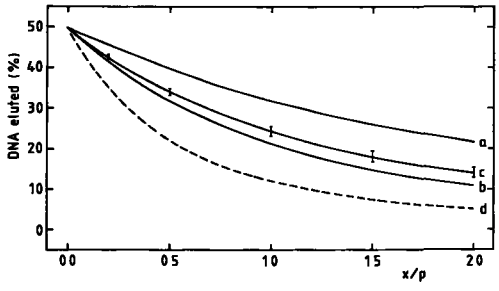

Figure A-1: Fraction of cross-linked and irradiated DNA eluted at the 50% elution point of irradiated non-treated DNA (equals $C_{0-m_{\frac{1}{2}}}$) as a function of x/p. Curve a: one-half of the links in molecules with molecular weight $m < m_{\frac{1}{2}}$ result in retention beyond the 50% elution point; Curve b: all links in molecules with $m < m_{\frac{1}{2}}$ result in retention up to the 50% elution point; Curve c: best estimate of the real curve on the basis of the subdivision of molecules with $m < m_{\frac{1}{2}}$ into 4 weight categories; bars: area between the upper and lower limit found in this approach; Curve d: curve according to the derivation of Ewig and Kohn 1978 and Kohn et al., 1976.

connected to one of category b, part of the products will be larger than $m_{\frac{1}{2}}$.

With the use of Equations C and D, we can calculate the weight fraction of fragments containing a link in the categories $m < \frac{1}{2}m_{\frac{1}{2}}$ (X_a) and $\frac{1}{2}m_{\frac{1}{2}} < m < m_{\frac{1}{2}}$ (X_b), as a function of x/p. Since the breaks are random, it can be considered a matter of change which 2 links (in different fragments) are connected together. This implies that 29.5% of the links in material of category b will be connected to fragments of the same class, and 20.5% to fragments of category a. The same applies to the links in material belonging to category a. Consequently, in the weight fraction X_b, at least 50% (linked to material with $m > m_{\frac{1}{2}}$) plus 29.5% of the links will be present in a cross-linked product with $m > m_{\frac{1}{2}}$. So, in the formula used for the calculation of X_b, x should be replaced by 0.795x in order to arrive at the fraction that certainly is no longer eluted before the 50% point. The same is true for 50% of the links in X_a; in X_a, therefore, x has to be replaced by 0.5x. Finally, 20.5% of the links in X_a are connected to other small fragments, and the products remain smaller than $m_{\frac{1}{2}}$, so that X_a with x replaced by 0.205x gives the cross-linked material that is expected to be eluted before the 50% point. Calculation of the sum of X_b (with 0.795x) and X_a (with 0.5x) for various values of x/p between 0 and 2 yields a corrected version of Curve a in Fig. A-1. In an analogous manner, Curve b can be corrected upwards by adding X_a (0.205x). In this way, the distance between the 2 graphs can be narrowed (from 4.0% at x/p=0.2 to 1.7%; 7.9% at x/p=0.5 becomes 3.4%; 10.5% at x/p=1.0, as well as 2.0 becomes 4.5%).

According to the same approach, the range between the 2 curves can be narrowed further, by subdividing the material with $m < m_{\frac{1}{2}}$ into more weight categories. Subdivision into 4 classes reduces the above-mentioned values to 0.9, 1.7, 2.3 and 2.3%, respectively. Line c in Fig. A-1 is constructed on the basis of these corrections as a fair approximation of the real curve (in the range x/p 0 - 2, this curve does not deviate more than 0.8% from the more easily constructed graph that is obtained by substituting 0.85x for x in Equation E; if a reconstruction of Curve c is desired, the following calibration points

can be used: x/p = 0.2, 42.4%; 0.5, 33.7%; 1.0, 24.0%; 1.5, 17.8% and 2.0, 13.7%). More refined calculations are without meaning because, in this approach, the possibility is ignored that more than 2 fragments are linked together when multiple links per fragment are present, which will lead to inaccuracies in particular at higher values of x/p.

For comparison the curve according to the approach of Ewig and Kohn (1978) and Kohn et al. (1976) has been drawn in Fig. A-1. It is clear that the latter will result in an underestimate of the number of cross-links of at least a factor of 2.

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4. INFLUENCE OF TREATMENT TEMPERATURE ON THE GENOTOXIC EFFECTS OF CISPLATIN IN CHO CELLS: CYTOTOXICITY, MUTAGENICITY AND INDUCTION OF LESIONS IN DNA.

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Summary

In cells exposed *in vitro* to the cytotoxic and mutagenic anti-tumor drug cisplatin (*cis*-$Pt(NH_3)_2Cl_2$), various adducts with nuclear DNA are formed. A comparative study was made of the influence of temperature variation during treatment of cultured Chinese hamster ovary cells with cisplatin on cytotoxicity, mutation induction and Pt-DNA-adduct formation. Before and after treatment (1 hr at 32°, 37° or 40°C) cells were kept at 37°C. Cytotoxicity increased with temperature; D_0 values were 29.6 ± 1.6, 21.1 ± 1.2 and 11.4 ± 0.6 μM at 32°, 37° and 40°C, respectively. Pt-DNA-binding to DNA at 40°C was 2.0 (± 0.3) times as high as at 32°C. This factor remained practically constant over a 24-hr post-treatment incubation of the cells, during which about 60% of DNA-bound Pt were removed. As the increase in cytotoxicity between 32° and 40°C roughly was in proportion to that in Pt-binding, no substantial changes in the spectrum of adducts appeared to occur. The induction of DNA interstrand cross-links, studied at 32° and 40°C, varied linear with dose. Influence of temperature was comparable to that on total Pt-binding. Amounts of cross-links strongly increased during 24 hr after treatment. Plots of cross-links against survival for 32° and 40°C almost coincided. Induction of 6-thioguanine-resistant (HGPRT) mutants at various cisplatin concentrations did not show a clear temperature dependency. Equitoxic treatments were

significantly more mutagenic at 32°C than at 40°C; the opposite of what has been reported for E.coli.

Introduction

Cisplatin (cis-diamminedichloroplatinum(II)) is used in the treatment of a wide variety of tumors (Von Hoff and Rosencweig, 1979; Hacker et al., 1984). The corresponding trans-stereoisomer does not show antineoplastic properties. The interactions of cisplatin and related cis-platinum coordination compounds with the nucleobases of DNA are held responsible for their antitumor properties (Prestayko et al., 1980; Roberts, 1981; Macquet et al., 1984). Cisplatin can bind to DNA either monofunctionally or bifunctionally (Reedijk et al., 1984). The bifunctional rather than the monofunctional DNA adducts are thought to account for the drug's action (Caradonna and Lippard, 1984; Marcelis and Reedijk, 1983).

Several bifunctional adducts have been found after reaction with DNA in vitro (Marcelis and Reedijk, 1983; Eastman, 1984; Fichtinger-Schepman et al., 1984a; 1984b) or after treatment of cultured cells (Hacker et al., 1984; Plooy et al., 1984b = Chapter 5). Chelation with one base has been found in vitro (Dehand and Jordanov, 1976), but not in cells. Several types of cross-links (DNA intrastrand, DNA interstrand and DNA-protein cross-links) have been detected in vitro as well as in cells (see Hacker et al, 1984). In cultured mammalian cells, the last two types of cross-links, which are also induced by the trans-platinum compounds, represent only a small part of the total amount of Pt-DNA-adducts (Plooy et al., 1984a = Chapter 3; Roberts and Friedlos, 1981). The DNA interstrand cross-links induced by cisplatin are rather persistent, much more than the ones caused by the trans-isomer. These adducts might be important for the genotoxic effects and for the antitumor activity of cis-platinum compounds (Zwelling et al., 1983; Plooy et al., 1984a = Chapter 3).

Recently, DNA intrastrand cross-links, which are exclusively induced by the cis-platinum compounds, have been discovered in mammalian

cells (Plooy et al., 1984b = Chapter 5); they were shown to represent a high proportion of the Pt-DNA-adducts. In E.coli, where these adducts also are induced in large quantities (Fichtinger-Schepman, personal communication), some of them generate mutants (Brouwer et al., 1981). Therefore, these lesions, too, could account for cisplatin's action.

The action of genotoxic agents on cells may be influenced substantially by the temperature of treatment (Bronk, 1976). Treatment of bacteria with cisplatin at an elevated temperature resulted in an enhancement of the cytotoxicity and a disproportionally strong increase of the mutagenicity (Brouwer et al., 1982). In cultured mammalian cells, a higher treatment temperature resulted in increased cytotoxicity (Meyn et al., 1980; Barlogie et al., 1980; Herman et al., 1982; Fischer and Hahn, 1982; Ishida and Mizuno, 1982; Herman, 1983). Hyperthermia has also been used in chemotherapy of cancer patient treated with cisplatin (Barlogie et al., 1980).

In order to obtain more detailed information on the influence of temperature on the genotoxic effects of cisplatin in mammalian cells, the cytotoxicity, mutation induction and Pt-DNA-binding in CHO cells at 32°, 37° and 40°C were investigated. In this study special attention was focussed on the induction and repair of DNA interstrand cross-links in an attempt to determine their relation to cytoxicity and mutagenicity.

Materials and Methods

Cell culture and cell treatment

Chinese hamster ovary (CHO) cells were cultured in monolayers (150 cm^2 flasks, Costar, Cambridge, Mass.) in Ham's F10 medium (Flow Laboratories, Cambridge, Mass.), supplemented with 15% fetal calf serum (FCS; Flow Laboratories), 1 mM L-glutamine (BDH, Poole, UK), penicillin (100 U/ml; Gist-Brocades N.V., Delft, Netherlands) and streptomycin (100 µg/ml; Gist-Brocades N.V.), at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in an incubator (Hereaus, Hanau,

BRD). All culture and treatment handlings were performed in a laminar down-flow system (Microflow Pathfinder Ltd., Fleet, UK) under yellow light (filtered T.L. light; Philips, Eindhoven, NL; wavelength > 525 nm). The cells were subcultured every 2-3 days. CHO cells to be treated were plated in petridishes (in densities indicated in the descriptions of the various assays) at least 4 hr before onset of the treatment. Cisplatin (cis-$Pt(NH_3)_2Cl_2$), synthesized by the group of Prof.Dr. J. Reedijk, State University, Leiden, Netherlands (Marcelis, 1982), was dissolved in DMSO just before treatment and a stock solution of 1 mg/ml was made in F10 medium containing 7.5% FCS instead of 15% FCS (concentration DMSO < 1%). The cells to be treated and bottles containing F10 + 7.5% FCS were placed in an incubator of the required temperature (32°, 37° or 40°C). After 0.5-1 hr of prewarming, the required amount of stock solution was added to the prewarmed medium to give the desired final concentration of cisplatin (in some experiments, stock solutions was added to the medium just before the prewarming period). Then, the medium covering the cells was replaced by the cisplatin-containing medium. Cells were treated for 1 hr at 32°, 37° or 40°C. Prewarming and treatment occurred in an atmosphere without CO_2. After treatment the medium was removed, the cells were washed twice with PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl) and given fresh medium (with 15% FCS) if post-treatment incubation (at 37°C in a 5% CO_2, 95% air incubator) was required.

Cell survival

Cells were trypsinized, counted and serially diluted with F10 medium supplemented with 15% FCS. Thereafter the cells were subcultured in 6-cm petridishes (Greiner GmbH and Co KG, Nürtingen, BRD) at a density of 500-10,000 cells per plate. At least 4 hr after seeding, the cells were treated as described above. After the addition of fresh medium the cells were incubated for 7 days at 37°C. Then, the cells were stained with 1% methylene blue and the colony-forming ability was determined. The plating efficiency of control cells (> 60%) was not affected by a 2-hr incubation at 32°, 37° or 40°C. All survival experiments were performed with 2 dishes for each dose used.

Mutation experiments

Determinations of mutation induction at the HGPRT gen (resulting in 6-thioguanine resistance) was performed as described (Plooy et al., 1984a = Chapter 3) but for the treatments, which were carried out as mentioned above, and for the media which contained FCS instead of new-born calf serum. Data were only taken into account when the background mutation induction was lower than 20 mutants/10^6 surviving cells and the plating efficiency of the cells was higher than 60%.

Alkaline elution experiments

The presence in the cellular DNA of interstrand cross-links was determined by the alkaline elution method described elsewhere (Plooy et al., 1984a = Chapter 3), except that the amount of radiation-induced breaks was lowered to 0.6 breaks per 10^9 dalton DNA. All samples were treated with proteinase K (Merck, Darmstadt, BRD, No. 24568) to remove proteins possibly cross-linked to DNA. The cross-links were calculated and expressed per 10^9 dalton DNA as discussed (Plooy et al., 1984a = Chapter 3).

Platinum determinations

Cells were plated in 14-cm petridishes (Greiner) at a density of 10^6 cells per plate. They were cultured (F10 + 15% FCS) until a density of 10^7 cells per plate was reached. Then the cells were treated with cisplatin as described, at 32°, 37° or 40°C, washed, collected and pelleted. Ten plates were used for each data point. The DNA of the 10^8 cells was isolated with hydroxylapatite columns according to Meinke et al. (1974). The DNA-containing fractions were pooled and dialysed for 48 hr against deionized water (3-4 times refreshed). The water was evaporated by freeze-drying or vacuum evaporation and the DNA dissolved in 1 ml deionized water containing 3% NaCl. DNA was precipitated with ice-cold ethanol, collected, washed twice with icecold ethanol and dissolved in 0.01 M phosphate buffer, pH 7.4. The DNA concentration was measured spectrophotometrically (1 mg/ml DNA gives a A_{260nm} of 20). The amount of platinum in the DNA samples was determined with neutron activation analysis by Dr.Ir. P.S. Tjioe at the In-

teruniversity Reactor Institute (Delft, Netherlands). The reaction $^{198}Pt\ (n,\gamma) \rightarrow {}^{199}Pt\ (t_{\frac{1}{2}} = 30\ min) \rightarrow {}^{199}Au\ (t_{\frac{1}{2}} = 3{,}5\ days,\ E_{\gamma} = 158$ and 208 keV respectively) was used. After chemical separation and measurement of ^{199}Au, the Pt-content of the sample was calculated (Tjioe et al., 1984). This content was expressed relative to the DNA concentration of the sample by calculation of the r_b value (number of Pt-atoms per nucleobase) according to the formula:

$$r_b = \frac{\text{Pt-concentration (M) x 20 x 330}}{A_{260}}$$

The Pt-determination, as performed, has an average standard deviation of about 6%. At the low Pt-content of our samples, however, the accuracy is less; it is roughly estimated to be $\pm$ 10%.

Results

The survival curves obtained when CHO cells were treated at 32°, 37° or 40°C with various doses of cisplatin, show a clear influence of the temperature (Fig. 1). All curves display the expected first order decline after a not very pronounced shoulder. From the slopes of the linear parts of the curves the D_0 values were computed (D_0 = dose-increment reducing survival to 37% of the original value). These amounted to 29.6 $\pm$ 1.6, 21.1 $\pm$ 1.2 and 11.4 $\pm$ 0.6 µM at 32°, 37° and 40°C, respectively. During the survival studies at the 3 temperatures it was observed that the morphology of cells and colonies was not affected by the hyper- or hypothermic treatment, nor was the plating efficiency of the control cells changed; hyper- or hypothermic conditions applied for 2 hr before or after a cisplatin treatment at 37°C had no influence on the cytotoxicity.

A strong influence was seen, however, when the moment cisplatin was added to the medium was varied. Routinely, the addition occurred to prewarmed medium, immediately before exposure of the cells, but in some experiments cisplatin was added at the onset of the 0.5 - 1 hr of prewarming at treatment temperature. This resulted in a much higher

cytotoxicity (not shown) with D_0 values of 11.7 ± 0.4, 8.1 ± 0.2 and 4.7 ± 0.1 at 32°, 37° and 40°C, respectively (overall increase 2.5 ± 0.1 times).

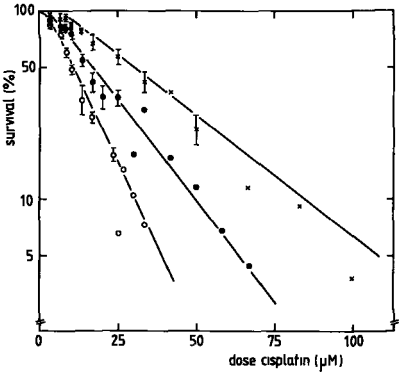

Figure 1: Survival of CHO cells after treatment with various concentrations of cisplatin at 32°, 37° and 40°C. Cells were exposed to cisplatin at the concentration indicated, for 1 h, at the temperature chosen. All preceding and subsequent handlings were at 37°C. Data are given of at least 4 independent experiments with 2 samples per data point ± s.e.m. For each temperature a straight line was fitted to the experimental points beyond the shoulder region, by regression analysis; the results were (intercept, slope ± s.d., number of points): (32°) 2.0928, 0.0146 ± 0.00079 (n = 29); (37°) 2.0233, 0.0205 ± 0.0011 (n = 32); (40°C) 2.0763, 0.0382 ± 0,0020 (n = 37). Symbols: X 32°C; ● 37°C; ○ 40°C.

To see whether the influences of temperature on cytotoxicity would coincide with differences in the number of Pt-DNA-adducts formed in the cells, the amount of Pt-atoms bound to the DNA was determined after treatment with cisplatin at the 2 extreme temperatures. Table I presents the amounts of platinum bound to DNA immediately after a treatment with 33 μM cisplatin for 1 hr, and after the cells had been given a repair period of 12 hr or 24 hr at 37°C after the removal of the drug. Without repair, the amount of DNA-bound platinum in the cells treated at 40°C was 2.0 ± 0.3 times the value found after treatment at 32°C. In cells given a repair period, the ratio's were 2.0 ± 0.3 (12 hr) and 1.8 ± 0.3 (24 hr). The constancy of this ratio over a 24-hr repair period, during which conditions were identical for

Table I Pt-DNA-binding in CHO cells treated with cisplatin at different temperatures, measured at 0, 12 and 24 h after treatment

	Pt-atoms bound per 10^6 nucleotides ($r_b \times 10^6$)		
	t = 0	t = 12	t = 24
32°C	25	14	11
40°C	49	28	20

Cells were treated with 33 μM cisplatin for 1 h at the temperature indicated. Post-treatment incubation was at 37°C. For each determination, DNA isolated from 10^8 cells was used. The Pt-content was determined by neutron activation analysis, the DNA concentration was measured spectrophotometrically. The values given in the table have a standard deviation in the order of 10% (see methods).

all cells and which resulted in removal of about 60% of the Pt-DNA-adducts, suggests that nature and distribution of the bulk of the lesions are the same, whether induction occurred at 32° or at 40°C. If this were true also for the cytotoxic adducts and if cytotoxicity were a direct function of the number of these lesions, the survival curve at 40°C should coincide with that at 32°C after a transformation of the X-axis in proportion to the ratio of adduct formation at the 2 temperatures. This means that the same ratio should be found with regard to shoulder width and D_0 as for Pt-binding. The values for shoulder width (2 and 6 μM) are too inaccurate to allow a significant comparison. The D_0 ratio amounts to 2.61 ± 0.20 (compared to 2.0 ± 0.3 for the Pt-binding), while a general comparison of the curves yields about the same ratio (at D_{37}: 2.69; at D_{10}: 2.66). This result is not inconsistent with the above assumptions, but also might indicate that, on the average, the adducts induced at 40°C are somewhat more harmful than those formed at 32°C.

The mutagenicity of cisplatin at different treatment temperatures was compared by studying the induction of HGPRT mutants in the cells at 32°, 37° and 40°C. In Fig. 2A the mutation induction (per 10^6 surviving cells) is plotted against the dose of cisplatin. Up to about 25 μM cisplatin, mutation induction is roughly proportional to dose, at higher concentrations the curves appear to level off. Little influence is seen of the treatment temperature, at least not in proportion to

the effect seen on adduct formation (Table I). As only during the 1-hr exposure period cells were subjected to different conditions, and not during the period mutations probably become expressed, this result suggests that at higher temperature the mutagenic lesions become less abundant among the total variety of Pt-DNA-adducts induced. In this respect the mutagenic lesions would be distinct from the cytotoxic adducts.

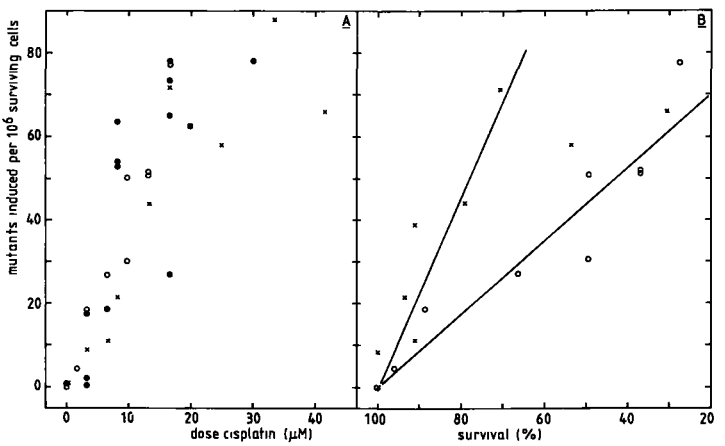

Figure 2: Induction of 6-thioguanine resistant mutants in CHO cells after treatment with different concentrations of cisplatin for 1 h at 32° (X), 37° (●) or 40°C (○). The amounts of mutants were calculated per 10^6 surviving cells after correction for spontaneous mutants. Experiments were accepted only if background was lower than 20 mutants/10^6 surviving cells and if plating efficiency was better than 60%. Mutation induction in relation to cisplatin concentration is given in Fig. 2A, and relative to survival of the cells in Fig. 2B (32° and 40°C only); survival was read from the curves in Fig. 1. Linear regression analysis was performed on the data points in Fig. 2B obtained at cisplatin concentrations below 25 μM; slopes are (32°) 2.16 ± 0.37 (n = 7) and (40°) 0.865 ± 0.113 (n = 9). Identical treatment of the 37°C results (not shown) yielded 1.16 ± 0.26 (n = 13); intercept at 100% survival: 3.3

This notion is supported by the graphs in Fig. 2B, where mutation induction has been plotted against survival, after identical treatments with cisplatin, for the 2 extreme temperatures (37°C results have been deleted to facilitate the comparison). It is clear that, in relation to survival, treatment at 32°C indeed is more mutagenic than

exposure at 40°C. According to the regression lines, the initial slopes differ by a factor of 2.49 ± 0.54. However, mutation induction values usually have a rather large standard deviation, and a satisfactory statistical treatment would require many more data points. It does not appear justified, therefore, to draw conclusions as to which extent exactly the 2 curves are different, but qualitatively the difference certainly is significant (covariant analysis).

The effect of temperature on the induction of DNA interstrand cross-links by cisplatin, which are rather persistent and, therefore, possibly involved in cytotoxicity and mutagenicity, was studied by comparing treatment at 32° and 40°C. The cross-links were determined with a sensitive, but indirect method, in which the elution of DNA through membrane filters under alkaline conditions is measured, after the induction of a known number of single-strand breaks by γ-rays. The retardation of the elution compared to that of DNA from cells not

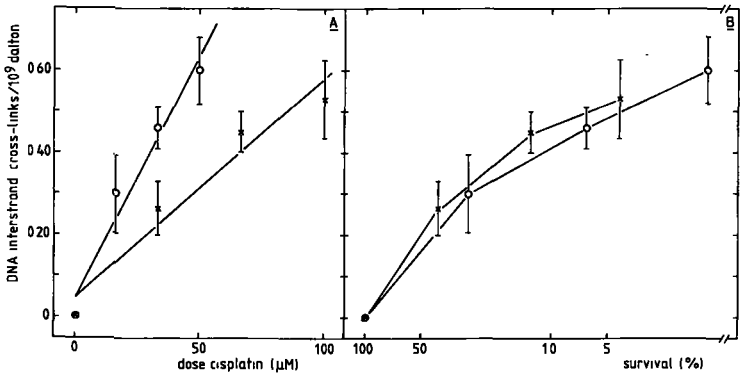

Figure 3: The amount of DNA interstrand cross-links in CHO cells after treatment with different concentrations of cisplatin for 1 h at 32° (X) or 40°C (O). The numbers of cross-links shown were determined after the cells had been given a 24-h post-treatment incubation at 37°C (immediately after treatment, only the values for cells treated at 40°C with 50 μM cisplatin (0.21 ± 0.08) significantly surpassed the detection limit). Cross-links ± s.e.m., expressed per 10^9 mol. weight of double stranded DNA, are plotted as a function of cisplatin concentration (3A) and against survival (read from graphs in Fig. 1) (3B). Slopes of the regression lines in 3A are: (32°) 0.00531 ± 0.00093 (n = 13); (40°C) 0.0118 ± 0.0017 (n = 12).

treated with cisplatin is a measure of the amount of cross-links. The cross-links were measured directly after the removal of the drug and after the cells had been kept at 37°C for 24 hr. After treatment at 32°C, no cross-links could be detected at t = 0; at 40°C, the highest concentration used caused a slight induction. After the 24-hr incubation, however, all treated cells contained measurable amounts of the adduct (Fig. 3A), in agreement with earlier observations that DNA interstrand cross-links show a delayed formation. The results are consistent with a linear dose-effect relationship. According to the slopes of the regression lines, induction at 40°C is 2.2 ± 0.5 times as high as at 32°C. This ratio is in agreement with the difference in total Pt-DNA-adduct formation (see Table I; at 33 μM, the concentration used in the measurements of total Pt, the cross-links ratio amounted to 1.72 ± 0.15). This result suggests that the formation of DNA interstrand cross-links is influenced by variation of treatment temperature in proportion to the effect on total adduct formation with DNA.

When the cross-links present at 24r h after treatment are plotted against survival after identical exposure, Fig 3B is obtained. The 2 curves are almost identical, which means that after equicytotoxic treatments the same amounts of DNA interstrand cross-links are present. This result supports the supposition that these cross-links are important lesions with regard to cell death. Because of the different influence of temperature when mutagenicity is compared with cytotoxicity, this result argues against a direct relationship between interstrand cross-links and mutation induction (Fig. 2B).

Discussion

A higher temperature during treatment of CHO cells with cisplatin results in a increased cytotoxicity of the drug, roughly in proportion to the increase in the number of Pt-DNA-adducts formed. This findings suggests that no substantial changes occur in the spectrum of DNA damages when the temperature is varied. However, owing to the possible

error in the various data, the results do allow also the interpretation that the lesions induced at 40°C are, on the average, somewhat more cytotoxic than those formed at 32°C. On the other hand, the fact that during a 24-hr repair period at 37°C adducts induced at 32° or 40°C are removed at the same rate, supports the former interpretation. Additional support comes from the study on DNA interstrand cross-links, which are assumed to be of biological importance: when treatment is at 40° instead of at 32°C, formation is enhanced in proportion to the increase in total Pt-binding as well as in cytotoxicity.

The results seem to contrast with findings by Meyn et al. (1980), who concluded that the fraction of DNA cross-links of the total Pt-DNA-adducts becomes smaller when treatment temperature is raised. However, in their study on the induction and repair, these authors did not discriminate between DNA interstrand and DNA-protein cross-links, which may explain the different outcome.

Recently, studies on the effects of temperature on the reaction of DNA with cisplatin in vitro, in which a variety of adducts were determined together comprising about 90% of total DNA-bound platinum, have shown that the spectrum of lesions remains unaltered between 37° and 50°C (Fichtinger-Schepman et al., 1984a).

In studies on mutation induction in E.coli, however, Brouwer et al. (1982) found strong indications for a selective increase in the formation of adducts on GCG sequences (but not on GAG) when treatment temperature was raised from 33° to 41°C. In this organism, therefore, a shift in the spectrum of lesions does appear to occur.

The induction by cisplatin of $HGPRT^-$ mutants in CHO cells did not show a strong influence of temperature. In relation to survival and adduct formation, however, treatments at 32°C appeared more mutagenic than those at 40°C. This result might indicate that in CHO cells, too, the spectrum of DNA-adducts induced is influenced by temperature, although not to such an extent that it became apparent from the other criteria investigated. Most likely, the Pt-DNA-adducts, responsible for the mutagenic effects in cells, are minor adducts in the whole spectrum of lesions formed in the DNA. The observed effect is in evident contrast to the results of Brouwer et al. (1982) in E.coli. The-

re, between 32° and 42°C the number of lacI mutants increased sharply with temperature, much more strongly than the cytotoxic effects. Apparently, the direction of the differential effects of temperature on the cytotoxicity and mutagenicity of cisplatin can change with the organism. The possibility cannot be excluded, however, that the mutations studied in E.coli and those determined in the present investigation originate from different lesions. Anyhow, it will be difficult, to draw general conclusions with regard to the influence of temperature on cisplatin treatments from the data that are presently available.

A remarkable observation was the effect of a preincubation (0.5 - 1 hr) of cisplatin with medium at the temperature of the subsequent exposure of the cells, which raised cytotoxicity by a factor of 2.5. This effect explains the difference between the D_0 (at 37°C) found in these experiments and the (lower) values of earlier studies, when the addition of cisplatin to medium before the prewarming period was customary (Plooy and Lohman, 1980; Plooy et al., 1984a = Chapter 3; Plooy and Lohman, 1984 = Chapter 2). The difference in cytotoxicity coincides with the effect on Pt-DNA-binding measured at 37°C. With freshly prepared solutions less Pt becomes bound to cellular DNA (Plooy et al., 1984b = Chapter 5) than with the preincubated solutions used in previous studies (Plooy et al., 1984a = Chapter 3; different by a factor of about 2.5). It is not clear which processes are responsible for the reactivity enhancement of cisplatin in a Cl^- and protein-rich environment (Prestayko et al., 1980).

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5. THE QUANTITATIVE DETECTION OF VARIOUS Pt-DNA-ADDUCTS IN CHINESE HAMSTER OVARY CELLS TREATED WITH CISPLATIN; APPLICATION OF IMMUNOCHEMICAL TECHNIQUES.

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Summary

With polyclonal antibodies raised against cis-$Pt(NH_3)_2$GuoGMP, small quantities of specific Pt-adducts could be detected in DNA from Chinese hamster ovary (CHO) cells treated with the antitumor agent cisplatin, after the DNA had been digested with nucleases and the degradation products separated by anion-exchange chromatography (FPLC). Directly after treatment with 83 μM cisplatin, resulting in 96 ± 3 x 10^{-6} platinum atoms bound per nucleotide, 35.9 ± 4.7% of the platinum was recovered as cis-$Pt(NH_3)_2$d(pGpG), derived from intrastrand cross-links on two neighboring guanines, 3.1 ± 1.6% as cis-$Pt(NH_3)_2$d$(GMP)_2$, the degradation product of interstrand cross-links on two guanines (0.07%, according to separate studies) and of intrastrand cross-links on two guanines separated by one or more bases. The immunochemical method was not sensitive enough for the detection of monofunctionally bound platinum on guanine residues. The amount of these adducts, present in the digests as $Pt(NH_3)_3$dGMP, could be established with atomic absorption spectroscopy (AAS) (38.5% of the total Pt-content of the DNA).

After a post-treatment incubation of the cells for 24 hr, the total amount of platinum decreased to 59 ± 0.5 x 10^{-6} atoms per nucleotide, indicating the removal of adducts. In the digests, cis-$Pt(NH_3)_2$d(pGpG) accounted for 46.6 ± 6.8% of the total Pt-content, cis-$Pt(NH_3)_2$d$(GMP)_2$ for 3.0 ± 0.9% (0.34% derived from DNA interstrand

cross-links). The amounts of monofunctional adduct had decreased to such an extent that the exact quantities (below 15%) could not be determined. According to AAS-asays, at the elution position of cis-Pt-$(NH_3)_2$d(pApG) a significant amount of Pt-product was present, both at t = 0 and 24 hr, but the signals did not allow quantitative evaluation (however, below 48% and 28%, respectively).

The possible role of the individual lesions in the DNA on the biological effects of this platinum compound in CHO cells is discussed.

Introduction

Cisplatin (cis-$Pt(NH_3)_2Cl_2$) is a potent genotoxic agent as well as an antitumor drug. It is used in the therapy of testicular and ovarian cancers, and of other tumors (Rosenberg, 1980; Carter, 1984). Both antitumor properties and genotoxicity of cisplatin are thought to result from the binding to cellular DNA (see reviews by Roberts and Thompson, 1979; Roberts, 1981); especially the N7 atoms of guanine and adenine are favorite target sites (Marcelis, 1982; Theophanides, 1980; Stone et al., 1974; Eastman, 1984; Macquet et al., 1984; Johnson, 1984; Marcelis and Reedijk, 1983; Fichtinger-Schepman et al., 1984a).

Cisplatin, with its two replaceable Cl^- ions, can form both mono- and bifunctional adducts. The bifunctional adducts, i.e. DNA interstrand cross-links, DNA-protein cross-links, DNA intrastrand cross-links, rather than the monofunctional adducts are believed to be responsible for the biological effects. DNA interstrand cross-linking takes place both in vitro (Girault et al., 1982) and in vivo (Zwelling et al, 1981; Pera et al., 1981; Roberts et al., 1982; Plooy et al., 1984a = Chapter 3; 1984b = Chapter 4). DNA-protein cross-links have been detected in eukaryotic cells (Pera et al., 1981; Kohn and Ewig, 1979; Banjar et al. 1984; Plooy et al., 1984a = Chapter 3) between DNA and histones (Lippard and Hoeschele, 1979; Scovell et al., 1984) and non-histone proteins (Filipski et al., 1983; Scovell et al., 1984).

Several types of DNA intrastrand cross-links have been detected

in DNA treated *in vitro*, such as binding of the platinum to two adjacent guanines (Fichtinger-Schepman *et al.*, 1982; 1984a; Caradonna *et al.*, 1982; Caradonna and Lippard, 1984), to adenine adjacent to guanine (Eastman, 1984; Fichtinger-Schepman *et al.*, 1984a; 1984c) and two guanines separated by one or more bases (Macquet and Butour, 1978; Johnson, 1982; Reedijk *et al.*, 1984; Fichtinger-Schepman *et al.*, 1984a). The induction of DNA intrastrand cross-links in cells, too, has been reported (Kelman and Buchbinder, 1978; Cohen *et al.*, 1980; Brouwer *et al.*, 1981; Caradonna and Lippard, 1984). Chelates of cisplatin within one base (the O^6 and N7 of guanine) have been found *in vitro* (Dehand and Jordanov, 1976), but there are no reports on chelation *in vivo*. Specific Pt-DNA-adducts probably are of essential importance for the antineoplastic and genotoxic action of cisplatin. Which are involved, and in which way, is still under investigation.

The detection of small amounts of Pt-adducts in DNA isolated from cells treated with biologically relevant doses of cisplatin, *i.e.* dosages allowing survival, demands very sensitive techniques. Recently, the required sensitivity has been approached with the application of specific antibodies directed to certain reaction products of DNA-damaging agents with nucleotides or nucleosides. Low levels of adducts were detected in DNA with the ELISA (enzym-linked immuno-sorbent assay) by using antibodies raised against such haptens coupled to immunoglobulins (Müller *et al.*, 1982). The method has become applicable to cisplatin-DNA adducts also, since antisera against cisplatin-treated DNA have become available (Malfoy *et al.*, 1981; Poirier *et al.*, 1982; Lippard *et al.*, 1983), as well as sera against Pt-containing haptens which mimic specific Pt-DNA-adducts (Fichtinger-Schepman *et al.*, 1984b).

With the rabbit antiserum, raised against the adduct *cis*-Pt-$(NH_3)_2$GuoGMP, fmol amounts of various Pt-DNA-adducts in DNA-digests can be measured, by using the competitive ELISA (Fichtinger-Schepman *et al.*, 1984b). The antibodies appear to be specific for platinum attached to two guanine residues, as both *cis*-Pt$(NH_3)_2$d$(GMP)_2$ and *cis*-Pt$(NH_3)_2$d(pGpG) could be detected at this low level, whereas the detection limit for cisplatin monofunctionally bound to guanine

($Pt(NH_3)_3dGMP$), as well as for the bifunctional adduct $Pt(NH_3)_2d(pApG)$ was about 1000 times higher. In digests of cisplatin-treated DNA, the first mentioned adduct may occur as the degradation product of DNA interstrand cross-links on two guanines and it may originate from intrastrand cross-links on two guanines separated by one or more bases ($\underline{cis}$-$Pt(NH_3)_2d(pG(pX)_npG)$); $\underline{cis}$-$Pt(NH_3)_2d(pGpG)$ is the degradation product of DNA intrastrand cross-links on two adjacent guanines.

In this paper the detection of various Pt-DNA-adducts in Chinese hamster ovary (CHO) cells treated with cisplatin is reported. After isolation and enzymatic digestion of the DNA, and after chromatographic separation of the degradation products, the Pt-containing (oligo)nucleotides were determined, both by the immunochemical method and by using atomic absorption spectroscopy (AAS). The results were compared with those of an earlier study in which we measured - via indirect procedures - the induction and repair of DNA interstrand and DNA protein cross-links in cells exposed to cisplatin (Plooy et al., 1984a = Chapter 3). On the base of this comparison the relevance of the detected Pt-DNA-adducts is discussed in relation to the biological effects found earlier (Plooy et al., 1984a = Chapter 3).

Materials and methods

Cells and cell treatment

Chinese hamster ovary (CHO) cells were cultured in monolayers in Ham's F10 medium (Flow, laboratories), supplemented with 15% newborn calf serum (NCS, Flow), and handled as described before (Plooy et al., 1984a = Chapter 3). Cells were plated in 15-cm Petri dishes (Greiner) and grown until they reached a density of 1-2 x 10^7 cells. Then they were treated in the dark, for 1 hr at 37°C, with cisplatin (synthesized in the department of Chemistry (State University Leiden, The Netherlands; stock solutions were freshly prepared in medium and diluted with F10 medium containing 7.5% NCS to the desired concentration). Subsequently, the cells were washed twice with PBS (phosphate buffered saline: 8.1 mM Na_2HPO_4 (Merck), 1.5 mM KH_2PO_4 (Merck), 0.14 M

NaCl (Merck), 2.6 mM KCl (Merck)) and fresh medium (with 15% NCS) was given. The cells were allowed to grow further if post-treatment incubation was required. After such a repair period, or immediately after treatment, the cells were scraped off from the dishes with a rubber policemen, collected, counted and pelleted by centrifugation. The freshly prepared (or deep-frozen) pellets were used for DNA isolation.

DNA isolation

Freshly (preferable to frozen) pellets (1-2 x 10^7 cells), freed from adhering supernatant by dripping, were homogenized in 100 µl of water by high speed vortexing (20°C, at 1000 rpm; Vortex-Genie; Bohemia, NY, USA). Simultaneously, 1 ml of methanol (Fisons) was added dropwise, very slowly, especially in the beginning. Then 1 ml chloroform (Fisons) was added to the cells and they were vortexed for 5 min, followed by the addition of another 3 ml methanol. The precipitated cellular macromolecules were collected by centrifugation, then resuspended homogeneously in 5 ml of methanol, and spun down again. The methanol was discarded, but complete drying of the pellet was avoided. One ml of buffer A (10 mM Tris-HCl (Baker), 150 mM NaCl, 10 mM Na_4EDTA (Siegfried AG), pH 7.5), supplemented with 2 mg/ml RNAse 1 (Sigma, heat inactivated by 5 min 80°C) was dripped onto the pellet under vigorous vortexing. The fine suspension was mixed with 10 µl of SDS (Sigma; 20% in water) by very mild shearing through a needle (Becton Dickinson; 1.1 x 40 mm). After 30 min at 37°C, 1 mg of proteinase K (in 20 µl deionized water; Merck, m24568) was added to the completely clear and viscous solution, which after shearing (to make a homogeneous mixture) was incubated for another 60 min at 37°C. Thereafter, the incubate was mixed with 2 ml of 2-propanol (Fisons) at 0°C, resulting in the immediate precipitation of the DNA. After centrifugation and washing with methanol:water (3:2; v/v), the DNA was dissolved in deionized water and dialysed overnight (37°C) against 100 mM NH_4HCO_3 (Merck) to prevent conversion of monofunctional Pt-DNA-adducts to bifunctional ones (Fichtinger-Schepman et al., 1982; 1984d; in the preceding steps the through-reaction was blocked by the high salt concentration). Subsequently, the DNA sample was dialysed for 24 hr

against deionized water (20°C), precipitated with 2-propanol at 0°C. The pelleted DNA was dissolved in 0.5 ml water and the DNA content (A_{260nm}) was measured with a Perkin Elmer (Lambda 5) spectophotometer.

Enzymatic degradation of DNA

To 180 µl DNA solution (250-400 µg DNA) 20 µl of buffer B (100 mM Tris-HCl, 40 mM $MgCl_2$ (Merck), 1 mM Na_4EDTA, pH 7.2) was added and supplemented with 4.8 µl 10 mM $ZnSO_4$ (Sigma), 4.0 µl DNAse 1 solution (1 mg/ml = 3000 U/ml; Sigma; E.C.3.1.21.1) and 20 µl P1 nuclease (1 mg/ml = 300 U/ml; Boehringer; E.C.3.1.4.-). This mixture was incubated at 37°C for 16 hr, whereafter 6 µl proteinase K (83 mg/ml) was added followed by incubation for another 2 hr at 37°C. Subsequently, the digest was heated for 3 min at 100°C and centrifuged in an Eppendorf centrifuge for 10 min. Under these conditions the DNA becomes optimally digested to nucleotides and Pt-containing (oligo)nucleotides (Fichtinger-Schepman et al., 1984a).

Fast-protein-liquid-chromatography (FPLC) on Mono Q

The digestion products of Pt-DNA samples were separated by anion-exchange column chromatography. An FPLC system (Pharmacia) with a Mono Q column (Pharmacia) was used, which was equipped with a detector of UV-absorbance (254 nm) and a Spectra Physics SP 4100 computing integrator. The elution, at 1 ml/min, was performed with mixtures of 12.5 mM Tris-HCl, pH 8.6 (Solvent A) and 12.5 mM Tris-HCl, 1 M NaCl, pH 8.6 (Solvent B) as follows: 1 min isocratic at 5% B; a linear gradient from 5 to 7.5% B in 3 min; 3.5 min isocratic at 7.5% B; a linear gradient from 7.5 to 10% B in 3 min and finally a linear gradient from 10 to 50% B in 2 min. After each elution the Mono Q column was washed with 100% B for 2.5 min and 100% A for 2.5 min at a flow rate of 1.75 ml/min (Fichtinger-Schepman et al., 1984a). Fractions of 0.2, 0.3 or 0.5 ml were collected. After calibration with commercial (Sigma) dCMP, dAMP, TMP and dGMP as reference nucleotides, the amounts of mononucleotides present in the injected digests of DNA from CHO cells (GC content of CHO DNA = 44%) were calculated from the peak area data (A_{254nm}) of the chromatograms.

Competitive ELISA

Salmon sperm DNA (NBC) which had been treated with cisplatin (75 µg/ml for 5 hr at 50°C) was heated for 10 min to 100°C and cooled down quickly, to make it single stranded. This single stranded DNA was used to coat Titertek polyvinyl chloride microtiter plates (96 wells, Flow lab): 100 µl of the DNA solution per well at a concentration of 1 µg/ml for 16 hr at 4°C (see Fichtinger-Schepman et al., 1984b). Thereafter, the microtiter plates were washed 5 times with 150 µl 0.05% T20 solution (T20 = polyoxyethylene sorbitan monolaurate; Sigma) with the aid of a Titertek microplate washer (Flow lab). Then, each cup of a plate was incubated with 125 µl PTF buffer (0.05% T20 in PBS supplemented with 1% foetal calf serum (FCS, Flow)) for 1 hr at 37°C. Subsequently, the plate was washed with 0.05% T20. Dilutions of the competitor, made in PTF buffer, were preincubated (150 µl) for 15 min at 20°C with 150 µl of a 1/50,000 dilution of the rabbit antiserum (W101; Fichtinger-Schepman et al., 1984b), added to the wells in duplicate (100 µl) and incubated for 90 min at 37°C. Then, the plates were washed 5 times with 0.05% T20 and incubated for another hour at 37°C after the wells have been provided with 100 µl goat-anti-rabbit serum, alkaline phosphatase-coupled (Miles, nr 61-275; 1/1000 dilution in 0.05% T20 + 0.1% gelatine in PBS, supplemented with 5% FCS). After washing 5 times with 100 µl 0.05% T20 and 3 times with 100 µl DEA solution (100 mM diethanolamine; Merck, pH 9.8), the wells were filled with 100 µl of the substrate PNP (p-nitrophenol phosphate-Na_2. $6H_2O$, 1 mg/ml in 10 mM DEA, 1 mM $MgCl_2$, pH 9.8; Boehringer). The plates were kept for 16 hr at 25°C in the dark. Then, the p-nitrophenol released by the enzymatic reaction in each of the cups was measured with a Titertek Multiskan Automatic Plate Reader (Flow lab; A_{405}nm). To determine the 0% and 100% inhibition values of the preincubate, in each microtiter plate 8 wells were incubated with PTF buffer without anti-Pt-antibodies (100% inhibition in preincubate) and 8 wells with the antibodies without competitor (0% inhibition). To calculate the amount of Pt-adducts present in the sample(s), a calibration curve was constructed with known amounts of reference Pt-adducts as competitor (Fichtinger-Schepman et al., 1984a); these calibration samples were

tested on the same microtiter plate as the unknown sample. Various dilutions of the experimental samples were used as competitors; from the results the dilution giving 50% inhibition was estimated. The corresponding amount of competitor was read from the calibration curve.

Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) was performed on the DNA solutions as described before (Plooy et al., 1984a = Chapter 3).

Results

In the evaluation of the immunochemical approach to the quantitative detection of cisplatin-induced DNA-adducts in mammalian cells, a treatment expected to result in well measurable amounts of Pt-containing products in the digests of DNA was determined. To this aim, CHO cells were exposed to 83 μM cisplatin at 37°C for 1 hr (approximately 1% survival). DNA was isolated either directly after the removal of the drug (0/83 DNA), or after the cells had been given an additional incubation of 24 hr at 37°C in the absence of cisplatin, to allow repair processes and the formation of bifunctional adducts to proceed (24/83 DNA). As a control for aspecific effects in the immunochemical detection, DNA from untreated cells was isolated and analysed (0/0 DNA). The still reactive monofunctional adducts possibly present in the DNA's were prevented from further reaction by treatment with NH_4HCO_3 (see Fichtinger-Schepman et al., 1984d). After enzymatic digestion with DNAse I and P1 nuclease, the mixtures of mononucleotides and Pt-containing (oligo)nucleotides were chromatographed on a high-resolution anion-exchange column. The total amounts of nucleotides injected on the column were calculated from the A_{254nm}-values measured for the various peaks in the eluate. In the isolated DNA's, the Pt-content was determined by AAS, and expressed relative to the DNA content (based on A_{260nm} measurements), in the r_b value (Pt-atoms per nucleotide). From the r_b value and the amount of nucleotides elu-

ted from the column, the total amount of platinum expected in the eluate of an injected sample was calculated.

Fig. 1A represents the A_{254nm} elution pattern of a digested 0/83 DNA sample; the peaks of dCMP (eluted at 5.2 min), dAMP and TMP (together at 6.0 min) and dGMP (10.9 min) are well recognizable. Comparable results were obtained with digests of 0/0 and 24/83 DNA's (not

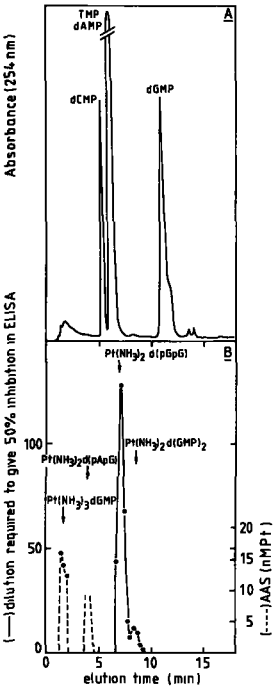

Figure 1: Chromatographic separation of mononucleotides and Pt-containing products present in digested DNA from cisplatin-treated CHO cells. CHO cells were treated with 83 µM cisplatin for 1 hr at 37°C. DNA was isolated and enzymatically degraded. The digest was chromatographed on an anion-exchange column (MonoQ; Pharmacia FPLC system). Elution was at 1 ml/min. Fractions of 0.2 - 0.5 ml were collected. The A_{254nm} of the eluate was monitored (panel A), showing the peaks of the four nucleotides. Fractions corresponding to the elution position of four identified Pt-products derived from known adducts in the DNA, were studied for the presence of these products by competitive ELISA, with an antiserum raised against cis-$Pt(NH_3)_2$GuoGMP, or by Pt-determination via AAS (panel B). The ELISA results are expressed as the dilution of the fractions giving 50% inhibition (left ordinate), the AAS results show the Pt-concentrations. For numerical results see Table I.

shown). The possible presence of Pt-DNA-adducts could not be discovered by their UV-absorbance, because of the very small quantities. However, their expected positions in the elution pattern could be established by calibrating the column with a digest of salmon sperm DNA that had been treated *in vitro* with a high dose of cisplatin (Fichtinger-Schepman *et al.*, 1984a): $Pt(NH_3)_3$dGMP eluted after 1.6 - 2.0 min; *cis*-$Pt(NH_3)_2$d(pApG) after 3.6 - 4.2 min; *cis*-$Pt(NH_3)_2$d(pGpG) after 6.0 -7.8 min and *cis*-$Pt(NH_3)_2$d$(GMP)_2$ after 8.2 - 9.4 min. The fractions of the eluates were screened for the presence of these Pt-DNA-adducts in the competitive ELISA, with the antiserum raised against *cis*-$Pt(NH_3)_2$GuoGMP. To eliminate the influence of possible differences in microtiter plates, corresponding fractions of the 0/0, 0/83 and 24/83 DNA-digests were tested on the same ELISA plate, together with suitable reference Pt-DNA-adduct(s). Various dilutions of each fraction were tested, to determine the concentration at which 50% inhibition was obtained.

The eluate of the digested DNA from untreated cells (0/0 DNA) did not inhibit at all. Some fractions of the 0/83 DNA and the 24/83 DNA digests, on the other hand, were clearly positive in the competitive ELISA. The positions of these fractions in the eluate fell in the region where *cis*-$Pt(NH_3)_2$d(pGpG) and *cis*-$Pt(NH_3)_2$d$(GMP)_2$ had been found to appear. In Figure 1B, the dilutions of the fractions of a 0/83 DNA-digest giving 50% inhibition have been plotted against the elution time. The 24/83 DNA digests (not shown) gave similar results. With the help of inhibition curves of the reference adducts, the amount of adduct present in each fraction of the peak region was determined; from the results, the amounts of the adducts in the DNA digests were computed. Table I summarizes the results obtained with one of the 0/83 DNA's.

The fractions collected in the first 4.5 min of the elution, supposed to contain $Pt(NH_3)_3$dGMP (1.6 - 2.0 min) and *cis*-$Pt(NH_3)_2$-d(pApG) (3.6 - 4.2 min), did not show competition in the ELISA. The Pt-content of those fractions was measured with AAS, because for these adducts AAS has a lower detection limit than the ELISA. In this way it was possible to detect and quantify the presence of the monofunctional

adduct on guanine in 0/83 DNA (see Figure 1B and Table I for detailed results). The fractions expected to comprise the adduct cis-$Pt(NH_3)_2$-d(pApG), however, gave such weak AAS signals that quantification was not possible although the response was significantly above the background. Since the quantitative detection of Pt in these materials has a lower limit of 10 nM, the total amount of the adduct on d(pApG) possible present in this "peak" (volume 1 ml) has to be less than 10 pmol. This means that in the 0/83 DNA, less than 34% of the DNA-bound platinum may have occurred as an intrastrand cross-link on the sequence pApG. However, the three quantitatively identified adducts together already account for 75.5% of the DNA-bound platinum, which reduces the maximal contribution of this cross-link to 24.5%.

In the digests of 24/83 DNA, too, cis-$Pt(NH_3)_2$d(pApG) was found present in amounts below the limit for quantitative detection. After the 24 hr repair period, the monofunctional adducts on guanine had

Table I Pt-DNA-adducts in CHO cells treated with 83 μM cisplatin, detected directly after the removal of the drug

	50% I [1] (fmol)	elution time [2] (min)	adduct [3] (pmol)	adduct [3] (%)	a/n [4] (x 10^6)
ELISA					
cis-$Pt(NH_3)_2$d(pGpG) [5]	4.9; 7.4 [6]	6.5 - 8.0	9.6	32.5	30.0
cis-$Pt(NH_3)_2(dGMP)_2$ [5]	9.0	8.0 - 9.4	1.44	4.8	4.4
AAS					
$Pt(NH_3)_3dGMP$ [5]		1.2 - 2.0	11.4	38.5	35.5
cis-$Pt(NH_3)_2$d(pApG) [5]		3.5 - 4.5	< 10 [7]	< 34 [7]	< 31 [7]

Pt-containing products present in digests of DNA from cisplatin-treated CHO cells were separated by anion-exchange chromatography and quantitatively determined by ELISA or AAS. For details, see legend to Fig. 1 and Methods section. The isolated DNA had an r_b value of 92 x 10^{-6}. The chromatographed sample contained 321 nmol of nucleotides (according to the A_{254nm} peaks in Fig. 1A) and 29.6 pmol of platinum compounds. 1) Amounts of the Pt-compound used for construction of the calibration graph required to give 50% inhibition; 2) Elution time of the material used for the determination (see Fig. 1); 3) The amount of Pt-compound in the total peak, according to ELISA or AAS-assay, absolute (pmol) and relative to total platinum injected (%); 4) a/n: amount of the adduct concerned per nucleotide in the DNA; 5) The four Pt-products identified in the eluate, originating from (top to bottom) intrastrand cross-links on two adjacent guanines; intrastrand cross-links on not adjacent guanines and interstrand cross-links on two guanines; monofunctionally bound platinum on guanine; intrastrand cross-links on adenine adjacent to guanine; 6) ELISA was repeated, with extended dilution range; 7) The AAS signal was too weak for reliable quantitative evaluation.

greatly decreased in number, to such an extent that quantitative detection was no longer possible. For both types of adducts, therefore, only maximal contributions could be determined.

A survey of the results obtained with the digests of 0/83 DNA's and 24/83 DNA's is given in Table II. In this table the results of a previous study are included, in which the DNA interstrand cross-links and DNA-protein cross-links induced in CHO cells by cisplatin were determined with a biophysical method. The data show that the main adducts present directly after treatment are: monofunctionally bound platinum on guanine (38.5%) and intrastrand cross-links on d(pGpG) (35.2%). Much smaller is the contribution of the interstrand cross-links on 2 guanines and the intrastrand cross-links on two guanines separated by one or more bases (together 3.1%). The intrastrand cross-links on the base sequence d(pApG) probably account for a large proportion of the remaining 23%.

During the repair period of 24 hr, the total amount of platinum bound to DNA decreased to about 60%, mainly due to the removal of monofunctionally bound platinum of which at least 76% disappeared. The amount of intrastrand cross-links on pGpG sequences decreased much less and, consequently, after 24 hr this adduct becomes the predominant lesion, representing about one-half of the total amount of Pt bound to DNA. For the other intrastrand cross-links, on pApG, no accurate figures can be given (less than 48%); probably they become the second important adduct after 24 hr. The contribution of cis-$Pt(NH_3)_2$-$d(GMP)_2$ remains at about the same level (3.0%).

According to the independent determinations of interstrand cross-links (see Table II), only a minute fraction of cis-$Pt(NH_3)_2d(GMP)_2$ may originate from these lesions (maximally 0.02 at t = 0). As these adducts are the only ones that increase in number during the post-treatment incubation, this proportion becomes much larger with time (0.11 at t = 24, if all interstrand cross-links connect two guanines). This result suggests that most of the cis-$Pt(NH_3)_2d(GMP)_2$ is derived from intrastrand cross-links on $d(pG(pXp)_nG)$ sequences, and that these cross-links are less persistent than the cross-links between the two opposite strands in the DNA helix (interstrand cross-links).

Table II Pt-DNA-adducts present in CHO cells directly after treatment with cisplatin (t = 0) and at 24 hr after treatment (t = 24)

	t = 0		t = 24	
	adduct (%)	a/n ($\times 10^6$)	adduct (%)	a/n ($\times 10^6$)
total amount of Pt-adducts [1]	100	92; 101	100	59; 58
cis-$Pt(NH_3)_2d(pGpG)$ [2]	35.9 ± 4.7	35.2 ± 4.8	46.6 ± 6.8	27.2 ± 4.1
cis-$Pt(NH_3)_2d(GMP)_2$ [2]	3.1 ± 1.6	3.0 ± 1.4	3.0 ± 0.9	1.8 ± 0.5
$Pt(NH_3)_3dGMP$ [1]	38.5	35.5	< 14.5	< 8.5
cis-$Pt(NH_3)_2d(pApG)$ [1]	< 34	< 31	< 48	< 28
interstrand cross-links [3]	0.07	0.07	0.34	0.20
DNA-protein cross-links [3]	0.11	0.11	0.13	0.07

The amounts of various Pt-DNA-adducts in DNA from CHO cells treated with cisplatin, were assayed on the basis of Pt-products present in the digests obtained after nucleolytic degradation. Treatments and assays were as described in the legends to Fig. 1 and Table I. After treatment with 83 μM cisplatin, the cells were either used directly for DNA isolation (t = 0), or they were incubated at 37°C for 24 hr with fresh medium before DNA was isolated (t = 24). The amounts of Pt-products determined in the different peaks of the eluates have been expressed relative to the total amount of Pt-adducts injected (= 100%) and relative to the total amount of mononucleotides present in the eluate (from A_{254nm}, see Fig. 1A) (a/n). The ELISA results obtained (for both t = 0 and t = 24) with 4 column eluates, derived from 2 batches of treated CHO cells, have been combined. The averaged values for a/n and % adducts are given ± s.d. 1) Results from AAS-assays. When the AAS signal was to weak for reliable quantitative evaluation, the maximal amount according to the conditions of the assay is given; 2) Results from ELISA; 3) Results obtained with a biophysical method, in DNA from identically treated CHO cells (data from Plooy et al., 1984a = Chapter 3). For the interstrand cross-links, these data were combined with those of other studies (Plooy et al., 1984b = Chapter 4; Plooy unpublished data). They pertain to DNA's from cisplatin-treated CHO cells with an r_b-value of 96×10^{-6} at t = 0 (by interpolation); the ranges for a/n were $(0.05 - 0.10) \times 10^{-6}$ (t = 0) and $(0.17 - 0.23) \times 10^{-6}$ (t = 24).

Discussion

The results presented in this paper demonstrate that - in principle - Pt-DNA-adducts present in mammalian cells that have been exposed to biologically relevant concentrations of cisplatin, can be de-

termined quantitatively, by a combination of chromatographic separation of the products after nucleolytic digestion of the DNA and immunochemical detection. For two products, the presently available antiserum already allows a rather sensitive detection (down to 1 adduct per 5 x 10^7 nucleotides when DNA from 10^7 - 10^8 cells is used). Improvement of the quality of the antiserum by replacing the polyclonal rabbit serum by high affinity monoclonal antibodies, is expected to lead to a substantial increase of this sensitivity. Development of antisera directed against other determinants will allow an equally sensitive determination of the other adducts. Our results bring within reach important practical applications, such as the monitoring of patients treated with cisplatin for the biological effectiveness of the treatment, by determination of the Pt-DNA-adducts in nucleated blood cells.

Furthermore, the ability to measure quantitatively Pt-DNA-adducts opens the possibility to study induction and repair of specific adducts in relation to biological effects, such as cell killing, induction of gene-mutations or chromosomal abnormalities, inhibition of DNA synthesis, etc.

The methods applied in the described experiments have been developed in studies with salmon sperm DNA treated *in vitro* with cisplatin, *e.g.*, with 33 μM for 2 hr, at 37°C, resulting in the high r_b value of 0.01 (Fichtinger-Schepman *et al.*, 1984a). In this DNA, the same adducts were found; the relative amounts, however, were different. Monofunctional adducts, being a major product in CHO cells (38.5% at t = 0) accounted for only 11% of ssDNA-bound platinum, whereas the intrastrand cross-links on two adjacent guanines were the dominating adducts (60 - 65%). An explanation might be that the second step in the reaction of cisplatin with DNA, the convertion of a monofunctional adduct into a cross-link, which appeared to proceed very quickly *in vitro*, is a much slower process inside the cell nucleus.

The approximately 3% *cis*-$Pt(NH_3)_2d(GMP)_2$ found in the CHO-DNA digests are composed of the degradation products of interstrand cross-links and of intrastrand cross-links on two not-adjacent guanines. Earlier results indicate that (at t = 0) DNA interstrand cross-

links amount to about 0.07% of the total amount of Pt-DNA-adducts. In vitro, cisplatin binds preferentially to guanines in DNA, especially in cross-linking reactions (Marcelis, 1982). Assumed that the preference exists also when the reaction occurs inside the cell, which appears plausible, the 0.07% interstrand cross-links will consist almost completely of connections between two guanines. Anyhow, about all cis-$Pt(NH_3)_2d(GMP)_2$ found at t = 0 is derived from the intrastrand connections. According to this reasoning, the amount of adducts per 10^6 nucleotides (a/n) of cis-$Pt(NH_3)_2d(pG(pX)_npG)$ drops from 2.9 $\pm$ 1.4 to 1.6 $\pm$ 0.5 between t = 0 and t = 24 (see Table II). Whether this reduction purely reflects the repair of the original intrastrand cross-links, or is the resulting effect of a combination of delayed formation and removal (as in the case of the interstrand cross-links; see Plooy et al., 1984a = Chapter 3) will have to be shown in more detailed time course experiments.

The question remains whether any conclusions are allowed with regard to the biological importance of the various Pt-DNA-adducts. Monofunctional adducts appear to be "repaired" rapidly. Moreover, the trans-analogue of cisplatin has been reported to induce many more monofunctional adducts, but has much less biological activity. Therefore, most of the effects will be caused by bifunctionally bound cisplatin. The cross-link on two adjacent guanines appears an important lesion, both in quantity (36% at t = 0, 47% at t = 24) and in persistence (over 50% still present after 24 hr). Possibly, the cross-links on d(pApG), though somewhat less abundant (< 24% at t = 0), are very similar in behaviour and in importance. Little is known about the noxiousness of these intratsrand lesions. They might be relevant for the biological effects of cis-platinum compounds, since - for steric reasons - the trans-isomers cannot form these adducts. In E.coli, cross-links on d(pGpG) appear not to be involved in the induction of base-pair substitution mutations (Brouwer et al., 1981), but this does not exclude other harmful effects.

A second type of DNA intrastrand cross-links, which do induce base-pair substitutions in E.coli (Brouwer et al., 1981) is the cross-link on two guanines separated by one base. Very likely, this lesion

is the main origin of the cis-$Pt(NH_3)_2d(GMP)_2$ found in CHO cells, although the exact amount cannot be calculated, since the contribution of intrastrand cross-links between somewhat more remote nucleotides is not known. However, it represents only a small fraction of the total Pt-DNA-adducts in the CHO cells (at most 3%). To see whether a relationship between this, probably mutagenic, lesion and the mutation induction at the HGPRT locus in CHO cells (see Plooy et al., 1984a = Chapter 3) could be found, the frequency of occurrence of both per HGPRT gene (supposed to exist of 2000 nucleotides, two DNA strands taken together) in cells treated with 83 μM cisplatin were calculated. Mutation induction will amount to about 150 mutants per 10^6 (surviving) cells - i.e., 0.15×10^{-3} per HGPRT gene -, the maximal number of cross-links on $d(pG(pX)_npG)$ sequences to 5.8×10^{-3} and 3.2×10^{-3} per gene, at t = 0 and t = 24, respectively. If these cross-links were responsible for the mutations that eliminate HGPRT activity, one in 20 - 40 lesions would be sufficient to cause this effect.

Other potential candidates for mutagenic lesions induced by cisplatin are the DNA-protein and DNA interstrand cross-links. Both have been shown to be rather persistent in CHO cells, in contrast to those induced by the trans-isomer (Plooy et al., 1984a = Chapter 3). The repair of DNA interstrand cross-links is thought to require some kind of post-replication repair (Fujiwara et al., 1977), because the damage is situated at nearly opposite bases of both sides of the DNA template. This process may very well induce errors during the required recombinational event, but unrepaired lesions, too, might give rise to mutations. The amount of DNA interstrand cross-links per HGPRT gene at t = 24 is about 0.40×10^{-3} (the level at t = 0 is roughly one-half this value, maximally (t = 6), this value is twice as much; see Plooy et al., 1984a = Chapter 3). Comparison with the mutation frequency suggests that these lesions might be highly mutagenic. However, the observation that the amount of interstrand cross-links induced by cisplatin at various temperatures (Plooy et al., 1984b = Chapter 4) did not coincide with the mutation induction, does not support this conclusion, but they seem to be related to the cytotoxicity (Plooy et al., 1984b = Chapter 4).

The DNA-protein cross-links also occur with about the same frequency as HGPRT mutations (ca. 0.1 x 10^{-3}). Since these lesions do not damage the genetic information in the opposite DNA strand, they are not expected to be extremely effective in the induction of point mutations. They may cause difficulties, however, at the time the cells come in mitoses (Houssier et al., 1983), so that the DNA-protein cross-links could account for all kinds of chromosomal abnormalities (Scovell et al., 1984), such as micronuclei induction (Bonatti et al., 1983).

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6. FORMATION AND REPAIR OF DNA INTERSTRAND CROSS-LINKS IN RELATION TO CYTOTOXICITY AND UNSCHEDULED DNA SYNTHESIS INDUCED IN CONTROL AND MUTANT HUMAN CELLS TREATED WITH CIS-DIAMMINEDICHLOROPLATINUM(II).

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Summary

A comparative study was performed with a variety of human cell lines on the effects of treatments with cis-diamminedichloroplatinum(II) (cisplatin) on cell survival and the induction of unscheduled DNA synthesis (UDS). In addition to control fibroblasts (Han; MB), cell lines defective in DNA repair were used (xeroderma pigmentosum, XP(A) and XP(F), and Fanconi's anemia, FA), as well as cells deficient in arylsulfatase A (79RD172; 75RD164). Ultraviolet light (UV) and mitomycine C were included in this study as model DNA-damaging agents. Furthermore, induction and repair of DNA interstrand cross-links caused by cisplatin were studied.

As for survival, only XP cells were abnormally sensitive to UV, and only FA cells to mitomycine C. To cisplatin, however, all mutants tested were more sensitive (2 to 5 times) than normal cells. UDS induction by UV was strong in all but the XP cells; the other two agents did not induce UDS. Induction of DNA interstrand cross-links by cisplatin was linear with dose. Formation continued up to 18-24 hr after treatment. During this period all cells but the RD mutants responded very similarly. In RD cells, much less cross-links were induced, which were repaired rapidly. In FA cells, accumulation continued for at least 96 hr, in the other cells (most of) the cross-links had been removed after that period.

The cisplatin-induced DNA interstrand cross-link is proposed as an important potentially lethal lesion, in view of its persistence in the highly sensitive FA cells. Furthermore, the possible involvement of certain steps of the long-patch excision repair pathway in the removal of this lesion is considered. The sensitivity of RD cells to cisplatin is attributed to cytoplasmic effects, rather than to chromosomal damage.

Introduction

Platinum coordination compounds, especially cis-diamminedichloroplatinum(II) (cisplatin), are very effective when applied in chemotherapy of a variety of human neoplasms (Burchenal, 1978; Prestayko et al., 1980). Beside antitumor activity, cisplatin possesses genotoxic properties. Its reactivity towards DNA in cells is thought to be responsible for the antitumor effects of cisplatin (Roberts and Thompson, 1979; Macquet et al., 1984). Cisplatin is a bifunctional agent that can form various reaction products with DNA, such as cross-links between DNA and protein (Houssier et al., 1983), and DNA inter- and intrastrand cross-links (Zwelling et al., 1979a; Marcelis and Reedijk, 1983). In addition, monofunctional Pt-DNA-adducts are formed, which are supposed to be an intermediate for bifunctional binding (Marcelis, 1982). The DNA interstrand cross-links probably are formed between two N7 sites of guanines (Marcelis and Reedijk, 1983). Only recently, the detection of DNA intrastrand cross-links induced by cisplatin in cells has become possible (Brouwer et al., 1981; Fichtinger-Schepman et al., 1984, Plooy et al., 1984b = Chapter 5). To what extent the various cross-links are important for the antineoplastic and genotoxic action of cisplatin is not fully clarified yet, and in some cases conflicting results have been reported (Zwelling et al., 1979b; Zwelling and Kohn, 1979; Zwelling et al., 1980; Pera et al., 1981; Roberts et al., 1982; Strandberg et al., 1982). In recent studies with Chinese hamster ovary (CHO) cells we found indications that it is not so much the initial level of DNA interstrand cross-links which correlates with cytotoxicity, but rather their persistence (Plooy et al., 1984a = Chapter 3).

Important information on the biological importance of certain DNA lesions can sometimes been obtained through the use of cells with a known deficiency in one of the systems for the repair of damaged DNA. Mutant cell lines of human origin that are relevant for the subject of this paper are cells isolated from xeroderma pigmentosum (XP) patients and those derived from persons suffering from Fanconi's anemia (FA). XP cells are thought to be defective in one or more genes coding for proteins involved in excision repair (Lehmann, 1982; Kraemer, 1983); they are extremely sensitive to UV-irradiation (Friedberg et al., 1979). Several complementation groups have been identified with different UV-sensitivities (Friedberg et al., 1979; Zelle, 1980; Lehmann and Karran, 1981). XP cells of complementation group A are more sensitive to UV than are group F XP cells, because they are more defective in their repair systems (Lehmann, 1982). Cells derived from FA patients are abnormally sensitive to cross-linking agents (Fujiwara et al., 1977; Ishida and Buchwald, 1982; Sognier and Hittelman, 1983). FA cells are thought to be defective in DNA interstrand cross-link repair (Sasaki, 1975).

Numerous cell lines exist which were isolated from patients with diseases caused by a deficiency in other factors than DNA repair. Two of these cell lines (79RD172; 75RD164) were included in this study. They were derived from mucolipidosis II patients and were shown to have an enzyme deficiency (arylsulfatase A) resulting in intralysosomal storage of possibly harmful compounds (Halley, 1980). RD cell lines behave like control lines with regard to UV-irradiation (Zelle, 1980) and were used as such, also in the present study. These cells became more interesting, however, when they appeared to be over-sensitive to cisplatin.

In the investigations presented here, the mutant cells and control human cells were compared with respect to the effects of cisplatin. Two model agents were included, UV as an inducer of intrastrand cross-links on adjacent bases (pyrimidine dimers; Hanawalt et al., 1979) and mitomycine C, which induces predominantly DNA interstrand cross-links (Iyer and Szybalski, 1964; Lown, 1979).

In the comparative study the cytotoxicity and the induction of unscheduled DNA synthesis (UDS) were determined after treatment of the

various cell lines with these agents. Furthermore, the induction and repair of DNA interstrand cross-links after treatments with cisplatin were studied.

Material and Methods

Cell lines

XP2CA (XP(A)), and XP126Lo (XP(F)) are xeroderma pigmentosum cell lines classified in complementation groups A and F, respectively (Zelle, 1980); The Fanconi's anemia (FA) cell line JaVo was kindly provided by dr. F. Arwert (Free University Amsterdam, The Netherlands); 79RD172 and 75RD164, both cell lines derived from mucolipidosis patients, were kindly provided by dr. A. Westerveld (Erasmus University Rotterdam, The Netherlands); 82MB2 (MB), obtained from dr. L. Roza (Medical Biological Laboratory TNO, Rijswijk, The Netherlands), and Han are cell lines originating from healthy persons. All cell lines were primary fibroblast cultures; the cells were not used beyond 25 passages.

Cell culture

The fibroblasts were cultured in flasks (75-cm^2, Costar) in Ham's F10 medium (Flow laboratories), supplemented with 1 mM L-glutamine (BDH, Poole, UK), penicillin (100 U/ml, Gist-Brocades NV, Delft, The Netherlands), streptomycine (100 µg/ml, Gist-Brocades NV) and 15% foetal calf serum (FCS, Flow laboratories) at 37°C in an incubator (Hereaus, Hanau, BRD) with a humidified atmosphere of 95% air and 5% CO_2. Cell handlings were performed in a laminar down-flow system (Microflow Pathfinder Ltd, Fleet, UK) under yellow light (filtered T.L. light; Philips, Eindhoven, NL; wavelength > 525 nm). The cells were subcultured every week in 1:2 or 1:3 dilutions by trypsinization.

Cell treatment

Cisplatin, synthesized by the group of Prof. dr. J. Reedijk (State University Leiden, The Netherlands; Marcelis, 1982), was dis-

solved in F10 medium containing 7.5% FCS just before use. The cells were treated with cisplatin in the dark, at 37°C for 1 hr. After this period the platinum-containing medium was sucked off and the cells were washed twice with PBS (phosphate buffered saline; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl). When post-treatment incubation was required, fresh medium (15% FCS) was added to the cells and incubation was continued for the time periods indicated (t = 0: directly after the removal of the drug). Cells were further processed as indicated in the various test protocols.

UV-irradiation at 254 nm with a 15 W low-pressure mercury, germicidal lamp (Philips, TUV lamp) was performed after the medium had been removed from the cells, at a dose rate of 0.38 J/m^2 per sec. Thereafter the cells were washed twice with PBS and handled further.

For treatments with mitomycine C (Kyowa Haggo Kogyo Ltd, Tokyo, Japan) the drug was dissolved in deionized water (1 mg/ml, stock solution, kept at 0°C) and dilutions were made in medium (7.5% FCS, 37°C) prior to use; treatments with mitomycine C were performed as described for cisplatin.

Cell survival

Cell survival experiments with human fibroblasts require feeder layers for the provision with necessary metabolites during the period that the inoculum is not yet selfsustaining. The same cell strain was used as feeder layer as for the test cells. Feeder layer cells were prepared from trypsinized cells (10 ml of 10^5 cells/ml) which were irradiated with ^{60}Co-γ-rays to a dose of 36 Gy (Gammacell 200, Atomic Energy of Canada, Canada dose rate 1.1 Gy/sec) at 0°C. After the irradiation the suspension was diluted with 235 ml Eagle's minimal essential medium (MEM; Flow laboratories; 20 ml MEM (10 times concentrated), 170 ml sterile deionized water, 6.4 ml sodium bicarbonate (7.5%), 2.5 ml L-glutamine (200 mM), 1.1 ml penicillin (100 U/ml), 1.1 ml streptomycine (100 µg/ml) and 34 ml FCS). 10- ml portions of this cell suspension were pipetted in 9-cm petridishes (Greiner; 4 dishes per data-point). Test cells were prepared from a stock solution (10^5 cells/ml; inoculum 1 ml) of freshly trypsinized cells cultured in F10

medium containing 15% FCS. Both feeder layer cells and test cells were incubated for 24 hr at 37°C before treatment, to allow attachment to the dishes. During this period no cell division of the test cells occurred. After this preincubation the test cells were treated with UV, mitomycine C or cisplatin, as indicated; subsequently they were trypsinized in 0,5 ml trypsine-EDTA (0.25% trypsine, 0.02% Na_2-EDTA in Sol A: 7.6 g NaCl, 0.2 g KCl, 1.8 g glucose, 1.2 g $NaHCO_3$ in 1 l deionized water, pH 7.4) for 3 min at 37°C. After trypsinization, 0.5 ml of medium (F10, 15% FCS) was added to each dish, the cells were suspended and, depending on the dose given, dilutions of 500-10,000 cells/ml were made in MEM medium. For each dose-point the treated cells were added to plates containing feeder layers cells. Subsequently, the dishes and 2 dishes containing only feeder layer cells (to check the inability of the feeder layer cells to form colonies) were placed in air-tight boxes. A beaker with 10 mg of a mixture of 468 mg sodium bicarbonate and 132 mg tartaric acid was placed in the box as well, and 30 ml of deionized water was added to this mixture just before closing the box. The box was sealed with air tight tape and placed in an incubator (37°C) for 17 days without disturbance. Then the dishes were taken out of the boxes, the cells were stained with 1% methylene blue and the colonies were counted. Each experiment was performed at least 3 times with 4 dishes per dose.

Unscheduled DNA synthesis (UDS)

In 3-cm petridishes 3 coverslips (1-cm^2) were placed aseptically. Cells (10^5 in F10, 15% FCS) were plated in these dishes and were allowed to attach to the coverslips for 48 hr (37°C). Then the medium was replaced by F10 with 15% FCS containing [^{3}H]-thymidine ([^{3}H]-TdR; Amersham, UK; 10 µCi/ml; spec. act. 24 Ci/mM) and the cells were incubated at 37°C for 2 hr, to label the S-phases. Thereafter the cells were washed twice with PBS and treated with UV, mitomycine C or cisplatin, as indicated. Then, the cells were given a 3-hr repair period by incubation with 1 ml of [^{3}H]-TdR (10 µCi/ml in F10, 15% FCS) at 37°C. UDS was visualized by autoradiography as described elsewhere (Lonati-Galligani et al., 1983; Zelle, 1979; see Chapter 2).

"Alkaline elution" (determination of DNA interstrand cross-links)

For each data-point two 3-cm petridishes were used in which 2.5 x 10^4 cells (per dish) were plated in F10 (15% FCS) medium. After a 24-hr attachment period (37°C), one plate (control cells) was given 0.1 ml [^{14}C]-thymidine ([^{14}C]-TdR, 0.25 μCi/ml; Amersham, UK; spec. act. 56.5 Ci/M) and the other plate (test cells) was given 0.1 ml [^{3}H]-thymidine ([^{3}H]-TdR, 2 μCi/ml; Amersham, UK; spec. act. 24 Ci/mM) for 24 hr (37°C). Thereafter the cells were washed twice with PBS, given fresh medium for 24 hr (37°C) and the test cells were treated with cisplatin. When post-treatment incubation was required, both control and test cells were incubated at 37°C for the desired time. Subsequently, the cells (on which 1 ml F10, 15% FCS was layered) were cooled to 0°C and irradiated with 3 Gy of ^{60}Co-γ-rays (Gammacell 100, Atomic Energy of Canada, Canada; dose rate 0.23 Gy/sec) producing 0.9 breaks per 10^9 dalton DNA. Control and treated cells were irradiated simultaneously. Several control dishes were used without irradiation in order to determine the background amount of breaks and to have an internal control for the experiments. The medium was replaced by PBS (0.5 ml, 0°C), the cells were scraped from the dishes, control and test cells were mixed and 10^6 Chinese hamster ovary (CHO) cells were added (in 1 ml PBS, 0°C) to provide for carrier DNA. Thereafter the alkaline elution was performed and the results were processed as described (Plooy *et al.*, 1984a = Chapter 3), resulting in the amount of DNA interstrand cross-links in break equivalents (amount of cross-links per 10^9 dalton of DNA).

Results

The survival of the various cell lines when treated with various dosages of UV, mitomycine C or cisplatin is given in Fig. 1. The XP cells showed to be extremely sensitive to UV (Fig. 1A), XP(A) more so than XP(F), which is consistent with the findings of Friedberg *et al.* (1979) and Lehmann (1982). XP(A) cells gave a completely linear survival curve, with a D_0 of 0.2 J/m^2, whereas in the curve for XP(F)

cells (D_0 = 0.6 J/m^2) a shoulder was clearly present (D_0: dose increment which reduces survival to 37% of the original value). Exposure of Han, MB, 79RD172 and FA cells to UV yielded survival curves with D_0 values which were not significantly different (MB, 79RD172 and FA: 1.8

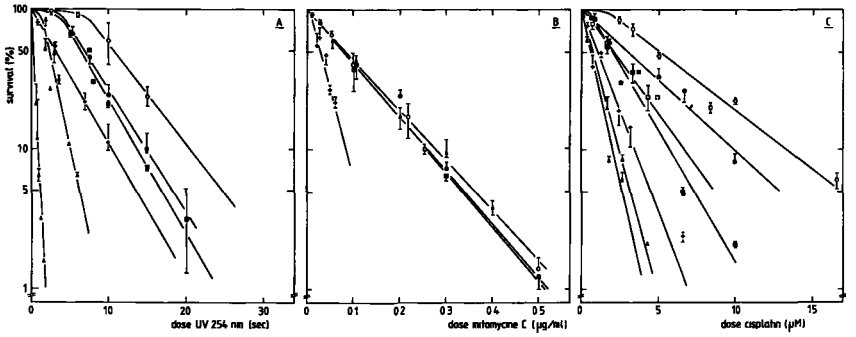

Figure 1: Survival of human fibroblasts of various cell lines after exposure to UV-irradiation at 254 nm (1A), mitomycine C (1B) or cisplatin (1C). UV-irradiation was at a dose-rate of 0.38 J/m^2 per sec, at 20°C. Treatments with mitomycine C or cisplatin were performed for 1 hr at 37°C. For experimental details see Materials and Methods. Data are given of at least 2 independent experiments in quadruplicate ± s.e.m. The D_0 values mentioned in the text were obtained from the linear parts of the graphs via regression analysis. Cell lines tested:
△ XP(A); ▲ XP(F); + FA; ■ 79RD172; □ 75RD164; ○ Han; ● MB.

J/m^2; Han: 2.2 J/m^2), but with different shoulder widths (absent for FA). The presence or absence of a shoulder and its shape is indicative for the mechanism of cell inactivation (e.g. one hit/one target or multi-hit/multi-target mechanism, or presence or absence of repair of sublethal damage).

After treatment with mitomycine C, none of the various cell lines showed a survival curve with a shoulder (Fig. 1B). From the results it is evident that only FA cells are abnormally sensitive to this compound. As XP cells behave like control cells, their repair deficiencies do not seem to affect the repair of mitomycine C-induced damage.

Fig. 1C gives the survival curves after cisplatin treatment. Almost all cell lines reacted differently to this agent. Usually, the shoulder in the survival curve is very small or absent. Only Han

cells, which showed the broadest shoulder after exposure to UV, display a broad shoulder after treatment with cisplatin. The other normal cell line, MB, has about the same D_0 value (5.2 and 4.8 μM for Han and MB, respectively), but has no shoulder. Both RD cell lines showed a distinctly higher sensitivity to cisplatin (D_0 values 2.8 and 2.3 μM for 79RD172 and 75RD164, respectively). This was rather surprising, because 79RD172 was initially considered as a control human cell line with respect to genotoxic treatments. The FA line and, in particular, the two XP cell lines appeared to be highly sensitive to cisplatin (D_0: 1.5, 1.1 and 0.9 μM for FA, XP(F) and XP(A), respectively).

In order to determine the ability of the various cell lines to perform repair synthesis, measurements of the induction of unscheduled DNA synthesis (UDS) as a result of treatment with UV, mitomycine C or cisplatin were carried out by means of autoradiography. This method is thought to detect mainly long-patch excision repair, in which stretches of 35-100 nucleotides are excised. Other repair reactions not involving (substantial) DNA synthesis are not picked up with this method, or with low sensitivity (short-patch repair). The results of the UDS measurements are given in Fig. 2. As expected, UV-irradiation (Fig. 2A) strongly induced UDS in FA, 79RD172 and Han cells, although

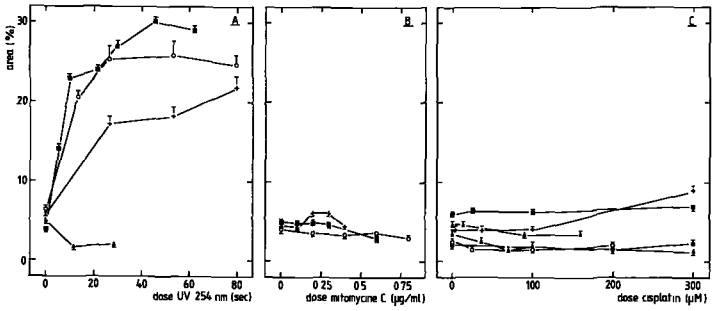

Figure 2: Induction of unscheduled DNA synthesis (UDS) in human fibroblasts caused by treatment with UV (2A), mitomycine C (2B) or cisplatin (2C). UDS was measured by autoradiography (automatic counter) by determining the fraction of the area above the nucleus that was occupied by silver grains in the photographic emulsion layered on top of the cell preparations. The number of silver grains is a measure of the amount of radioactive precursors incorporated in the DNA owing to repair synthesis. The values are given of at least 25 nuclei ± s.e.m. Cell lines used: △ XP(A); ▲ XP(F); + FA; ■ 79RD172; ○ Han; ● MB.

the level of repair synthesis in FA cells is less than that in the other two cell lines. 79RD172 cells showed a UDS induction comparable to that observed for control cell lines, which is in agreement with their normal survival after UV-irradiation (Fig. 1A). XP(A) cells did not show UDS after UV-irradiation, which confirms earlier results (see Zelle, 1980).

Mitomycine C did not induce UDS in FA, Han or 79RD172 cells (Fig. 2B); XP cells were not included in this experiment. Apparently, mitomycine C-induced lesions are not repaired *via* a mechanism involving UDS.

Fig. 2C shows the results obtained after treatment with cisplatin. None of the cell lines tested showed a significant level of UDS, with the possible exception of FA cells at a very high (and toxic) dose of cisplatin (300μM).

The induction and repair of one type of Pt-DNA-adduct, *viz* the DNA interstrand cross-link, was studied in more detail. For the cell lines 79RD172, MB, FA and XP(A), the amount of cross-links present at

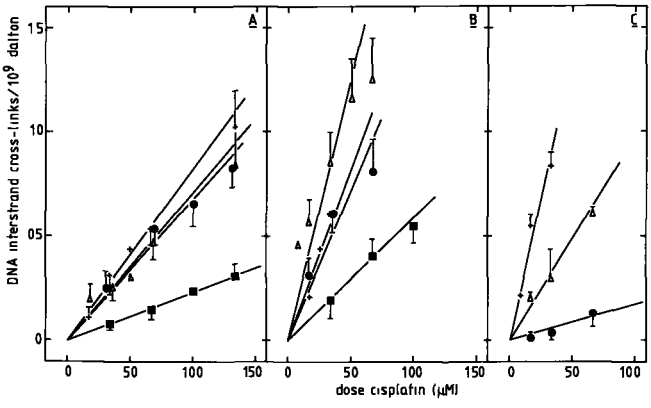

Figure 3: The induction of DNA interstrand cross-links in various cell lines by treatment with different dosages of cisplatin (1 hr, 37°C). Cross-links were measured with the alkaline elution technique and expressed per 10^9 molecular weight of DNA. Determinations were performed after post-treatment incubation of the cells for various periods; data given here refer to samples taken directly after drug removal (3A), or after a subsequent repair period of 24 hr (3B) or 96 hr (3C). Data are mean values of at least 3 independent experiments ± s.d. Cell lines:
△ XP(A); + FA; ■ 79RD172; ● MB.

various times after treatment with different dosages of cisplatin was determined (at 0, 12, 18, 24, 48, 72 and 96 hr after treatment for most cell lines). In Fig. 3 representative examples of dose response curves (t = 0, 24 and 96 hr) are given. In general, the results were consistent with a linear dose-response relationship. The effect of a post-treatment incubation on the number of interstrand cross-links is summarized in Fig. 4A, which shows the results of time course experiments after treatments with 33 μM of cisplatin. Immediately after treatment, the FA, XP and normal cells contained about equal amounts of DNA interstrand cross-links (see also Fig. 3A), whereas the 79RD172 cells were exceptional in showing much less induction of this lesion. In all cases the formation of the cross-links continued after termination of the treatment, for 18-24 hr. Thereafter, a gradual decrease occurred, but not in FA cells where formation appeared to continue at a low rate (Fig. 4A). When the graphs are considered as a whole, a distinct difference is seen between RD, FA and normal cells. In RD cells, less cross-links are induced which appear to be repaired within a short period, whereas in FA cells induction appears to be normal, but repair does not seem to occur. For XP cells, the results suggest a slightly stronger induction and/or slower repair of cisplatin-induced interstrand cross-links than in normal cells, since all XP points fall above those of the MB cells (see also Fig. 3). When the results after a repair period of 96 hr are compared, it is evident that XP cells contain less cross-links than FA cells, but more than MB cells. In general, the results obtained at other dosages of cisplatin confirmed this picture (see also Fig. 3).

In order to see whether a direct relationship might exist between the induction (or persistence) of DNA interstrand cross-links and the cytotoxicity of cisplatin, a comparison was made at equal toxicity. To this end, the dose-response curves obtained after various post-treatment incubation times were used to calculate the number of cross-links present after treatments resulting in 1% survival of the cells; the corresponding dosages were obtained from the graphs in Fig. 1C (D_1: 11.6 , 26.0, 7.5 and 4.6 μM for 79RD172, MB, FA and XP(A), respectively). (Extrapolation instead of direct measurement at D_1 was preferred in view of the sensitivity limit of the cross-link assay). The

results are shown in Fig. 4B. At equal toxicity level, normal human fibroblasts (MB) appear to tolerate the induction of more interstrand cross-links than any of the other cell lines. The FA cells, known to be deficient in cross-link repair, tolerate less of these lesions according to this comparison. At moments late after treatment, however, the number of cross-links in FA cells surpasses that present in normal cells. It appears justified to relate the high cytotoxicity of cisplatin for these cells to the induction of persistent cross-links. The XP(A) cells appear to tolerate even less cross-links than the FA cells, although the XP(A) cells are not really deficient in cross-link repair. Nevertheless, the removal is slow, and at 96 hr after treatment the initially large difference between XP(A) and normal cells has disappeared. The level of cross-links in XP(A) cells is lo-

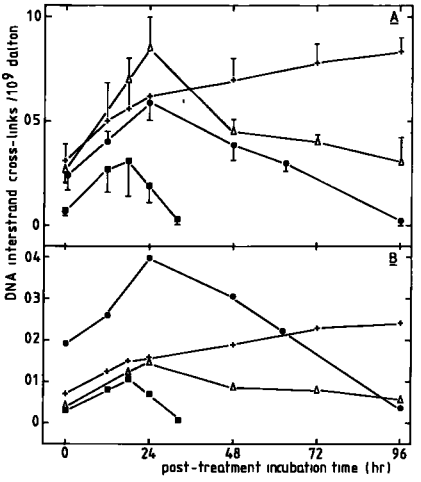

Figure 4: Induction and repair of DNA interstrand cross-links in cells treated with cisplatin. 4A: the amount of cross-links per 10^9 dalton DNA as function of the post-treatment incubation time, after treatment with 33 μM cisplatin for 1 hr at 37°C. The data points (± s.d.) were taken from experiments where the amount of cross-links in the cells after variable post-treatment incubation times were determined as a function of the dose of cisplatin. Data are mean values of at least 3 independent experiments ± s.d. 4B: the amount of cross-links present in the cells after treatment with equitoxic doses of the drug (D_1, resulting in 1% survival). The D_1 values were read from the survival curves in Fig. 1C. The data were extrapolated from the dose-response curves obtained after several post-treatment incubation times. Cell lines:
△ XP(A) (D_1 = 4.6 μM); + FA (D_1 = 7.5 μM); ■ 79RD172 (D_1 = 11.6 μM); ● MB (D_1 = 26.0 μM).

wer than that of FA cells, in particular at moments late after treatment. If the cross-links are considered as the predominant lethal lesion in FA cells, it does not appear justified, therefore, to hold the interstrand cross-links fully responsible for the cytotoxicity of cisplatin in XP cells.

In relation to cytotoxicity, 79RD172 cells appear to tolerate very few cross-links. In view of the fact that the few ones induced disappear within 48 hr, a causal relation with cell death seems rather improbable. More likely, in these cells cisplatin acts in a different manner, possibly unrelated to a damaging of DNA. In that case the low level of DNA cross-link induction, not only at equal cytotoxicity, but also at equal molar dose of cisplatin, might be attributed to limitations in the proportion of the agent reaching the cell nucleus.

Discussion

The comparative study of the effects of UV, mitomycine C and cisplatin on normal and mutant human cell lines resulted in a rather complicated picture. As for survival, the XP cells showed the expected high sensitivity to UV, attributable to a deficiency in certain steps of the long-patch excision repair (Lindahl, 1979; Lehmann and Karran, 1981). With regard to mitomycine C, XP cells appeared no more sensitive than normal human fibroblasts, which confirmed the findings of Fujiwara *et al.* (1977). Very likely, therefore, the O^6-guanine adduct induced by this agent (Lown, 1979) is not removed *via* long-patch excision repair, which is consistent with the absence of UDS in cells treated with this compound (Fig. 2B). The removal might proceed *via* short-patch excision repair as has been found with other - albeit monofunctional - alkylating agents (Lindahl, 1979).

The extreme sensitivity of both XP cell lines tested to cisplatin suggests that a substantial fraction of the Pt-DNA-damage is removed *via* long-patch excision repair. This notion is refuted, however, by the observation that cisplatin does not induce UDS in any of the cell lines. To explain these seemingly contradictory observations, it might

be postulated that in human cells the removal of cisplatin-induced DNA damage involves certain steps of the incision-excision repair pathways (those that are defective in XP cells of complementation group A and F), but does not result in the excision of long patches of DNA. Another possibility could be that the bulk of cisplatin-induced lesions is rather harmless and only a small proportion is involved in lethality. If only lesions of the latter type are repaired *via* the long-patch excision pathway, it might remain unnoticed at the present sensitivity of the UDS assay.

FA cells appear to be proficient in long-patch excision repair, because they react almost normally to UV-irradiation (both in sensitivity and UDS induction). It has been assumed that FA cells are (partly) deficient in short-patch excision repair (Fujiwara *et al.*, 1977), which might explain their high sensitivity to mitomycine C if mitomycine C-induced damaged were repaired *via* this pathway. FA cells are also abnormally sensitive to cisplatin. This would seem to indicate that the repair of damage induced by this agent requires the short-patch excision repair system that is defective in these cells, which is in conflict, however, with the fact that XP cells are highly sensitive for cisplatin but not for mitomycine C. It has to be assumed, therefore, either that a variety of potentially lethal lesions is induced by cisplatin, some requiring short-patch excision repair and some removed *via* a pathway of which steps are defective in XP cells, or that the most important potentially lethal Pt-DNA-adduct needs steps from both pathways for its removal. The latter situation might offer an explanation for the fact that only in FA cells a small but significant UDS-induction by cisplatin was observed, if we furthermore assume that only a small amount of long-patch repair synthesis takes place, *e.g.*, because of the limited number of the lesions involved. In that case, only in FA cells UDS becomes noticeable, because the long-patch repair system continues to act on lesions which remain present since the steps resulting in complete removal are not performed.

The notion that removal of cisplatin-induced lesions in mammalian cells involves some repair synthesis of DNA - be it (very) limited - is supported by the results obtained with CHO cells (Plooy and Lohman, 1984 = Chapter 2).

A model in which two types of excision repair are involved in the repair of DNA interstrand cross-links in mammalian cells has been proposed earlier, by Fujiwara and coworkers (1977) and Cleaver (1978). In this model, the two ends of the cross-link are regarded not to be equivalent and are supposed to be recognized by different enzymes. One arm of the cross-link would be unhooked by an enzyme involved in short-patch excision repair (defective in FA cells?) and the other arm would be removed by a long-patch excision repair enzyme (defective in XP cells?).

A plausible candidate for the potentially lethal Pt-DNA-adduct appears to be the interstrand cross-link. It is a relatively rare lesion, as it amounts to not more than 1% of the total amount of Pt that has reacted with nuclear DNA. Furthermore, in the highly sensitive FA cells this adduct remains unrepaired, in contrast to normal cells (Fig. 1A). However, the fact that XP cells are even more sensitive to cisplatin seems to argue against the interstrand cross-links as the most important lesion, since in these cells removal of this adduct seems not to be blocked, only to proceed somewhat less rapidly (Fig. 4). This may be misleading. It appears conceivable that in XP cells repair of these cross-links does not go to completion: possibly, only one arm of the interstrand connection is unhooked. After that, the lesion is no longer detected as a cross-link by the method used, but it remains present as a very bulky DNA adduct, which may be quite harmful for the cell if no further repair (long-patch excision) takes place. However, the possibility cannot be ruled that other lesions are responsible for the high cytotoxicity for XP cells.

RD cells behave like normal cells after exposure to UV or mitomycine C, but are exceptional in their reaction to cisplatin. In our opinion, the increased sensitivity to this agent has nothing to do with adduct formation with nuclear DNA, nor with defective repair. The relatively low induction of interstrand cross-links (see Figs. 3 and 4) suggests that at the same external concentration of cisplatin, the nuclear DNA is less exposed in RD cells than in the other cells. This might indicate a hindered penetration of the compound into the nucleus. In view of the tendency of RD cells to accumulate certain compounds, it appears attractive to attribute both the reduced permeation

into the nucleus and the high cytotoxicity of cisplatin to intralysosomal storage of this agent or its metabolites. This means that lethality should be due to cytoplasmic effects of the drug. It might be wondered whether a high level of adduct formation with mitochondrial DNA could occur and cause the death of the cells.

In conclusion, the results of this investigation can be seen as an underlining of the importance of DNA interstrand cross-links among the lesions induced by cisplatin: although only a small fraction of the Pt-DNA-adducts belongs to this category, the biological effects appear to be rather significant.

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7. CONCLUSIONS

7.1. Structure-activity relationships

The ten platinum coordination compounds used in the studies described in this thesis were selected to investigate the influence of the stereoconfiguration of the ligands around the Pt-atom, the charge of the Pt-atom and the chemical nature of the ligands (see Chapters 1 and 2).

In order to study the role of the stereoconfiguration, pairs of compounds were synthesized in which the inert ligands (ligands that are stably bound to the central Pt-atom) were either in cis- or trans positions around the Pt-atom. In the described assays with mammalian cells in vitro, both trans-compounds: trans-diamminedichloroplatinum-(II) (tII) and trans-diamminetetrachloroplatinum(IV) (tIV) were very little cytotoxic and not mutagenic, whereas the corresponding cis-compounds were severely toxic and mutagenic to these cells. These results coincided with the effects observed in vivo (as discussed in Chapter 2).

The second criterion for the selection of the platinum compounds has been the charge of the Pt-atom. Platinum coordination compounds can carry either 4 or 6 ligands, depending on the charge of the Pt-atom (Pt(II) versus Pt(IV)). In a cis-geometry 2 of the ligands (Cl) are the most reactive in both types of compounds. The cis-Pt(II) compounds: cII, $Pt(en)_2Cl_2$, Pt(DMDAP)mal and $Pt(DACH)Cl_2$ and the cis-Pt(IV) compounds: cIV and $Pt(DMDAP)Cl_4$, all were cytotoxic and mutagenic in the mammalian cell systems used (Chapter 2), in agreement with the effects found in bacteria and in mice (for references see Chapter 2). On the whole, the responses of Pt(II) and Pt(IV) compounds did not differ dramatically; Pt(IV) compounds seemed to be only slightly more cytotoxic than the Pt(II) compounds

The nature of the ligands appeared to have drastical effects on the action of the drug (Chapter 2). The compounds with non-leaving, non-cyclic ligands possessing a proton at the N-atom coordinated to Pt, when placed around the Pt atom in cis-configuration, were found to be comparable to cisplatin in their action. Compounds with cyclic non-

leaving groups showed remarkable differences in their activities, both with respect to cells in culture as to mice tumor systems. The compound with cyclic ligands missing a hydrogen at the N coordinated to Pt ($Pt(py)_2Cl_2$) was found to be moderately cytotoxic and non-mutagenic in CHO cells; it did not show antitumor activity. Of the two compounds with cyclic ligands having such a hydrogen, however, one ($Pt(DACH)Cl_2$) was cytotoxic and mutagenic in CHO cells and had antitumor activity *in vivo*, whereas the other ($Pt(ACH)_2Cl_2$) showed to be slightly cytotoxic and non-mutagenic, but with this compound a rather specific antitumor activity was observed (see Chapter 2). This suggests that very specific adducts may be formed by some compounds which have killing properties only in special tumor cells, or that particularly lethal adducts are formed only in special tumor cells.

The two leaving ligands (malonate or Cl^-) did influence the cytotoxicity and Pt-DNA-binding in CHO cells; the influence on mutagenicity in these cells and on antitumor activity in mice was much less (Chapter 2).

7.2. Interaction of platinum compounds with DNA

The possibility that the non-cytostatic action of *trans*-compounds and of the non-mutagenic, hardly-toxic *cis*-compounds could be attributed to the fact that these compounds do not reach the DNA in cells, or do not react with it, has been ruled out, because all ten tested compounds were shown to react with DNA and to inhibit the semi-conservative DNA synthesis in mammalian cells in culture up to at least 24 hr after treatment (Chapter 2). This might be taken to suggest that the toxicity and mutagenicity of the other *cis*-compounds should be ascribed to other effects than the formation of adducts with DNA. This is refuted, however, by the outcome of the experiments described in this thesis, which strongly supports the hypothesis that both the cytostatic and the genotoxic action of the platinum compounds, in general has to be attributed to the induction of specific lesions in the DNA of the cells (chapters 2, 3 and 5). On the other hand, there are indications that also other causes may contribute to the cytotoxic action of the mutagenic *cis*-compounds, beside their interaction with

nuclear DNA (Chapter 6). The unusual response to treatment with cisplatin (cII) observed with the human mutant cell lines derived from mucolipidosis patients who are suffering from intralysosomal storage of possible harmful products, suggests that part of the cytotoxic action may be due to interactions with other macromolecules in these particular cells (Chapter 6).

Attempts were made in this study to identify the specific DNA adducts ("key lesions"), which are held responsible for the genotoxic effects in mammalian cells treated with the interesting cis-platinum compounds (Chapters 2, 3, 5 and 6). A first observation was that in mammalian cells in culture the total amount of Pt-DNA-lesions (as measured by Pt-DNA-binding) did not correspond with the cytotoxicity nor with the mutagenicity of the ten tested compounds (Chapter 2). By searching for possible specific lesions it was found that the biological activity of the platinum compounds cannot be attributed to the induction of double- or single-strand breaks in the DNA, because these lesions were not found in the DNA of cells treated with platinum compounds (Chapter 2). No evidence was observed that any of the ten compounds intercalated with DNA and it is unlikely, therefore, that the cytotoxic action is related to such a process, as has been proposed for other cytostatic drugs such as bleomycine. Covalent binding to DNA-constituents appears more probable. In many cases covalent binding of genotoxic agents destabilizes the polymeric structure of DNA which results in strand breaking when the DNA is treated with alkali (alkali labile sites, ALS). However, such ALS were not found in mammalian cells treated with any of the ten platinum compounds (Chapter 2), and, therefore, the nature of the adducts should be different in comparison to, for instance, those found after treatment of cells with alkylating agents (see Chapter 2).

Both cis- and trans-complexes can react with DNA either monofunctionally or bifunctionally. The bifunctional adducts are supposed to be formed via a through-reaction of the monofunctional ones. In CHO cells, the class of monofunctional adducts (merely on guanine) induced by cisplatin is a major lesion (Chapter 5). In DNA reacted with cisplatin in vitro (discussed in Chapter 5), however, the monofunctional

adducts seemed to be minor lesions, indicating that *in vitro* the conversion of monofunctional adducts to bifunctional ones occurs faster than in CHO cells.

It is likely that the toxic and mutagenic properties of cytostatic *cis*-compounds should be explained on the assumption that they induce other Pt-DNA-adducts than the *trans*-compounds. *In vitro*, the non-cytostatic *trans*-compound, tII, induces much more monofunctional adducts *in vitro* than its stereoisomer cII (see discussion Chapter 5). If a similar difference exists with respect to the reactions within the cell, this would be an indication that monofunctional lesions are less important for the biological action of the cytostatic platinum compounds than bifunctional adducts, which comprise DNA intrastrand cross-links, DNA interstrand cross-links and DNA-protein cross-links.

Several types of DNA intrastrand cross-links induced by cisplatin have been detected *in vitro* (discussed in Chapter 5) and in mammalian cells in culture (Chapters 3 and 5): intrastrand cross-links on the base sequence pGpG, on the base sequence pApG and on two guanines separated by one or more bases (*i.e.*, on $pG(pX)_npG$ base sequences; see Chapter 5). The formation of "short distance" intrastrand cross-links connecting 2 nucleotides in the same DNA strand that are adjacent or are separated by one nucleotide, appears unlikely for the *trans*-compounds because of steric reasons. This difference between *cis*- and *trans*-compounds with regard to the induction of these intrastrand cross-links makes these lesions to likely candidates for the genotoxic adducts.

The characterization of the various DNA intrastrand cross-links in CHO cells treated with cisplatin was made possible by a new and sensitive immunochemical detection method (see Chapter 5). With this method it was found that the intrastrand cross-links on the base sequence pGpG account for about 1/3 of the total amount of Pt-DNA-adducts measured directly after treatment of CHO cells with cisplatin. In the same cells intrastrand cross-links on the sequence pApG were present, but these seemed to be less abundant than those on adjacent guanines. Evidence in *E.coli* (as discussed in Chapter 5) revealed the existence of a possible third type of DNA intrastrand cross-links be-

tween two guanines separated by one base (i.e., on pGpXpG base sequences). In E.coli this DNA-adduct appeared to be of importance for the mutagenic action of cisplatin. This adduct might also be present in CHO cells, although the current immunochemical method did not allow an exact assessment of the amount, since the degradation product cis-$Pt(NH_3)_2d(GMP)_2$ was determined, which may originate from interstrand cross-links (a small fraction) and from intrastrand cross-links on $pG(pX)_npG$ base sequences, where n can be 1 or more. The cross-links on pGpXpG can only form a small fraction of the total amount of Pt-DNA-adducts in CHO cells (at most 3%, the total percentage of the degradation product mentioned; see Chapter 5).

The formation of DNA interstrand cross-links and DNA-protein cross-links were measured with sensitive physical/biochemical methods (alkaline filter elution; Chapters 3, 4 and 6). In both human and CHO cells, DNA interstrand cross-links were found to be induced by cisplatin. In CHO cells cisplatin was compared with its Pt(IV) analogue and their trans-stereoisomers. All four induced these lesions, however their kinetics seem to be different. The cells treated with the cis-compounds showed a delayed formation of interstrand cross-links, which was also observed, albeit to a far less extent, with the trans-compound, tIV, whereas in case of tII the maximum number of cross-links was found immediately after treatment. Even at the time that these lesions reached their highest amounts, 6 to 12 hr after treatment for the cis-compounds, they were only minor reaction products (less than 1% of the total).

In CHO cells, DNA-protein cross-links also have been found to represent only a small fraction of the total amount of Pt-DNA-adducts. This was found after treatment with cisplatin, its Pt(IV) analogue and with their trans-stereoisomers.

7.3. The repair of Pt-DNA-adducts.

When the repair of Pt-DNA-damage was studied in mammalian cells by following the removal of the DNA-bound platinum from the cells, it was found that the majority of the lesions is removed in a relatively short period (40-60% within 24 hr; Chapters 4 and 5). The most exten-

sively studied DNA repair process in mammalian cells is excision repair (see Chapter 1). Excision repair is a multi-enzyme repair process, in which one of the steps is the resynthesis of DNA stretches after the removal of lesions (DNA repair synthesis). Two types of excision repair are known: i) long-patch repair synthesis, as observed after irradiation of mammalian cells with UV and ii) short-patch repair synthesis, that is found after treatment of mammalian cells with e.g. alkylating agents. Since the UV-induced pyrimidine dimer is a DNA intrastrand cross-link, it might have been expected that the repair of intrastrand cross-links as formed by cisplatin (approximately 60% of the total amount of Pt-DNA-adducts in CHO cells; Chapter 5) would result in long repair patches in mammalian cells. However, neither in human cells treated with cisplatin (Chapter 6) nor in hamster cells treated with any of the ten platinum compounds (Chapter 2) substantial induction of long-patch repair (measured as unscheduled DNA synthesis) could be detected.

On the other hand, the extreme sensitivity of xeroderma pigmentosum (XP) cell lines for cisplatin suggested that an important contribution to the removal of the Pt-DNA-damage comes from the long-patch excision repair (Chapter 6). It remained to be explained, however, why cisplatin did not induce UDS in any of the normal human cell lines tested. It could mean that only certain rare, but extremely lethal lesions are repaired via the long-patch excision pathway; a small amount of long-patch excision repair will remain unnoticed at the present sensitivity of the UDS assay.

Fanconi's anemia (FA) cells appeared to be proficient in long-patch excision repair, because they reacted almost normally to UV-irradiation (Chapter 6). It has been suggested that FA cells are (partly) deficient in short-patch excision repair (as discussed in Chapter 6). Like XP cells, also FA cells were extremely sensitive to cisplatin (Chapter 6). This seemed to indicate that the repair of key lesions induced by this agent requires both the short-patch and long-patch excision repair system. If both systems have to act on the same lesions (e.g., interstrand cross-links), this may offer an explanation for the fact that only in FA cells a small but significant UDS-induc-

tion by cisplatin was observed: only in FA cells UDS will become noticeable, because the long-patch repair system continues to act on lesions which are not removed because of the absence of short-patch excision repair. Such a model in which two types of excision repair are involved in the repair of DNA interstrand cross-links in mammalian cells, has been proposed (see discussion in Chapter 6). In this model, the two ends of a cross-link are regarded not to be equivalent and may be recognized by different enzymes: one arm of the cross-link is unhooked by an enzyme involved in short-patch excision repair (defective in FA cells) and the other arm is recognized by a long-patch excision repair enzyme (defective in XP cells) (see Chapter 6).

Which repair processes are involved in the removal of the majority of the Pt-DNA-adducts in mammalian cells is not clear yet. However, significant evidence was found in this study that repair of many of the platinum lesions takes place and that the kinetics of the repair process is dependent on the kind of Pt-DNA-adduct induced (Chapter 3 and 5).

The repair systems discriminate between various types of Pt-DNA-adducts. Monofunctional adducts are repaired quickly, since after a repair period of 24 hr less than 1/4 of the original amount (36% of the total amount of Pt-DNA-adducts at t = 0) was still present in the cisplatin-treated mammalian cells (Chapter 5).

Also the bifunctional adducts are repaired. It is not certain, however, whether the observed reduction of the absolute amounts of the intrastrand cross-links purely reflected the repair of the original lesions or that it was due to a combination of delayed formation and removal (as in the case of the interstrand cross-links, see above and Chapter 3).

The cross-link on two adjacent guanines appeared to be repaired more slowly than the overall rate of the Pt-DNA-adducts, since they represented 36% of the total adducts at t = 0 and 47% at t = 24. In absolute numbers, approximately 3/4 of these lesions were still present after 24 hr, so that they are rather persistent (Chapter 5). Possibly, the cross-links on d(pApG), though somewhat less abundant (< 24% at t = 0), are very similar in behaviour and in importance.

Intrastrand cross-links on $pG(pX)_npG$ base sequences appeared to be repaired at about the same rate as the overall amount of Pt-DNA-adducts, since both at t = 0 and t = 24 hr 3% of the lesions were found in this form (Chapter 5).

The amount of DNA interstrand cross-links induced by the cis-compounds reached a maximum at 6 to 12 hr after the removal of the drug, probably because the continuing formation of the cross-links over compensated the disappearance caused by repair. Thereafter repair exceeded formation, however, up to 48 hr after treatment they were still detectable. So, the cis-platinum induced interstrand cross-links are very persistent lesions (Chapter 3).

Beside DNA-DNA interstrand cross-links, also the DNA-protein cross-links induced by the cis-compounds showed a persistent character, in contrast to those induced by the trans-compounds (Chapter 3).

These results indicated that the cellular repair systems are able to distinguish between lesions induced by cis- and trans-platinum compounds, respectively. The trans-platinum induced DNA interstrand and DNA-protein cross-links were repaired faster than those induced by the cis-compounds (Chapter 3).

7.4. Modification of the Pt-DNA interactions by temperature or other factors.

The spectrum of DNA lesions induced in mammalian cells by cisplatin can be varied by changing conditions of treatment (Chapter 4). Modifications were induced: i) by varying the treatment temperature and ii) by activation of cisplatin by means of preincubation in medium.

Influence of temperature

In studies on mutation induction in E.coli (as discussed in Chapter 4), strong indications were found for a selective increase in the formation of adducts on pGpCpG sequences (but not on pGpApG) with temperature when the bacteria were treated with cisplatin at temperatures ranging from 33° to $41^{\circ}C$. Such an effect of the temperature on

the whole spectrum of Pt-DNA-lesions was not found after treatment of isolated DNA with cisplatin in vitro (see Chapter 4).

The survival and adduct formation in CHO cells strongly depend on the temperature: at 32°C, cisplatin is less toxic and induces fewer lesions in CHO cells than at 40°C. Both differed a factor 2.0 to 2.5 (Chapter 4), suggesting that temperature enhancement does not influence the spectrum of lesions, but rather increases the level of Pt-DNA-binding. The amount of interstrand cross-links present in the cells, 24 hr after the removal of cisplatin, differed also a factor 2.5 between the two temperature treatments. When incubations resulting in the same cell killing were compared, the amount of cross-links (t = 24) was equal. This again is an indication that the spectrum of lesions induced at both temperatures is not changed.

The induction of $HGPRT^-$ mutants in CHO cells treated with cisplatin was not so much influenced by the temperature. Consequently, when the mutation induction was compared at equitoxic dose levels, it appeared that treatment at 32°C induced more mutants than at 40°C. This result may indicate that in CHO cells the spectrum of lesions is slightly changed, however, not so much that it became apparent from the other determined parameters (see above).

It is not yet clear what the influence of temperature may be on the cytostatic action of platinum compounds: if the cytostatic action is related to the mutagenic potential of a drug (Chapter 2), a temperature rise may result in decreased cytostatic action of the drug, despite the larger amount of platinum bound to the DNA of the cells.

Influence of preincubation

A remarkable observation was the effect that preincubation (0.5 - 1 hr) of cisplatin in medium at the temperature of the subsequent exposure of the cells, increased the cytotoxicity by a factor of 2.5 (see Chapter 4). This effect explains the differences between the D_0 values (at 37°C) found in experiments reported in Chapter 4 and the (lower) values of earlier studies, when the presence of cisplatin in the medium during the prewarming period was customary (Chapter 2 and

3). The difference in cytotoxicity coincided with the effect on Pt-DNA-binding: with freshly prepared solutions less Pt became bound to cellular DNA (Chapter 4 and 5) than with the preincubated solutions (Chapter 2 and 3; about factor 2.5). It is not clear which process is responsible for the reactivity enhancement of cisplatin in a Cl^- and protein-rich environment. However, it seems that the replacement of the Cl^- groups of cisplatin by more reactive groups is not prevented by high chloride concentration when other molecules than Cl^- are present in the solution (see Chapter 4).

7.5. Relevance of the detected Pt-DNA-adducts

To what extent leads the study on platinum compounds described in this thesis to a further understanding of the relation between the induction of "key lesions" and the biological effects (cytotoxicity, mutagenicity, DNA synthesis inhibition, SCE induction and antitumor activity)?

As described, monofunctional Pt-DNA-adducts were rapidly repaired. In addition, the biologically inactive trans-analogue of cisplatin has been reported to induce many more monofunctional adducts (see Chapter 5). This suggests that the biological effects of the platinum compounds cannot be related to monofunctionally bound cisplatin, but rather to the bifunctional ones.

Little is known about the noxiousness of the intrastrand cross-links on neighboring guanines and on adenine adjacent to guanine. Together they represented approximately 60% (t = 0) to 80% (t = 24) of the total amount of lesions (Chapter 5). When it is also taken into account that the trans-isomers cannot form these adducts - for steric reasons - they seem to be relevant for the biological effects of cis-platinum compounds. In E.coli, cross-links on d(pGpG) appeared not to be involved in the induction of base-pair substitution mutations (as discussed in Chapter 5), but this does not exclude other harmful effects. Because of their large amount, they may be responsible for the observed DNA synthesis inhibition (Chapters 2 and 5).

A second type of DNA intrastrand cross-links, which do induce base-pair substitutions in E.coli (see Chapter 5 for references) is

the cross-link on two guanines separated by one base. Most likely, these lesions were also present in CHO cells treated with cisplatin (see above) and might represent up to 3% of the total amount of Pt-DNA-adducts. To see whether a relationship may exist between these, probably mutagenic, lesions and the mutation induction at the HGPRT locus in CHO cells, the frequency of the occurrence of these lesions per HGPRT gene (supposing one double stranded HGPRT gene, consisting of 1000 nucleotides each, is present per cell) in cells treated with 83 μM cisplatin was estimated. The maximal number of cross-links on $d(pG(pX)_npG)$ sequences was 5.8×10^{-3} and 3.2×10^{-3} per gene, at t = 0 and t = 24 hr, respectively (see Chapter 5); mutation induction amounted to about 150 mutants per 10^6 (surviving) cells, i.e., 0.15×10^{-3} per HGPRT gene (see Chapter 3). If these cross-links are responsible for the mutations that prevent expression of HGPRT activity, one in 20 - 40 lesions would be sufficient to cause this effect.

Other potential candidates for mutagenic lesions induced by cisplatin are the DNA-protein and DNA interstrand cross-links. Both have been shown to be rather persistent in CHO cells, in contrast to those induced by the trans-isomer (Chapter 3). As described in Chapter 2, an error-prone repair pathway may be involved in the repair of interstrand cross-links. The amount of DNA interstrand cross-links per HGPRT gene at t = 24 is about 0.40×10^{-3} (the level at t = 0 is roughly half this value, while at t = 6 it is twice as much; see Chapter 3). A numerical comparison with the mutation frequency (0.15×10^{-3}) suggests that these lesions might be highly mutagenic. However, the observation that the variation in the amount of interstrand cross-links (t = 24) induced by cisplatin at various temperatures does not coincide with the effect of temperature on mutation induction, suggests that this lesion is not related to the mutagenicity.

In contrast to DNA-protein cross-links, the interstrand DNA cross-links cause a mutilation of the genetic information in both DNA strands at (approximately) the same position. In order to circumvent this double block on the template, recombination with homologous chromosomes appears to be necessary. Such a process is thought to be involved in the induction of SCE. The amount of DNA interstrand

cross-links per cell (t = 24 hr) and the amount of SCE per cell were similar, indicating that the persistent interstrand cross-links may be also related to the formation of SCE (Chapter 3). However, it is not clear yet, if the interstrand cross-links are responsible for both mutagenicity and SCE induction, because then these two processes should have one or more steps in common.

Furthermore, the amount of DNA interstrand cross-links after 24 hr post-treatment incubation induced by cisplatin at various temperatures coincide with the increased cytotoxicity (Chapter 4). It is tempting, therefore, to consider the DNA interstrand cross-links also important in relation to cytotoxicity. This hypothesis is further supported by the experiments with FA cells (Chapter 6): differences in the repair of this minor lesion have a pronounced influence on the biological effects of the drug in these cells.

The DNA-protein cross-links also occur with about the same frequency as HGPRT mutations (ca. 0.1 x 10^{-3}/gene). Therefore, they cannot be ruled out as potential mutagenic lesions. Furthermore, these type of adducts might cause difficulties at the time the cells come in mitoses, resulting in chromosomal abnormalities, such as micronuclei induction (see Chapter 2 and 5).

ABBREVIATIONS

DNA	deoxyribonucleic acid
ssDNA	single-stranded DNA
RNA	ribonucleic acid
dCMP	deoxycytidine 5'-phosphoric acid
dAMP/dpA	deoxyadenosine 5'-phosphoric acid/salt
TMP	thymidine 5'-phosphoric acid
dGMP/dpG	deoxyguanosine 5'-phosphoric acid/salt
TdR	thymidine
BUdR	5-bromo 2-deoxyuridine 5'phosphoric acid
CHO	Chinese hamster ovary cells
FA	Fanconi's anemia cells
XP	xeroderma pigmentosum cells
MB	control human cell line, isolated at the Medical Biological laboratory TNO
RD	cell line originating from mucolipidosis II (aryl-sulfatase A deficient) patients, isolated at the Erasmus University, Rotterdam.
FCS	foetal calf serum
NCS	newborn calf serum
F-10	Dulbecco's modified culture medium
MEM	Eagle's minimal essential medium
UDS	unscheduled DNA synthesis
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
SCE	sister chromatid exchanges
AAS	atomic absorption spectroscopy
ALS	alkali labile sites
SSB	single-strand breaks
DSB	double-strand breaks
ELISA	enzym linked immunosorbent assay
SDS	sodium dodecylsulfate
DMSO	dimethyl sulfoxide
HPLC	high performance liquid chromatography
FPLC	fast protein liquid chromatography
D_n	dose at which n% of the cells survive
D_0	dose increment in the linear part of the survival curve reducing survival to 37% of the original value
B_0	shoulder in a survival curve (B_0 = dose where the linear part of the survival curve intercepts with y = 100%
LD_0	maximal tolerable dose (0% of the animals are killed)
LD_{50}	dose resulting in killing of 50% of the animals
ID_n	dose at which n% of the tumor is regressed

T.I.	therapeutic index, LD_{50}/ID_{90}, measure of the selectivity of a drug
T/C	ratio of the tested over the control survival expressed as %
ADJ/PC6	solid tumor in mice
L1210	liquid tumor in mice
TNO	Dutch organisation for applied scientific research
cisplatin, cII	cis-dichlorodiammineplatinum(II)
tII	trans-dichlorodiammineplatinum(II)
cIV	cis-tetrachlorodiammineplatinum(IV)
tIV	trans-tetrachlorodiammineplatinum(IV)
$Pt(py)Cl_2$	cis-dichlorobis(pyridine)platinum(II)
$Pt(en)Cl_2$	cis-dichloro(1,2-diaminoethane)platinum(II)
Pt(DMDAP)mal	cis-malonato(2,2-dimethyl-1,3-diaminopropane)-platinum(II)
$Pt(DMDAP)Cl_4$	cis-tetrachloro(2,2-dimethyl-1,3-diaminopropane)-platinum(IV)
$Pt(DACH)Cl_2$	cis-dichloro(1,2-diaminocyclohexane)platinum(II)
$Pt(ACH)_2Cl_2$	cis-dichlorobis(aminocyclohexane)platinum(II)
r_b	platinum atoms per nucleotide (in DNA)
EDTA	ethylenediaminetetraacetate
Tris	tris(hydroxymethyl)aminomethane
PBS	phosphate buffered saline
rpm	revolutions per minute
dpm	desintegrations per minute
U	unit
UV	ultraviolet
AP	apurinic/apyrimidinic
TCA	trichloroacetic acid
BSA	bovine serum albumine
T20	polyoxyethylene sorbitan monolaurate
DEA	diethanolamine
PNP	p-nitrophenol phosphate
A	absorption
E	extinction
w/v	weight per volume
v/v	volume per volume
ENNG	N-ethyl-N'-nitro-N-nitrosoguanidine
s.d.	standard deviation
s.e.m.	standard error of the mean
Ci	curie
Gy	gray

SUMMARY

DNA is the hereditary material of all living cells, coding for all processes inside the cell and in the whole organism. Therefore, the maintenance of its integrity is of utmost importance for the normal functioning and reproduction of an organism. DNA can be damaged by a variety of physical and chemical agents, leading to DNA alterations which might interfere with the coding properties of the DNA. DNA alterations can be recognized and repaired by repair mechanisms in almost all organisms, including man.

In the higher multicellular organisms, the proliferation of individual cells is strictly regulated. Tumor cells are cells which for some reason have escaped the overall control; they usually proliferate at an abnormally high rate. In most cases tumor cells are more sensitive to DNA-damaging agents (cytostatic agents) than are normal cells. This phenomenon has led to protocols with which patients suffering from neoplasms are treated with DNA-damaging agents to cure their illness. Cisplatin (cis-diamminedichloroplatinum(II)) is such a cytostatic agent. It is effective in the treatment of certain cancers, but has considerable side-effects. The way tumor cells are preferentially killed by cisplatin is not known, nor has the cause of the severe toxic side-effects on normal organs and tissues in man been established. In order to improve this situation it is important to understand the working mechanism of cisplatin and related platinum compounds (for an overview: see Chapter 1). Therefore, a study was initiated on the effects of platinum coordination compounds with and without antitumor activity in mammalian cells at the molecular level.

In Chapter 2, a comparative study on the effects of ten platinum coordination compounds on Chinese hamster ovary (CHO) cells is described. The compounds differ in their ligands, the valence of the central Pt-atom and their configuration; antitumor-active, as well as inactive, compounds were included. All compounds tested inhibited the semi-conservative DNA synthesis, induced some, but very little DNA repair synthesis, and did not induce single- and double-strand breaks and alkaline labile sites in the DNA of the cells. The cytotoxicity

showed large differences. When the mutagenicity (HGPRT mutants) was compared, six compounds were found to be mutagenic. In general, the mutagenicity in CHO cells coincided with the antitumor activity observed in mammals. On the basis of these findings together with those reported for bacteria (Mattern *et al.*, 1983; van Kralingen, 1979), the mutagenicity rather than cytotoxicity seems to predict the cytostatic properties of the platinum compounds. All compounds were found to induce Pt-DNA-adducts; at equal concentration the little-toxic, non-mutagenic compounds were more effective in this respect than the more harmful ones. The experiments suggested that the active antitumor compounds, which are cytotoxic and mutagenic, induce very specific, probably noxious, Pt-DNA-adducts.

The possible nature of such specific Pt-DNA-adducts was further investigated in CHO cells (Chapter 3). In this study, 4 platinum compounds were compared *viz.* *cis*- and *trans*-diammine(di/tetra)chloroplatinum(II, respectively, IV). The two *cis*-compounds were antitumor active, whereas the *trans*-compounds were not. After equitoxic treatments, it was found that the *cis*-Pt-DNA-adducts were much more cytotoxic and more efficient inducers of sister chromatid exchanges (SCE) than the *trans*-Pt-DNA-adducts. Only the *cis*-Pt-DNA-complexes could be related to the mutagenic activity of the compounds.

When the nature of the various Pt-DNA-adducts were further analyzed, it was found that the *cis*-platinum complexes induced DNA interstrand cross-links, with a delayed formation; such lesions were also rather persistent. The *trans*-compounds induced (DNA-protein and interstrand) cross-links which are rapidly eliminated during post-treatment incubation of the cells. It appeared that the repair systems operational in CHO cells could discriminate between *cis*- and *trans*-Pt-DNA-adducts. Unrepaired *cis*-induced DNA interstrand cross-links and DNA-protein cross-links might be responsible for cytotoxicity, mutagenicity and antitumor activity; SCE induction too might be related to the persistent DNA interstrand cross-links.

Studies on the effects of the temperature (32° - 40°C) during treatment of CHO cells with *cis*-diamminedichloroplatinum(II) (cII, cisplatin) are described in Chapter 4. It was found that the cytotoxicity was enhanced by a high-treatment temperature. This enhancement

could be explained by a higher amount of platinum bound to the DNA of the cells. The level of DNA interstrand crosslinks (upto 24 hr after treatment) was equal, when equitoxic dosages of cisplatin were used at the various temperatures; this suggested that DNA interstrand cross-links might be related to the cytotoxicity. At equitoxic dosages, the mutagenicity caused by cisplatin was higher at 32°C than at 40°C. This indicates that the spectrum of lesions induced by cisplatin is influenced by the temperature treatment, although not so much that it became apparent from the other criteria tested in this investigation.

In these studies it was observed that preincubation of cisplatin in medium before treatment resulted in an increased level of Pt-DNA-binding and, consequently, an increased cell killing. The reason why cisplatin acts more strongly after a preincubation period is not yet understood.

New and very sensitive immunochemical detection techniques were used in the experiments described in Chapter 5, which allowed the detection of Pt-DNA-adducts in CHO cells after treatment with cisplatin at relatively mild dosages. The immunochemical procedure followed was particularly useful in the detection of DNA intrastrand cross-links; directly after the treatment of the cells intrastrand cross-links on adjacent guanines, cis-$Pt(NH_3)_2d(pGpG)$, accounted for 36% of the total amount of lesions; intrastrand cross-links on two not adjacent guanines cis-$Pt(NH_3)_2d(pG(pX)_npG)$ amounted to 3%. The study described in Chapter 3 revealed that 0.1% consisted of interstrand cross-links and 0.1% of DNA-protein cross-links. Furthermore, monofunctional adducts on guanine, isolated as $Pt(NH_3)_3dGMP$, could be detected with atomic absorption spectroscopy to represent 39% of the total amount of lesions. Intrastrand cross-links on pApG basesequences possibly account for most of the remaining 23% of the adducts. Comparing the results in CHO cells with those reported on DNA treated in vitro, it appeared that the same types of adducts are probably formed in vitro and in vivo, only the ratio between the monofunctional adducts and the cross-links on d(pGpG) seemed to be different (more cross-links in vitro).

After a post-treatment incubation period of 24 hr, the total amount of DNA-bound platinum was reduced by 40% to 60%; the spectrum of lesions was found to be: 47% cross-links on d(pGpG); 2.6% on d(pG-

$(pX)_n pG$) (n is one or more); 0.4% DNA interstrand cross-links and 0.1% DNA-protein cross-links. The monofunctional-Pt-DNA adducts had decreased to less than 15% of the total amount of adducts; cross-links on d(pApG), which could not be measured accurately, might account for, at the most, 48% of the total platinations.

Combining the biological effects with the adducts detected in the cells, it can be concluded that: all bifunctional Pt-DNA-adducts might be responsible for cytotoxicity, mutagenicity and antitumor activity of cisplatin, although there are reasons to believe that the DNA-protein cross-links are involved in chromosomal aberrations only. The intrastrand cross-links on pGpG base sequences might be responsible for most of the DNA synthesis inhibition. Cross-links on d(pGpXpG) and the DNA interstrand cross-links seem to be mutagenic; the DNA interstrand cross-links might as well be related to the cytotoxicity and to the SCE induction.

In Chapter 6, the effects of cisplatin (and model DNA-damaging agents) on mutant and control human cells are described. From the results on the survival and the induction of repair synthesis in XP, FA, and control cells it could be concluded that short-patch and, probably, also long-patch excision repair is involved in the removal of cisplatin damage.

FA cells are found to be deficient in DNA interstrand cross-link repair, which resulted in a high sensitivity to cisplatin. XP cells, too, appeared very sensitive to cisplatin. It is assumed that they are able to unhook at least one side of the cross-link induced by cisplatin, but possibly the unhooking of the other side requires steps that are deficient in XP cells. Otherwise, the high sensitivity of XP cells should be attributed to unsufficient repair of other Pt-DNA-adducts. In human cells, the major part of the Pt-DNA-damage seems to be repaired via the small-patch excision repair pathway, but in the repair of DNA interstrand cross-links, which are rare lesions, long-patch excision repair might be involved.

Further investigation is needed to relate the observed Pt-DNA-adducts to the biological effects of cisplatin and related platinum compounds and to explore the effects of the repair system on the removal of intrastrand and monofunctional adducts.

SAMENVATTING

DNA is het erfelijke materiaal van alle levende cellen en het bevat alle informatie die nodig is voor het funktioneren van de cel en het gehele organisme. Het DNA is opgebouwd uit twee DNA-strengen, die d.m.v. waterstofbruggen met elkaar verbonden zijn. Eén DNA-streng bestaat uit een keten van suikers (deoxyribose) en fosfaatgroepen, die om en om gerangschikt zijn. Aan de fosfaatgroepen zijn de basen adenine (A), guanine (G), thymine (T) en cytosine (C) gebonden, de erfelijke informatie wordt door de volgorde van de basen in de DNA-streng bepaald. Voor het normaal funktioneren en voortplanten van een organisme is het noodzakelijk, dat de informatie in het DNA onveranderd gehandhaafd blijft. DNA kan beschadigd worden door velerlei fysische of chemische stoffen, wat kan leiden tot veranderingen in de koderende eigenschappen van dit makromolekuul. Gelukkig, echter, bezit vrijwel elk organisme, inklusief de mens, reparatiesystemen die deze veranderingen in het DNA kunnen herkennen en herstellen.

De deling van individuele cellen in hogere (veelcellige) organismen is aan strikte regels gebonden en de delingssnelheid is over het algemeen laag. Tumoren bestaan uit cellen die, om de één of andere reden, aan het regelsysteem zijn ontsnapt en normaliter delen zij met een ongewoon hoge snelheid. Veelal zijn tumorcellen gevoeliger voor DNA-beschadigende stoffen dan normale cellen. Dit heeft geleid tot de introduktie van DNA-beschadigende agentia bij de behandeling van patiënten met tumoren (cytostatika), met het doel de tumorcellen te doden, zodat de patiënt overleeft. Cisplatin (cis-diamminedichloroplatina(II)) is zo'n cytostatikum. Waarom de tumorcellen sneller gedood worden door het cisplatin dan gewone cellen, en wat de oorzaak is van de ernstige giftige (=toxische) neveneffekten die optreden in sommige gezonde cellen is nog steeds niet bekend.

Om de werking van het cisplatin te verbeteren en de neveneffekten te verhelpen is het belangrijk het werkingsmechanisme van cisplatin en verwante platinaverbindingen te leren kennen (zie Hst. 1 voor een overzicht). Hiertoe zijn de, in dit proefschrift beschreven, effekten van tien verschillende platinaverbindingen op moleculair niveau onderzocht in gekweekte zoogdiercellen.

Hst. 2 beschrijft een vergelijkende studie over de effekten van de tien platinacoordinatieverbindingen in gekweekte ovariumcellen van de Chinese hamster (CHO). De onderzochte platinaverbindingen verschillen in: 1) de valentie van het centrale platina-atoom (=Pt) (2- of 4-waardig); 2) de stereokonfiguratie (cis- of trans-, dwz. de reaktieve groepen zijn, respektievelijk, naast elkaar rond het Pt-atoom gerangschikt of zij bevinden zich tegenover elkaar, zie Fig. 1.1.; Hst 1), 3) de aard van de niet-reaktieve groepen. Onder deze platinaverbindingen zijn zowel antitumor-aktieve als -inaktieve verbindingen aanwezig.

Alle verbindingen vertraagden de normale (semi-konservatieve) DNA-synthese en veroorzaakten zeer kleine hoeveelheden DNA-synthese, ten gevolge van herstelwerkzaamheden. Er werden geen enkel- of dubbelstrengs breuken en alkaligevoelige plaatsen in het DNA van de cellen geïntroduceerd. Het vermogen tot celdoding (cytotoxiciteit) vertoonde grote verschillen tussen de tien platinaverbindingen. Zes van de tien verbindingen waren in staat om blijvende veranderingen (mutaties) in het DNA, die leidden tot abnormale cellen (mutanten), aan te brengen. Dit bleek uit het ontstaan van mutaties in het HGPRT gen (=bepaalde plaats in het DNA). In het algemeen hebben de verbindingen die mutageen zijn, ook antitumoraktiviteit in zoogdieren. Het verband tussen cytotoxiciteit en antitumorwerking is niet zo duidelijk. Alle platinaverbindingen reageren met het DNA in de cel en vormen covalent gebonden Pt-DNA-adducten (reaktie produkten van cisplatin met DNA); bij koncentraties, die leiden tot hetzelfde percentage overlevende cellen (equitoxische doses), lijken de weinig-toxische, niet-mutagene verbindingen beter in staat adducten te vormen dan de toxische verbindingen. De experimenten suggereren, dat de antitumor-aktieve verbindingen, die ook cytotoxisch en mutageen zijn, zeer specifieke en waarschijnlijk zeer schadelijke Pt-DNA-adducten vormen.

Zoals beschreven is in Hst. 4, blijkt dat, wanneer cisplatin vóórdat het werd toegevoegd aan de cellen een tijdje (0.5 -1 uur) in medium (=voedingsoplossing voor de cellen) bij 37^{o}C werd bewaard, dit resulteerde in een verhoogde hoeveelheid DNA-gebonden platina. De cy-

totoxiciteit in de CHO-cellen nam hierdoor met een zelfde factor (=2.5) toe.

De mogelijke aard van de specifieke Pt-DNA-adducten werd eveneens onderzocht in de CHO-cellen (Hst. 3). Deze studie beschrijft experimenten waarin vier platinaverbindingen werden vergeleken, nl: cis- en trans-diamminedichloroplatina(II); cis- en trans-diamminetetrachloroplatina(IV). De 2 cis-verbindingen waren mutageen en antitumor-aktief, terwijl de 2 trans-verbindingen dat niet waren. Na behandeling van de cellen met equitoxische doses kon gekonkludeerd worden dat de cis-Pt-DNA-adducten veel meer cytotoxisch zijn en effektiever 'sister chromatid exchanges' (SCE, een soort chromosoomschade) induceren dan de DNA-adducten gevormd door de trans-verbindingen.

Bij het zoeken naar de mogelijke vorming van DNA interstrand cross-links (kruisverbindingen tussen twee DNA strengen, zie Hst. 1) door de vier platinaverbindingen, werd ontdekt dat de *cis-Pt-complexen* deze DNA interstrand cross-links vertraagd vormen. Deze cross-links en de eveneens geïnduceerde DNA-eiwit cross-links (verbinding tussen één DNA streng en een eiwitmolekuul) werden slechts langzaam hersteld. De trans-verbindingen daarentegen, induceerden DNA interstrand en DNA-eiwit cross-links die snel gerepareerd werden. De herstelsystemen van de cel lijken in staat te zijn onderscheid te maken tussen cis- en trans-Pt-DNA-schade. Uit deze proeven bleek dat een zekere relatie tussen de niet-gerepareerde, door cis-Pt-verbindingen gevormde, DNA interstrand cross-links en de geïnduceerde cytotoxiciteit, mutageniteit, antitumoraktiviteit en de SCE vorming mogelijk is.

Studies aangaande de effekten van temperatuur (32°, 37° en 40°C) tijdens de behandeling van CHO-cellen met cis-diamminedichloroplatina(II) (cisplatin, cII) zijn beschreven in Hst 4. Bij verhoging van de behandelingstemperatuur nam de cytotoxiciteit van cisplatin toe. Deze toename kon verklaard worden door een grotere hoeveelheid aan DNA-gebonden platina. Na behandeling van de cellen bij de verschillende temperaturen met equitoxische doses van cisplatin, bleek dat het niveau van de DNA interstrand cross-links (24 hr na de behandeling) in alle gevallen gelijk was, hetgeen opnieuw een aanwijzing is dat de DNA

interstrand cross-links gerelateerd zijn met de cytotoxiciteit. Er lijkt echter geen relatie te zijn tussen de DNA interstrand cross-links en de mutageniteit bij de verschillende temperaturen, want na equitoxische koncentraties van cisplatin kon ook vastgesteld worden, dat de mutageniteit hoger was na een behandeling bij 32°C dan bij 40°C (terwijl de hoeveelheid cross-links gelijk was). Dit duidt erop, dat het spektrum van de door cisplatin geïnduceerde adducten beïnvloed wordt door de temperatuur.

Nieuwe en gevoelige immunochemische detectiemethoden werden gebruikt in de experimenten beschreven in Hst. 5. Deze methoden maakten de detectie van enkele specifieke Pt-DNA-adducten in het DNA geïsoleerd uit CHO-cellen mogelijk nadat deze behandeld waren met een vrij lage dosis cisplatin (nl., dosis resulterend in 1% overleving). Op t=0 (meteen na de behandeling van de cellen) bestond 36% van de totale hoeveelheid Pt-DNA-adducten uit intrastrand cross-links (kruisverbinding tussen twee basen in één DNA streng) op twee naast elkaar gelegen guanines (cis-$Pt(NH_3)_2$d(pGpG)) en 3% uit intrastrand cross-links op twee guanines gescheiden door een of meer basen (cis-$Pt(NH_3)_2$d(pG-$(pX)_n$pG)). Vervolgens kon 39% geïdentificeerd worden (m.b.v. atomaire absorptiespektroskopie) als monofunktioneel gebonden platina op guanine ($Pt(NH_3)_3$dGMP). De overige 23% van het totaal aantal Pt-DNA-adducten bestond uit DNA interstrand cross-links (0.1%), DNA-eiwit cross-links (0.1%) en verder waarschijnlijk uit intrastrand cross-links op naast elkaar gelegen adenine en guanine. Bij vergelijking van de resultaten in CHO cellen met die gevonden in in vitro (in de reageerbuis) behandeld DNA (zie Hst. 5), kon gekonkludeerd worden, dat het spektrum van adducten in beiden hetzelfde was, maar dat de percentages van de adducten verschilden. In CHO-cellen waren veel meer monofunktioneel gebonden Pt-DNA-adducten en minder intrastrand cross-links op pGpG basevolgordes dan in vitro behandeld DNA.

Werd het DNA uit, met cisplatin behandelde, cellen geïsoleerd nadat zij 24 uur doorgekweekt waren, voor de DNA-isolatie uit de cellen, werd gevonden dat de totale hoeveelheid DNA-gebonden platina met 40-60% gereduceerd was. Het spektrum van adducten was: 47% intrastrand cross-links op naburige guanines; 2.6% intrastrand cross-links op

$pG(pX)_npG$ basevolgordes (X kan zowel A, G, T of C zijn); 0.4% DNA interstrand cross-links en 0.1% DNA-eiwit cross-links. Monofunktioneel gebonden platina vertegenwoordigde, ten hoogste, 15% van het totaal aantal lesies en de intrastrand cross-links op pApG basevolgordes, ten hoogste 48%.

Bij het kombineren van resultaten uit cellen behandeld met cisplatin (Pt-DNA-adducten versus biologische effekten) kon vastgesteld worden, dat alle bifunktionele adducten verantwoordelijk zouden kunnen zijn voor de cytotoxiciteit, mutageniteit en de antitumoraktiviteit van cisplatin. Er waren evenwel redenen om aan te nemen dat de DNA-eiwit cross-links alleen betrokken zullen zijn bij chromosomale afwijkingen. De intrastrand cross-links op pGpG (en eventueel ook die op pApG) basevolgordes zouden, vanwege hun grote hoeveelheid, de DNA synthese remming kunnen veroorzaken. Cross-links op pGpXpG basevolgordes en DNA interstrand cross-links leken (numeriek) te korreleren met de mutatie-induktie. Zoals beschreven in Hst. 4, lijken de DNA interstrand cross-links echter niet geheel verantwoordelijk te zijn voor het ontstaan van mutanten; zij kunnen echter wel verantwoordelijk gesteld worden voor de cytotoxiciteit en de induktie van SCE.

In Hst. 6 worden de effekten van cisplatin op gekweekte menselijke cellen beschreven. Naast cellen geïsoleerd uit stukjes (spier) weefsel van gezonde mensen werden ook cellen van mensen met bepaalde erfelijke aandoeningen (xeroderma pigmentosum (XP); Fanconi's anemia (FA); en mucolipidosis-II-patienten (RD)) gekweekt en op de effekten van cisplatin onderzocht. Ter vergelijking werden deze cellen ook onderworpen aan stoffen waarvan de werking (redelijk) bekend was.

FA cellen waarvan gedacht wordt dat zij de mogelijkheid tot het herstellen van DNA interstrand cross-links missen, bleken zeer gevoelig te zijn voor cisplatin, evenals de XP cellen, die gestoord zijn in het vermogen om schade, veroorzaakt door ultravioletlicht (bv. zonlicht), uit hun DNA te verwijderen. De hypothese wordt geopperd, dat voor het herstel van de DNA interstrand cross-links geïnduceerd door cisplatin, welke verantwoordelijk zijn voor de celdood, twee herstelsystemen nodig zijn. Eén daarvan is afwezig in FA cellen ('short-patch' excisieherstel), het andere is afwezig in XP cellen ('long-

patch' excisieherstel). Aan de andere kant zou de hoge gevoeligheid van XP cellen voor cisplatin veroorzaakt kunnen worden door onvolledig herstel van andere Pt-DNA-adducten. Uit metingen van de DNA-herstel-synthese in deze mensecellen leek het erop dat het grootste deel van de schade hersteld werd via het 'short-patch' excisieherstel.

Verder onderzoek is nodig om de aangetoonde Pt-DNA-adducten te relateren aan de biologische effekten van de platinaverbindingen en om de effekten van de verschillende DNA-herstelsystemen op deze adducten na te gaan.

CURRICULUM VITAE

22 januari 1954	geboren te Schiedam
juni 1971	eindexamen HBS-B aan de Rijksscholengemeenschap te Schiedam
september 1971	inschrijving aan de Rijksuniversiteit te Leiden, studierichting biologie
juni 1975	kandidaatsexamen biologie, 2e hoofdvak scheikunde, start doktoraal studie - hoofdvak genetika, o.l.v. dr. F.M.A. van Breugel - hoofdvak plantengenetika, o.l.v. dr. K. Planqué - onderwijsbevoegdheid biologie
september 1978	doktoraal examen biologie

In de studiejaar 1974 t/m 1978 was ik, als student-assistent, in dienst van de Rijksuniversiteit Leiden bij de subfakulteit biologie, afdeling genetika, ter assistentie bij de cursus genetika voor 1e jaars biologen (1e drie jaren) en op de cursus genetika voor 3e jaars chemici (laatste jaar). Van 1 oktober 1978 tot 1 januari 1979 ben ik werkzaam geweest (research fellow) in het Biological Research Center te Szeged, Hongarije, op de afdeling plantengenetika van dr. P. Maliga, o.l.v. dr. L. Márton. Dit onderzoek werd gesubsidieerd door UNESCO en de Hungarian Academy of Science. Van 15 juni 1979 tot 1 februari 1984 ben ik als wetenschappelijk medewerkster werkzaam geweest in het Medisch Biologisch Laboratorium TNO te Rijswijk, binnen de afdeling biochemie, o.l.v. dr. F. Berends, in de sectie genetische toxicologie o.l.v. dr. ir. P.H.M. Lohman, alwaar het in dit proefschrift beschreven onderzoek werd verricht.

NAWOORD

Zonder de experimentele hulp van Margriet van Dijk was dit proefschrift niet of veel later tot stand gekomen, vooral de experimenten van het laatste uur waren onmisbaar. Anne Marie Fichtinger-Schepman, Rob Baan, Govert van der Schans, Paul Lohman en Frits Berends hebben zeer veel tijd opgeofferd en veel moeite besteed aan het bekritiseren van mijn manuscripten. Verder wil ik een aantal mensen, die aan de tot stand koming van dit proefschrift hebben bijgedragen, met name noemen:

- Angelique Venema, die mij, als stageaire, enige tijd heeft bijgestaan;
- de medewerkers van de afdeling fotografie (o.l.v. Michel Boermans), die op sublieme wijze zorg hebben gedragen voor alle tekeningen en foto's;
- de medewerkers van de keuken (o.l.v. Jan van der Linden), die het reinigen van al het glaswerk hebben verzorgd;
- Ria Engelen en Henny Wilhelmi, die mij geholpen hebben bij het gebruiken van de tekstverwerkers;
- de medewerkers van de werkplaats (o.l.v. Jan Engelen) en alle andere personen die op enigerlei wijze hebben bijgedragen aan mijn werk;
- de deelnemers van de platinawerkgroep, die de bijeenkomsten tot stimulerende gebeurtenissen maakten;
- Ton Marcelis en collega's, die de platinaverbindingen hebben gesynthetiseerd;
- Puck, die mij geregeld van dropjes heeft voorzien;
- alle vrienden, die maandenlang de vorderingen en tegenslagen hebben willen aanhoren.

De afdeling biochemie van het MBL-TNO, speciaal de sectie genetische toxicologie, zal ik altijd een warm plekje in mijn hart toedragen, de fijne werksfeer was voor mij onmisbaar. Ik heb dagelijks veel plezier beleefd aan de gezellige, soms dolle autoritten van en naar Leiden, die ik met Rein en Ton heb gemaakt. Last but not least, dient het KWF genoemd te worden dat mij de kans heeft gegeven dit onderzoek uit te voeren.