DETERMINANTS OF CISPLATIN SENSITIVITY IN HEAD AND NECK CANCER

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The research described in this thesis was performed at the Departments of Genetic Toxicology, Medical Biological Laboratory, TNO, Rijswijk (currently TNO Nutrition and Food Research Institute, Zeist), and Otolaryngology/Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

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aan mijn ouders
voor Peter en Anika

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Chapter 1

General Introduction.

1. Platinum compounds

In 1969 Rosenberg accidentally discovered the capability of *cis*-diamminedichloroplatinum(II), also called cisplatin (Figure 1), to block cell division in bacteria (1). Further studies revealed the antineoplastic properties of cisplatin and these encouraging results led to its introduction as an anticancer drug. Since then, cisplatin and more recently also its analogue carboplatin [*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum (II)] (Figure 1) have been proven to be useful chemotherapeutic agents against a broad range of human solid tumors. With a variable success rate, they are used for the treatment of testicular, ovarian, bladder, cervical and head and neck cancers. Over 90% of the patients with testicular tumors are cured with cisplatin therapy (2, 3). In case of head and neck squamous cell carcinoma (HNSCC) only 30-50% of the patients are responding to the cisplatin treatment, however, without improvement of their long-term survival (4, 5). One of the reasons is the occurrence of a second primary tumor in the head and neck region. Such a tumor is seen in about 15-30% of the patients, dependent on the site of the first tumor and the length of the follow-up (6).

The contribution of the platinum (Pt) compound itself to the risks of second cancers is difficult to establish since Pt-containing antitumor agents are generally administered to cancer patients in combination with other cytostatics. So far no cases have been published with strong indications for a carcinogenic potential of Pt compounds in humans.

However, there are two main factors that limit the broad application of Pt compounds, namely, the occurrence of severe side effects and the development of resistance to the drugs.

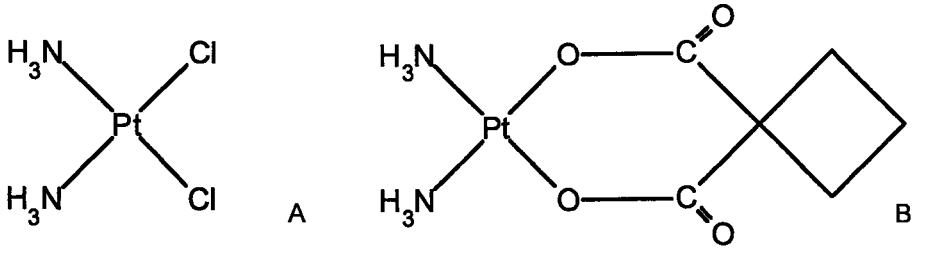

Figure 1. Structural formula of cisplatin (*A*) and carboplatin (*B*).

1.1. Side effects of Pt-containing drugs

Cisplatin and carboplatin show different dose-limiting side effects. Cisplatin is administered by infusion or bolus injection in doses of 20-100 mg/m^2. Due to the fact that a large amount of the administered dose is cleared within a few hours by the kidneys, these organs are targets for toxicity. Beside this major, dose-limiting, side effect cisplatin can cause nausea and vomiting, ototoxicity and neurotoxicity (7, 8).

Carboplatin is a so-called second-generation Pt compound, which is also widely used in the clinic (9). Other new second- and third-generation Pt-containing drugs like tetraplatin [d/1-tetrachloro-1,2-diaminocyclohexane platinum(IV)], iproplatin [dichlorodihydroxo-bis-2-propanamine platinum(IV)], lobaplatin [(1,2-diaminomethyl-cyclobutane) platin(II) lactate], oxaliplatin [oxalato-trans (1-1,2-diaminocyclohexane) platinum(II)] and the orally administered Pt compound JM216 [bis-acetato (ammine) dichloro (cyclohexylamine) platinum] are still in phase I or II clinical trials. Carboplatin can be administered in higher dosages (300-1200 mg/m^2) than cisplatin and is not leading to renal toxicity, possibly due to the slower formation of its hydrolyzed species. Also the clearance of carboplatin is slower than that of cisplatin (8, 10). The most important dose-limiting side effect of carboplatin is myelosuppression. Because of these non-overlapping toxicities, cisplatin and carboplatin can be combined during therapy.

To circumvent renal toxicity in patients due to cisplatin treatment, the drug can be administered in hypertonic saline to prevent hydrolysis. Also the diuresis can be enhanced. The hematological toxicity caused by carboplatin can be lowered by the administration of colony-stimulating factors and/or with autologous bone marrow or peripheral stem cell transplantation (11). Another approach is the use of rescue agents such as sodium thiosulfate and amifostine (WR2721). The advantage of amifostine is its selective protection of normal tissues in comparison to tumor tissues (12, 13). Due to these modulating agents, which reduce the toxic side effects, it is possible to increase the doses of the Pt drug to be administered.

1.2. Resistance to Pt compounds

A major problem in the clinic is the resistance of patients to Pt-based regimens, which can be intrinsic or acquired during chemotherapy (14). The acquisition of resistance against cisplatin has extensively been studied in *in vitro* selected cell lines (15). In these studies the cells have been exposed for a long time period to a supra-physiological dose of cisplatin before resistance is induced. Subsequently, these cells can be cultured in the absence of the drug while resistance is maintained. The advantage of this approach is that mechanisms leading to drug resistance in these cells can be compared with those in the sensitive parental cells.

Various factors have been reported to be involved in the resistance against cisplatin: reduced drug accumulation, enhanced detoxification, reduced levels of DNA damage, increased DNA repair and/or tolerance of DNA damage. In most studies, the obtained resistance appeared to be determined by a combination of factors.

The first barrier for cisplatin is the entrance into the cell. It is generally believed that the drug enters cells by passive diffusion, but some evidence has been found for active transport across the cell membrane (16). The fact that the intracellular amount of cisplatin (free or bound) increases linearly with the exposure dose and is not

saturable are arguments in favor of the passive diffusion theory. However, the discovery of modulators of Pt-drug accumulation and energy-dependent transport systems is more in agreement with an active transport mechanism during influx and efflux of Pt drugs. By reducing the accumulation of the drug, cells can become resistant. It has been reported that some cisplatin-resistant cells are able to maintain a low intracellular Pt-drug level, either by reduced uptake and/or a higher efflux of the Pt drug. The influx is dependent on the permeability of the cell membrane and on the involvement of possible channels or pumps (16). The efflux of many anticancer drugs is based on an active transport mechanism, which is known as the multidrug-resistance (MDR) phenomenon. The best known MDR-related proteins are P-glycoprotein (Pgp), multidrug-resistance associated protein (MRP) and lung-resistance protein (LRP), which are responsible for active transport of drugs out of the cell. Whether one or more of these proteins are able to transport cisplatin has not been clearly established.

Chemotherapeutic agents such as cisplatin can bind to glutathione (GSH) and/or metallothionein, a process catalysed by the enzyme glutathione-S-transferase (GST). The GSH-conjugate can subsequently be actively transported by the so-called glutathione-X-conjugated (GS-X) export pump (17, 18). It has been reported that the GS-X pump is the same as the MRP, although results of De Vries and colleagues (19) implied that transport of GSH-conjugated cisplatin is not the major function of the MRP. It is mainly transporting the unbound drug out of the cells. Another pump, recently described, is the SQM1 protein, which was found to be reduced in a cisplatin-resistant HNSCC cell line (20). The canalicular multispecific organic anion transporter (cMOAT), also known as MRP2, was found to be overexpressed in a cisplatin-resistant human HNSCC cell line (21). The definitive role of these two new transporter molecules in cisplatin resistance has still to be elucidated.

In vitro cultured cells, which were made resistant to cisplatin, often contain increased GSH levels (17, 22). However, investigations of the cisplatin-GSH conjugate in the presence of isolated DNA have shown that this binding is reversible (23, 24), and this suggest that the cisplatin-GSH complex is a transient entity from which cisplatin can be released to bind to the DNA. In addition to GST, enzymes such as glutathione peroxidase, glutathione synthetase, glutathione reductase and dipeptide gamma-glutamylcysteine synthetase (γ-GS) have a potential role in the detoxification of drugs (25, 26). It has been reported by Kurokawa *et al.* (26) that in cisplatin-resistant cells increased levels of γ-GS resulted in increased activity of the GS-X pump.

Finally, when cisplatin has bound to DNA and Pt-DNA adducts have been formed (see below), the cells can respond in various ways: either they undergo growth arrest followed by DNA repair or the cells die, *e.g.* through apoptosis, or the cells are tolerant to DNA damage and are still able to proliferate.

Increased DNA repair has indeed been found in cisplatin-resistant cell lines (15). Cisplatin-DNA adducts can be repaired via the nucleotide excision repair (NER) pathway (27), which consists of a number of steps: recognition of the DNA damage, removal of the adduct by incision and excision, filling of the gap by complementation of the single strand by DNA polymerase I, and finally, ligation of the repaired strand (28). Also other mechanisms of DNA repair may be involved in the removal of cisplatin-DNA damage, such as mismatch repair (29), base excision repair by DNA glycosylases (30) and postreplication repair (31). DNA damage is usually repaired before cells continue to divide. However, when the repair is not sufficient or absent and the cell is still proliferating, the damage can lead to mutations, which are then transferred to the daughter cells.

When the cells have too much damage in their DNA they may undergo programmed cell death, the so-called apoptosis (32, 33). Cell-cycle control proteins such as p53 in the late G1-phase, are involved in this process. Cisplatin-induced apoptosis has been described in human ovarian carcinoma, melanoma and germ-cell tumor lines (34-36). In case of an inactivating mutation in p53, cells are able to escape apoptosis, which results in a reduced susceptibility towards DNA damaging agents (37).

Recently, an increasing amount of evidence has been provided that the ability of cells to tolerate high levels of DNA damage is also a potential mechanism of resistance (38-41). Despite that damage, such resistant cell lines are still able to proliferate, suggesting that the enzyme polymerase, needed for DNA replication, is mutated or altered because it is no longer obstructed by the Pt-damage on the template. This phenomenon is called replicative bypass (42). Also increased mRNA levels of polymerase (43) and damage recognition proteins (44, 45) have been reported to be involved in this tolerance phenomenon.

2. Platinum (Pt)-DNA adducts

2.1. Formation of Pt-DNA adducts

In the cell cisplatin and carboplatin must undergo hydrolysis before reaction with the nucleobases in DNA takes place and a stable chelate is formed (Pt-DNA adduct). It is hypothesized that these adducts are responsible for the antitumor activity of the Pt drugs (46, 47). Because of this modification of DNA, Pt-containing agents have been classified in the group of alkylating agents. Due to the binding of the Pt drug the DNA double helix becomes distorted and forms 'kinks' of about 40° in the direction of the helical axis (48). These conformational changes are recognized by damage recognition proteins such as the high mobility group (HMG) proteins (45).

Pt-containing drugs react preferentially with nitrogen atoms of the nucleobases guanine (G) and adenine (A) in the DNA, which leads to the formation of various

types of adducts (Figure 2). After hydrolysis of one chloride, cisplatin forms a monoadduct through reaction with the N7 of guanine (abbreviation Pt-G). Most of these monoadducts are subsequently converted to bifunctional adducts after hydrolysis of the second chloride. At the end of these reactions the relative occurrence of the monoadduct is about 12%, while the major adduct is present in the form of the bifunctional adduct Pt-GG (about 65%) in which cisplatin is bound to two adjacent guanines. In other intrastrand crosslinks cisplatin is bound to guanine and a neighbouring adenine (Pt-AG; about 14%) or to two guanines separated by one or more nucleobases (G-Pt-G; about 9%) (49). A minority of the adducts are present as interstrand crosslinks G-Pt-G, in which the Pt drug is bound to two guanines on separate DNA strands (about 2%). Carboplatin is able to form the same types of adducts, but its reaction rate is much slower. A striking difference with cisplatin, however, is the ratio between the adduct types formed; the occurrence of the adduct Pt-GG after carboplatin treatment is only about 31%, while G-Pt-G is the major adduct formed (about 37%) (50). The amounts of the Pt-G and Pt-AG adducts are similar as found in cisplatin-treated DNA (about 16% each).

Comparison of the adduct levels induced by cisplatin and carboplatin after treatment of Chinese hamster ovary cells with equitoxic dosages of these drugs, suggested that the intrastrand, bifunctional Pt-AG adduct might be responsible for the cell killing effect (51).

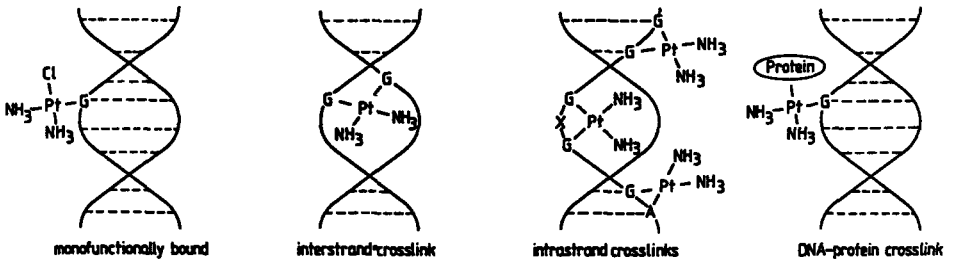

Figure 2. Schematic representation of the known adducts formed upon treatment of DNA with cisplatin or carboplatin.

2.2. Detection methods of Pt-DNA adducts

Because it is generally accepted that Pt-DNA adducts are responsible for the antitumor activity of Pt-antitumor agents, it is very important to be able to study the intracellular levels of these lesions. For this purpose, several methods have been developed to determine the cisplatin-DNA adducts, but these differ in sensitivity, application and amount of DNA needed.

Various methods have been used to determine total Pt levels such as adsorptive voltammetry (52), pulse polarography (53) and atomic absorption spectroscopy (AAS). With these methods both the free Pt as well as the Pt bound to DNA or proteins in blood and urine of treated patients can be quantified; these assays are sensitive enough for such applications. In general, AAS is used to detect Pt-containing species in white blood cells (WBC) or other cells and tissues (54-56); this method can also be used to determine adduct levels in DNA samples isolated from patient blood. The disadvantage is that large DNA samples (>200 mg) are needed to determine adduct levels in the low fmol/μg DNA range. An advantage, however, is that this method measures the Pt atom itself and is thus applicable for adducts of all types of Pt-containing compounds.

An assay to specifically determine the various cisplatin-DNA adducts is the competitive enzyme-linked immunosorbent assay (ELISA) as published by Fichtinger-Schepman *et al.* (57). After digestion of the DNA to nucleotides, the various adducts are separated from the unmodified nucleotides by anion-exchange column chromatography (49). Then, the adducts are separately quantified by the use of various rabbit antisera raised against cisplatin-containing (di)-nucleotides. The amount of DNA required for this assay is still rather large, *i.e.* about 100 mg in order to detect the low platination levels of about 0.5 - 2 fmol/μg DNA encountered in patient WBC or tumor tissues. The advantages of this method compared to AAS are that it is more sensitive and that it can distinguish between the various adduct types. Disadvantages are that the assay is rather laborious and that the availability of the antisera is limited. Furthermore, this assay is applicable for cisplatin and carboplatin adducts but probably not for those formed by other Pt compounds like iproplatin and lobaplatin due to the specificity of the antibodies.

A method for the detection of Pt-DNA adducts directly in cells is immunocytochemistry, in which *e.g.* the polyclonal antibody NKI-A59 raised against cisplatin-modified DNA is used (40, 58). The availability of this antiserum is also limited, and the sensitivity of the assay is sometimes not high enough to analyse cells obtained from patients treated with Pt-containing agents. An advantage, however, is that with this assay the adduct levels can be measured at the single cell level and that, in combination with cell-type specific immunostaining, extra information can be obtained on adduct levels in specific cell types.

The fact that clinical samples such as tumor biopsies are often quite small and contain DNA with low platination levels is a reason to develop more sensitive and accurate detection methods. In this thesis, an approach to solve this problem is described, *viz.* by modification of the ^{32}P-postlabelling assay for Pt-containing DNA adducts as first described by Blommaert and Saris (59). In their method, the adducts Pt-GG and Pt-AG are purified by a strong-cation exchanger (SCX) after digestion of the DNA to nucleosides. Subsequently, by incubation with sodium cyanide (NaCN) the Pt is removed to increase the labelling efficiency of the resulting dinucleotides

GpG and ApG. Then, after ^{32}P-labelling and separation by thin-layer chromatography, the radioactivity of the products is determined by use of autoradiography (see also Chapter 5).

3. Predictive assays for tumor responses to Pt compounds

A method to prospectively determine the efficacy of Pt-containing chemotherapy is important because it would provide the opportunity to individualize the treatment. Prediction of the clinical response will enable clinicians to establish an optimal dose of the drug when the tumor appears to be responding, or to choose an alternative treatment when it is revealed that the tumor is not responding. The ultimate goal is to improve the response rates.

So far, a number of reports have shown a correlation between the tumor response and the level of Pt-DNA adducts in WBC from treated patients with different types of cancer (54, 56, 60-64). However, other studies could not demonstrate such a correlation (65-67). These inconsistent results can probably be explained by the fact that the treatment regimens and the methods of adduct determination were different. The use of these WBC for predicting tumor response, is only valid when the Pt drug is equally available for these cells as compared to the tumor cells and when these cells deal with the ensuing DNA damage in the same way as the tumor cells do during the time period studied after the drug treatment. The correlation that has been found in several studies between the clinical response and the DNA damage in this easily obtainable cell type suggests that the induction and processing of DNA platination in the eventual target, *i.e.* the tumor, is indeed similar.

Already in 1987, Fichtinger-Schepman and colleagues demonstrated a linear correlation between the platination levels present in WBC after *in vitro* treatment with cisplatin and those in WBC from the same patient after chemotherapy. However, no comparison was made between these adduct levels, after *in vitro* and *in vivo* cisplatin exposure, and the tumor response (68). Armstrong and coworkers reported a relation between Pt-DNA adduct levels in *in vitro* treated bone-marrow aspirates and the extent of remission in adult acute nonlymphocytic leukemia (69). In addition, Oshita *et al.* found a correlation between the DNA platination level in WBC treated *in vitro* and the tumor response to cisplatin treatment in patients with non-small cell lung cancer (NSCLC) (70).

Investigators also used *in vitro* sensitivity tests on tumor cells to predict the clinical response to cisplatin treatment. It has been shown that a 'histoculture drug-response assay' can be used to predict clinical outcome in cancer patients (71, 72). Shaw and colleagues selected the chemotherapy combination per patient

prospectively, on the based of the *in vitro* drug sensitivity testing results of cell lines derived from the patient's small cell lung cancer (SCLC) or NSCLC tumor (73). However, the overall response to this individualized chemotherapy was not significantly better than the response rate following standard cisplatin-containing regimens. There was also no difference in survival time after treatment between these two patient groups, which illustrates the poor predictive value of this *in vitro* test (73). More encouraging findings were reported by Yamaue *et al.* (74). They individualized the chemotherapy combination for patients with colorectal cancer on the basis of the *in vitro* sensitivity of purified tumor cells by use of the MTT assay. The clinical response was better than in patients on standard treatment, suggesting that individualization of chemotherapy may be worthwhile.

Also several other factors, besides platination levels and drug sensitivity, have been found to be significantly correlated to the response of cisplatin-based chemotherapy. High GST-pi expression in NSCLC patients was related to low response rates (75). Also accumulation of and missense mutations in the cell-cycle control protein p53 is predictive of a poorer clinical outcome of ovarian carcinoma and NSCLC after cisplatin-based regimens (76, 77).

4. Head and neck squamous cell carcinoma (HNSCC)

One tumor type for which it is very important to be able to predict the tumor response is HNSCC since the response rates to cisplatin treatment are rather low. HNSCC affects over 500,000 persons per year worldwide. This cancer accounts for 10% of all cancer cases and for more than 5% of all cancer deaths (5). HNSCC arises in the mucosa of the upper aerodigestive tract including the oral cavity, pharynx, larynx, nasal cavity and paranasal sinuses.

Epidemiological data indicate that the incidence of this malignancy is increasing due to exogenous risk factors, primarily the use of tobacco and alcohol (78, 79). The combination of these two risk factors has been shown to result in a more than additive relative risk. A major factor that determines the development of HNSCC, however, may be the intrinsic susceptibility of individuals to DNA damage (80).

The treatment of HNSCC is dependent on the tumor site and the stage of the disease (5). The most common approach is surgery and/or radiation, which results in cure rates of over 60% in early-stage disease. Patients with advanced disease have a poor prognosis due to local recurrence and the development of metastatic disease. Chemotherapy in these patients with cisplatin as a single agent or in combination with other drugs shows an increased therapeutic activity with partial and complete response rates between 30 and 50% (5). However, despite the initial responses, the long-term survival of these patients has not improved due to tumor regrowth (81).

5. Outline of the thesis

As described above, HNSCC patients treated with cisplatin show large interindividual variation in tumor response. It is interesting to investigate why only 30 to 50% of the HNSCC patients are responding to the therapy and what mechanisms are involved herein. This study was performed to establish a possible correlation between Pt-DNA adduct levels in the tumor cells and the tumor response and a correlation between the adduct levels obtained after *ex vivo* treatment and the *in vivo* response. This will open the possibility to develop an assay to predict the response of a tumor to cisplatin treatment already before the actual chemotherapy has started, as well as to optimize the treatment for each individual patient.

So far, little is known about factors determining the response to cisplatin in patients. Therefore, the first aim of the work described in this thesis was to study the mechanisms underlying cisplatin sensitivity. As an *in vitro* model system, a panel of human HNSCC cell lines with various sensitivities towards cisplatin was used. The sensitivity of the cultured cells to cisplatin was determined by use of a colorimetric method and correlated with various cellular parameters: the cellular doubling time, the DNA index and the degree of differentiation, the latter determined with antibodies directed against cytokeratins. An additional parameter was the intracellular Pt-drug level determined with AAS over a given time period (Chapter 2).

An increased level of GSH has been described as one of the underlying factors of resistance to cisplatin therapy. Therefore, these levels were determined in the HNSCC cell lines and correlated with the *in vitro* sensitivity. An additional aspect of this study concerned conjugation and transport of the Pt-containing drugs. Cisplatin bound to GSH can be exported from the cells by the GS-X pump (see above). It is thought that this pump is similar to MRP, however, conflicting opinions have also been reported. For this reason the relevance of MRP and two other MDR-related proteins, Pgp and LRP was investigated. The levels of these proteins were determined by immunohistochemistry and correlated with the response of the cultured cells to cisplatin exposure (Chapter 3).

The previously mentioned studies address the factors involved in the sensitivity of HNSCC cells to cisplatin treatment. HNSCC originates from stem cells in the basal layer of the mucosa in which mutations have been induced, possibly by genotoxic compounds. Protection of this layer is of crucial importance. It is likely that this protection is provided by keratinocytes in the surface (parabasal and superficial) layers. It is hypothesized that these keratinocytes, the normal healthy counterpart of the tumor cells, have a function as a barrier to absorb mutagenic and carcinogenic compounds. They can respond in three ways to this exposure, either by growth arrest followed by DNA repair, by dying in a programmed manner through apoptosis, or by tolerating the DNA damage and hold up the barrier. In *in vitro* assays we

studied how these normal oral keratinocytes deal with the DNA damage induced by cisplatin. The growth inhibition by cisplatin was determined and correlated with the intracellular Pt levels and the number of cells undergoing apoptosis. These results were also compared with those obtained from the HNSCC cell lines (Chapter 4).

It is generally accepted that the antitumor activity of cisplatin is based on the specific interaction of this drug with DNA, which results in the formation of Pt-DNA adducts. Therefore, it is important to investigate the adduct levels in tumor tissues. Unfortunately, the methods available to determine the Pt-DNA adduct levels were not sufficiently sensitive and accurate for the monitoring of adduct levels in the usually small tumor specimens, which also contain low adduct levels. Therefore, as shown in Chapter 5, a recently developed ^{32}P-postlabelling method for the detection of the two major cisplatin adducts, *viz.* Pt-GG and Pt-AG, has been modified for the accurate quantification of the adduct levels in small DNA samples.

In order to investigate whether the adduct levels in the tumor itself are correlated with the tumor response, HNSCC-bearing nude mice were treated with cisplatin intravenously and the responses to therapy were determined. At various time points after the start of the treatment the tumors were removed and analyzed with respect to cisplatin accumulation and DNA adduct levels. These data were related to the *in vivo* tumor responses. To investigate the value of *in vivo* studies for the prediction of the *in vivo* response, the cisplatin accumulation, the DNA adduct levels and the survival after cisplatin treatment of the cultured HNSCC cells were compared with those obtained after the *in vivo* treatments in nude mice carrying HNSCC xenografts (Chapter 6).

Finally, attempts were made to set up a predictive test as a tool to individualize the treatment with cisplatin. With such a test, on one hand unnecessary treatment can be prevented when the tumor appears to be insensitive, whereas on the other hand an optimal dose can be established if the tumor is sensitive. It is to be expected that the use of protective agents (bone marrow growth factors or rescue agents) will allow the maximal tolerated dose to be increased. This will open perspectives to obtain more complete responses or even cures. The used hypothesis was that the adduct levels in *in vitro* treated tumors could be predictive of the *in vivo* tumor response. In order to investigate this hypothesis, complete human HNSCC tumors as well as tumor fragments were treated in the laboratory with cisplatin. The resulting Pt-DNA adduct levels were related to the response of the same HNSCC tumors in cisplatin-treated nude mice. In addition, tumor biopsies from patients were treated with cisplatin and cisplatin-DNA adduct levels were determined. Because three of these patients were treated with cisplatin after the biopsy was taken, the responses could be compared to the adduct levels in the biopsies (Chapter 7).

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Chapter 2

Relationship between the parameters cellular differentiation, doubling time and platinum accumulation and cisplatin sensitivity in a panel of head and neck cancer cell lines.

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Abstract

Patients with head and neck squamous cell carcinoma (HNSCC) treated with cisplatin show a large inter-individual variation in tumor response. Little is known about factors that contribute to this variation. The aim of our study was to correlate the sensitivity to cisplatin with a number of cellular parameters using a panel of 10 human HNSCC cell lines. A 7-fold variation in response after 72 hr of exposure to cisplatin as determined in a colorimetric proliferation assay was observed. The IC_{50} values did not correlate with the DNA index, the cellular doubling time or the expression of differentiation markers. Intracellular platinum (Pt) concentrations were measured by atomic absorption spectroscopy after exposing the cells to 10 μM cisplatin for 1- 72 hr. The intracellular Pt levels increased up to 24 hr. One cell line, derived from the tumor of a patient previously treated with radiotherapy, accumulated much more Pt than the other cell lines. For these other cell lines, a significant positive correlation was found between Pt accumulation and sensitivity. In conclusion, cisplatin-induced growth inhibition in HNSCC *in vitro* is generally positively correlated with cellular Pt levels. However, the fact that occasionally cancer cells can survive despite high intracellular Pt levels indicates that additional parameters are needed to explain a response unequivocally.

Introduction

Head and neck squamous cell carcinoma (HNSCC) affects over 500,000 persons a year worldwide and accounts for 10% of all cancer cases and for more than 5% of all cancer deaths (1). This cancer arises in the mucosa of the upper aerodigestive tract including the oral cavity, pharynx, larynx, nasal cavity and paranasal sinuses (1). Using surgery and radiation, a cure rate of over 60% is obtained in patients with early-stage disease. However, patients with advanced disease have a poor prognosis: fewer than 30% can be cured (1). Therefore, there is still ample scope to optimize present modalities and introduce new strategies.

With cisplatin as a single agent or in combination with other drugs, encouraging results in chemotherapy of HNSCC have been obtained. However, in spite of an initially good response rate after monochemotherapy in 30-50% of the patients, long-term survival has not improved (2). Cisplatin in combination with 5-fluorouracil gives very good results in neoadjuvant therapy of HNSCC (1). The efficacy of this treatment, however, has still to be confirmed in well-designed randomized clinical trials.

An explanation for the inter-individual variation in tumor response upon cisplatin chemotherapy is resistance at the cellular level, which can be either intrinsic or acquired due to earlier treatments (3). Mechanisms that may explain cisplatin resistance are increased growth rate, decreased platinum (Pt) accumulation, increased cisplatin inactivation by glutathione (GSH) or metallothionein, decreased DNA-adduct formation and enhanced DNA repair (3, 4). Interestingly, large inter-individual differences have been determined with respect to DNA-adducts in white blood cells of cisplatin-treated patients, which may be predictive for tumor response (5, 6). An association between cisplatin sensitivity and the cytokeratins (cks) 14 and 18 has been described. The cisplatin-resistant tumor cells showed a decreased expression level of these cks (7, 8). Cks are a family of intermediate filament proteins and specific for epithelial cells. Besides the newly reported function in drug transport, the expression of cks depends on the type of epithelium, degree of differentiation and transformation (9).

In most studies that aim to explain differences in cisplatin sensitivity, cell lines have been used that were made resistant *in vitro* by exposing the cells for months to unrealistically high concentrations of cisplatin. These cell lines often show a 10- to 100-fold lower sensitivity when compared with the parental cell lines from which they were derived (3, 4). It is questionable whether the results of such an approach justify conclusions about variable cisplatin sensitivity *in vivo*.

We now describe a study on a panel of HNSCC cell lines derived from untreated and previously treated patients for which the sensitivity was correlated with various cellular and pharmacokinetic parameters.

Material and Methods

Cell cultures

Human HNSCC cell lines UM-SCC-11B (11B), UM-SCC-14C (14C), UM-SCC-22A (22A), UM-SCC-22B (22B), and UM-SCC-35 (35), were obtained from Dr T.E. Carey (Ann Arbor, MI). Cell lines 92VU040T (040T), 93VU096aT (096aT), and 93VU120T (120T) were established at the laboratory of Human Genetics, *Vrije Universiteit*, Amsterdam, The Netherlands (10). The cell lines VU-SCC-RO (RO) and VU-SCC-OE (OE) were established from xenografts. The latter were obtained after implantation of fresh human HNSCC biopsies into athymic nude mice. RO originates from the oropharynx and OE from a metastasis of the oral cavity. The corresponding cell lines RO and OE were established after 41 and 32 passages *in vivo*, respectively, and could be kept in culture for a further 12 passages in case of RO and for over 90 passages in case of OE. The site, stage and histological features of the established cell lines and the gender, age and survival data of the patients from whom the tumors originated are listed in Table I (10).

Cells were routinely grown at 37°C and 5% CO_2 as subconfluent monolayers in 25- or 75-cm^2 flasks (Nunclon, Nunc, Roskilde, Denmark). RO and 096aT were used between passage 5 and 10. Experiments with the other cell lines were performed within 20 passages starting from 5 to 25. The HNSCC cell lines were regularly checked for mycoplasma infection using Hoechst 33258 (according to the method described by the supplier, Sigma, St.Louis, MO) and found to be free of contamination. Cell lines 11B, 14C, 22A, 22B and 35 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Breda, The Netherlands), supplemented with 5% heat-inactivated FCS (Flow, Irvine, UK) and 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco-BRL). The RO, OE, 040T, 096aT and 120T cell lines were cultured in minimal essential medium (MEM, Gibco-BRL) supplemented with 10% FCS, 1% non-essential amino acids (Gibco-BRL) and penicillin/streptomycin.

Sensitivity to cisplatin

To determine inhibition of cell growth in response to cisplatin exposure, the semi-automated proliferation assay with sulforhodamine B (SRB, Sigma) was used, as described previously (11). Briefly, 1,000-3,000 cells in 150 µl medium were plated in the wells of 96-well plates (Greiner, Friekenhausen, Germany) and incubated at 37°C. After 72 hr in lag phase, 50 µl of medium containing cisplatin (Platinol; Bristol Meyers Squibb, Woerden, The Netherlands) at the desired concentration and 2.5% FCS was added to the wells and the cells were further incubated for 72 hr. The optimal cell density at the start was cell line dependent and was chosen to ensure exponential growth for these last 72 hr. The cells were then fixed with 25% TCA and the cellular proteins stained with SRB solution (0.4% SRB in 1% acetic acid). Absorbance was measured at 540 nm, using a micro-plate reader (Multiskan, Helsinki, Finland), after solubilization of the bound dye with 10 mM Tris (pH 10.5). The data were corrected for the amount of protein in the medium. The sensitivities of the cell lines to cisplatin were expressed as IC_{50} values, *i.e.* the concentration of the drug causing 50% growth inhibition when compared with values of untreated control cells.

Cellular doubling time

The time HNSCC cells needed to double their number was determined by the SRB assay, as described above. For this purpose, cells were seeded on several plates and allowed to grow for 1-7 days. For the calculation of the doubling time, only data from cells in the exponential growth phase were used.

Table I. Characteristics of HNSCC cell lines.

characteristic	cell line									
	11B	14C	22A	22B	35	040T	096aT	120T	RO	OE
sex	n.k.	n.k.	n.k.	n.k.	n.k.	F	M	F	M	M
age[1]	n.k.	n.k.	n.k.	n.k.	n.k.	65	42	67	70	61
primary tumor site	larynx	floor of mouth	hypopharynx	hypopharynx	tonsillar fossa	tongue	trigonum retromolare	tongue	oropharynx	oral cavity
specimen site[2]	primary (resect)	local recurrence	primary	metastasis of 22A	primary	primary	primary	primary	primary	local recurrence neck node
TNM[3]	T2N2a	T2N0	T2N1	T2N1	T4N1	T3N0	T4N1	T3N1	T3N2*b*	*n.a.*
degree of differentiation[4]	n.k.	n.k.	n.k.	n.k.	n.k.	mod./well	mod.	mod.	mod.	mod.
prior therapy[5]	CT	SU/RT/CT	none	none	none	none	none	none	none	SU/RT
patient status[6]	DWD	DWD	DWD	DWD	DWD	A	A	A	DWD	DWD

[1]Age of the patient at the time of biopsy. n.k., not known.
[2]Specimen site, site of tissue from which the cell line or xenograft originated.
[3]TNM, tumor staging according to the American Joint Commission on Staging (27).
[4]Degree of differentiation of the tissue at the time of biopsy as determined upon pathohistological examination. *mod., moderately differentiated.*
[5]Prior therapy, therapy given to the patient before biopsy. CT, chemotherapy; SU, surgery; RT, radiotherapy.
[6]A, alive; DWD, death with disease.

Cellular differentiation

To determine the degree of differentiation and the DNA index of the cultured cells, pellets of 10^6 cells were resuspended in 300 µl of phosphate-buffered saline (PBS) to which 700 µl of absolute ethanol (-20°C) was slowly added. The samples were stored at -20°C until use. The fixed cells were washed with PBS containing 1% BSA to prevent cell clotting and divided in portions of 10^5 cells. The cells were characterized for the presence of differentiation-related cks 8, 10 and 19 by incubation for 1 hr at room temperature with mouse monoclonal antibodies (MAb) M20 (1:10 dilution in PBS/1% BSA, a gift from Dr G.N.P. van Muijen, University of Nijmegen, The Netherlands), RKSE60 (diluted 1:10, a gift from Dr F.C.S. Ramaekers, University of Maastricht, The Netherlands) and 170.2.14 (diluted 1:20, Boehringer, Mannheim, Germany), respectively. As a positive control, an antibody against a broad spectrum of human cks was used (MNF-116, diluted 1:100; Dakopatts, Roskilde, Denmark) and as negative control a MAb recognizing myoscint (diluted 1:250; Centocor, Leiden, The Netherlands), which is specific for striated muscle cells was used. Thereafter, the cells were washed twice with PBS/1% BSA and incubated for 1 hr at room temperature with fluorescent (FITC)-labeled goat-anti-mouse antibody (Southern Biotechnology, Birmingham, AL), diluted 80 times in PBS/1% BSA, again followed by 2 wash steps. The cells were then resuspended in PBS containing 0.1% Triton X-100, 3 mM EDTA, 20 µg/ml RNAse A (made DNAse free by incubation for 15 min at 80°C; Boehringer) and 50 µg/ml propidium iodide (PI, Sigma) to stain the DNA. After incubation for 30 min at 37°C, the number of FITC positively stained cells (above background), among a total of 5,000-10,000 cells, was measured by a multichannel flow cytometric system (FACScan; Becton Dickinson, Sunnyvale, CA) equipped with an argon laser (488 nm).

DNA index

To determine the DNA content of the cultured HNSCC cells, ethanol-fixed cells (see above) were suspended in PBS with Triton X-100, EDTA and RNAse. DNA was stained with PI as described above. As an external standard for the DNA index determination, human lymphocyte samples isolated on Ficoll-Paque (Pharmachemie, Haarlem, The Netherlands) from 9 different healthy individuals were used, which were assumed to be diploid (DNA index is 2.0) (12). The DNA content in the cells was subsequently measured on the basis of the PI fluorescence by FACScan flow cytometry at an excitation wavelength of 488 nm. The ratio of the mean channel number of the G_0/G_1 peak of the tumor cells to that of the human lymphocytes was taken as the DNA index.

Number of chromosomes

For determining the number of chromosomes in the tumor cells, metaphase spreads were prepared. Cultured cells were arrested at metaphase by adding colcemid (Sigma) to a final concentration of 1 µg/ml followed by incubation for 1 hr at 37°C and then harvested by trypsinization and centrifugation. Subsequently, the cell pellets were resuspended and incubated in hypotonic KCl (0.06 M) for 10-15 min at room temperature, washed twice with methanol/acetic acid (3:1) and suspended in this solution to a concentration of 10^6 cells/ml. Then, using a Pasteur-pipette, the fixed cells were dropped on water-moistened microscope slides from a height of about 0.5 m. The slides were air-dried and the chromosomes stained for 7 min at room temperature with Giemsa (Merck, Darmstadt, Germany; 0.76% in methanol/glycerol [1:1 v/v]) diluted 20 times in PBS. In about 50 cells the numbers of chromosomes were counted using a light microscope (magnification 400x).

Cellular platinum accumulation

To investigate whether the cellular platinum (Pt) accumulation is different for HNSCC cell lines with various sensitivities to cisplatin, cells were treated continuously with 10 µM cisplatin. At various time points cell samples were harvested and washed twice with cold PBS (4°C), and the number of cells in each sample was counted with a haemocytometer (Fuchs Rosenthal). Untreated cells were exponentially growing during the whole experiment. Pellets of 5 x 10^6 cells were dried at 65°C for about 2 hr followed by suspension in 100 µl PBS. After addition of 200 µl 2 M NaOH, the cells were destroyed during a 2-hr incubation at 55°C. After neutralization of the samples with 100 µl 4 M HCl,

the Pt contents were quantified by atomic absorption spectroscopy (AAS, Perkin Elmer, Norwalk, CT; model 4000, HGA-500) and expressed as pmol Pt/10^6 cells (± standard deviation) (13). K_2PtCl_6 solutions were used for calibration (14).

Statistical analysis

Correlations between the several cellular parameters and the sensitivity of the cultured cells were determined by linear regression analysis (Statgraphics, Manugistic, Rockville, MD), calculating the correlation coefficients (cc) and the *p* value (two-sided test). Only *p* values below 0.05 were considered to indicate significant correlation.

Results

Sensitivity to cisplatin

The cisplatin-induced growth inhibition was determined in HNSCC cell lines after continuous exposure with the drug for 72 hr (Table II). The IC_{50} values were determined for 10 cell lines and ranged from 0.4 µM for 096aT to 2.8 µM cisplatin for 120T, indicating a 7-fold difference between the least and most sensitive cell line.

Cellular doubling time

The cellular doubling time of these 10 HNSCC cell lines ranged between 27 and 48 hr (Table II). Comparing these doubling times with the IC_{50} values after 72 hr of exposure to cisplatin showed no correlation (Table III).

Cellular differentiation

Cultured HNSCC cells were analyzed for the presence of ck 8 and 19, which are markers of suprabasal and basal cells in normal oral mucosa tissue, respectively. A high expression is indicative of a low degree of cellular differentiation. Characterization was also performed with ck 10, which is expected to be present only in well-differentiated keratinized cells. The percentage of ck 8- and 19-positive cells within the total population of the various cell lines varied from 19 to 90% for ck 8, and from 42 to 92% in the case of ck 19 (Table II). The extent of ck 10 staining was less and ranged between 2.2 and 28% of positively stained cells. The negative control, for which the irrelevant antibody against myoscint was used, also showed some staining (2.6-19%), whereas the positive control MNF-116 yielded staining percentages from 57 to 98%. Overall, the degree of differentiation, assessed as the number of positively stained cells, did not correlate with the sensitivity to cisplatin (Table III).

Table II. Cellular characteristics of HNSCC cell lines.

cell line	IC_{50} (μM cisplatin)[1]	doubling[2] time (hr)	expression level[3]					DNA index[4]	
			myoscint	ck 8	ck 10	ck 19	MNF-116	DNA content[5]	no. of chromosomes
11B	2.2 ± 0.6	31 ± 3	n.d.[7]	n.d.	n.d.	n.d.	n.d.	n.d	46-51[6]
14C	2.7 ± 0.7	27 ± 4	2.6 ± 1.7	47 ± 42	2.2 ± 0.6	42 ± 34	98 ± 1.2	3.1 ± 0.2	42-70
22A	1.3 ± 0.3	31 ± 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	46[6]
22B	1.2 ± 0.3	34 ± 2	5.3 ± 0.4	77 ± 16	4.1 ± 0.8	51 ± 7	96 ± 1.8	2.2 ± 0.5	41-49
35	0.9 ± 0.8	38 ± 2	15 ± 13	19 ± 14	5.9 ± 2.8	42 ± 16	71 ± 6.4	3.0 ± 0.1	48-58
040T	2.0 ± 0.5	27 ± 3	19	80	22	87	85	2.6 ± 0.3	46
096aT	0.4 ± 0.1	41 ± 3	17	27	28	92	92	3.2 ± 0.6	54-64
120T	2.8 ± 1.0	34 ± 3	5.9	67	6.6	83	78	2.5	44-50
RO	2.4 ± 0.8	43 ± 4	8.0 ± 5.6	90 ± 2.0	7.7 ± 6.0	55 ± 20	86 ± 14	3.7 ± 0.2	54-68
OE	2.3 ± 0.9	48 ± 3	4.0 ± 2.2	44 ± 20	3.0 ± 2.5	60 ± 18	57 ± 20	3.5 ± 0.3	64-74

[1]The sensitivities to cisplatin of cultured HNSCC cells were determined in at least 3 independent experiments. The IC_{50} values (±SD) are the concentrations of the drug causing 50% growth inhibition. The exposure was continuous for 72 hr and proliferation was measured with the SRB assay.
[2]Doubling time, time the cells needed to double their number (±SD), measured with the SRB assay.
[3]Percentage of positively stained cells (±SD) using antibodies directed against cytokeratins 8 (ck 8), 10 (ck 10) and 19 (ck 19) as determined by FACS analysis. Myoscint was used as an irrelevant antibody, and MNF-116 was used as a positive control.
[4]DNA index determined by 2 different methods, namely, the DNA staining measured by FACS analysis and the number of chromosomes counted in metaphase spreads.
[5]Significantly correlated with the number of chromosomes counted (cc=0.96; p=0.0007).
[6]Data from Bradford *et al.* (19).
[7]n.d., not done.

DNA content

The DNA indices of the HNSCC cells were not affected by the passage number of the culture. Except for the 120T cells, which were measured in one passage only, the DNA index for each of the cell lines was determined 3 times in cell samples that were more than 10 passages apart. From this, we conclude that the DNA indices of these cultured cells are stable, at least over the time period studied. As can be seen in Table II, most of the tumor cell lines were not diploid but more or less triploid. The indices, as found with the FACS method, showed a positive correlation with the average number of chromosomes counted in metaphase spreads (cc=0.96, p=0.0007). The DNA indices were found not to correlate with the IC_{50} values (Table III).

Table III. Correlation coefficients between sensitivity (IC_{50} values) and various cellular parameters.

parameter	correlation coefficient[1]
cellular doubling time	0.40 (0.29)
cellular differentiation[2]	
ck 8	0.58 (0.18)
ck 10	0.49 (0.27)
ck 19	0.11 (0.81)
DNA content[3]	0.04 (0.93)
number of chromosomes[4]	0.05 (0.90)
cellular Pt-accumulation[5] (hr)	
1	0.70 (0.08)
6	**0.83 (0.02)**
24	**0.77 (0.04)**
48	0.71 (0.07)
72	**0.74 (0.05)**

[1]Correlation coefficients for the relations between the various parameters and the IC_{50} values are shown. The IC_{50} values were determined after continuous exposure to cisplatin for 72 hr. Correlations were determined for 9 cell lines; the discordant data for cell line OE was omitted from all calculations. p values of the correlation coefficients are given in parentheses. Significant correlations are given in **bold**.

[2]Expressed in percentages of positively stained cells using antibodies against cytokeratin 8 (ck 8), 10 (ck 10) and 19 (ck 19); (n=7).

[3]DNA content determined by FACS analysis; (n=7).

[4]Number of chromosomes determined in metaphase spreads by light microscopy; (n=9).

[5]Pt content in cells treated continuously with 10 μM cisplatin for the time indicated; (n=7).

Cellular platinum accumulation

Cellular Pt contents were determined at various time points during continuous cisplatin exposure. The data (Figure 1), indicate that the Pt accumulation differed considerably between the 8 cell lines tested. Two cell lines, 096aT and RO, could not be tested because of failure of growth. The results after 48 and 72 hr of incubation show that the OE cells, derived from a pretreated patient (Table I), accumulated Pt to a much higher extent than all other cell lines tested in this study. When the discordant data for the OE cell line were excluded, significant negative correlations were found between the Pt accumulation data and the 72 hr IC_{50} values of the remaining 7 HNSCC cell lines, as can be seen in Table III and Figure 2. This means that there is a significant positive correlation between Pt accumulation and cisplatin sensitivity in 7 of 8 cell lines studied. Furthermore, Figure 1 also shows, again with the OE line being an exception, that the Pt accumulation rate is decreasing in time. A relatively long period of time is apparently needed to reach an equilibrium between the intra- and extracellular compartments. The retention of the drug was also studied by exposing the cells to 30 µM cisplatin for 1 hr and analyzing the Pt content after a drug-free period that varied from 0 to 71 hr. The cell lines did not differ with respect to drug retention; thus this did not correlate with the antiproliferative activity (data not shown).

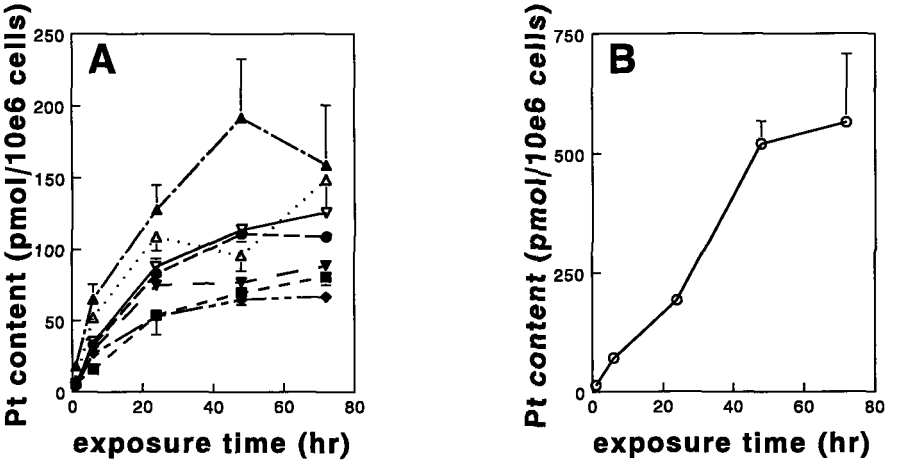

Figure 1. (*A*) Cellular Pt content (±SEM) measured in 7 HNSCC cell lines after continuous exposure to 10 µM cisplatin for the time periods indicated. Cell line 14C (■); 22B (Δ); 35 (▲); 22A (●); 11B (▽); 040T (◆); 120T (▼). (*B*) Cellular Pt content (±SEM) determined in cell line OE. Note the differences in y-axis scaling.

Discussion

To determine which factors may explain the sensitivity of cultured HNSCC cells to the chemotherapeutic drug cisplatin, a variety of cellular parameters were measured and related to the cisplatin-induced growth inhibition. Most studies on differences in cisplatin sensitivity have been performed with cells that have been made resistant *in vitro* by exposing them to unrealistic concentrations and exposure times. It is questionable whether such a system reflects the situation in untreated patients. Using a panel of 10 HNSCC cell lines, of which 8 have with certainly not been exposed to cisplatin before, we have observed a significant relatively large variation in sensitivity to cisplatin.

The sensitivities of the HNSCC cell lines to cisplatin were compared with the cellular doubling times (Tables II, III). No correlation was found between the doubling time and the sensitivity measured after 72 hr of exposure to cisplatin. These results suggest that cisplatin can affect cells irrespective of their position in the cell cycle, but it is more likely that the exposure time is long enough to eliminate differences related to doubling time. The ultimate response to the damage, however, is dependent on the cell cycle phase in which the cells are at the moment of treatment (15, 16).

The HNSCC cells were also analyzed with respect to the presence of cks, whose expression is related to differentiation and transformation (9). Cornified epithelia show specific expression of cks 1, 2, 10 and 11. Noncornified and respiratory epithelial cells express cks 8 and 18, and usually also 7 and 19 (9). In addition, cks are associated with drug transport interacting with cisplatin sensitivity (7, 8). The HNSCC cell lines studied here showed variable expression of the different cks (Table II). The very low expression of ck 10 and the relatively high expression of the simple markers cks 8 and 19 are in agreement with earlier reports and are indicative of malignancy (9). Comparing the sensitivities of the various cell lines to cisplatin determined after 72 hr of continuous exposure with the ck expression patterns did not reveal any correlation. The expression pattern of individual cks within one cell line is very heterogeneous, which is probably the reason for the relatively high standard deviations (Table II). This has also been reported by Koldovsky *et al.* (17), indicating that ck expression is reflecting a finely tuned balance. Altogether, these data do not provide support for the studies showing that expression of ck is correlated with a response to cisplatin (7, 8).

Beside the cks as markers for differentiation, the DNA index was also determined. The two methods used for this purpose, namely, the determination of the DNA content with flow cytometry and the counting of chromosomes in metaphase spreads, showed a good correlation (cc=0.96, p=0.0007), indicating that both methods can be used to compare DNA content in cultured cell lines (Table II). The flow cytometric method, with human peripheral lymphocytes as a control (12), is

a very fast technique, in which more than 5,000 cells can be measured. Using this method, the DNA indices determined in tumor cell lines 14C and 22B were in agreement with those given by Roa *et al.* (18) and Bradford *et al.* (19). The DNA content in the tumor cells proved to be stable over multiple passages during a long period. This was also found for the UM-SCC cell lines by Roa *et al.* (18). It may be anticipated that aneuploidy is related to the stage of malignancy and that the prognosis may become poorer along with a high DNA index. Cytogenetic analysis of the non-UM-SCC cell lines, performed by Hermsen *et al.* (10), showed a wide range of chromosomal aberrations and abnormalities, indicating the complexity of the changes in the DNA of HNSCC tumor cells. Such chromosomal abnormalities, however, did not appear to correlate with the degree of differentiation or malignancy grade (10). In the present study no correlation could be established between the sensitivity of HNSCC cell lines to cisplatin and the DNA content measured with both methods described (Table II, III). This is in agreement with the results of Sark *et al.* (20). Furthermore, in the present study, the DNA index was not related to the degree of differentiation or to other cellular parameters.

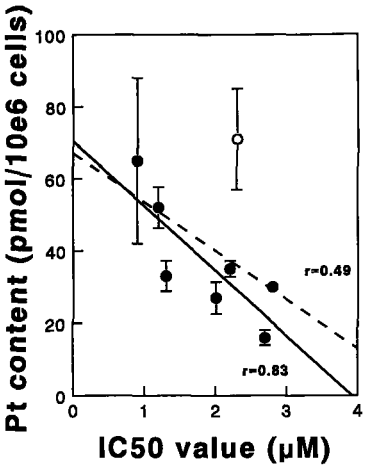

Figure 2. Correlation between IC_{50} value determined after 72 hr of cisplatin exposure and the Pt accumulation levels (±SD) in the cells after 6 hr of treatment with the drug. Cell line OE (O). Regression lines are shown with (dotted line) and without the OE cell line.

The most important finding of our study is that a significant correlation pertains between cellular Pt levels and sensitivity. This is true for almost all time points studied (Table III). These correlations could only be established when the discordant data for cell line OE were omitted. A positive correlation between cisplatin

accumulation and sensitivity has often been described, in particular in studies in which cells have been made relatively insensitive to cisplatin (21, 22). Our data indeed indicate that drug accumulation is not only important for the explanation of variations in acquired but also in their intrinsic sensitivity. It is generally believed that the toxic lesion of cisplatin is the binding of this drug to the DNA, thus forming DNA-adducts, which finally results in cell death (23). Our data indicate that the relatively crude measurement with AAS of intra-cellular levels of Pt, free or bound to nucleic acids and protein, after exposure to a relatively high concentration of cisplatin can provide important information. This opens the way to clinical application. If the correlation between total Pt levels and the response to cisplatin treatment is present *in vivo*, predictive tests become possible. After *ex vivo* exposure (treating a tumor biopsy in the laboratory with cisplatin), the total Pt levels measured would predict whether the tumor will respond to the therapy given to the patient.

The OE cell line was exceptional in the sense that relatively high levels of Pt could be tolerated without expectedly high sensitivity. What is the reason for this moderate sensitivity despite high Pt levels in these OE cells? This cell line was obtained from a tumor of a patient pretreated with radiotherapy. The genetic make-up of these cells may thus have been altered. As described by Hill *et al.*, (24), X-irradiation is able to induce resistance to cisplatin in human teratoma cells associated with enhanced tolerance of Pt-DNA damage. It is possible that these cells have an increased repair system or that their DNA damage detection system is impaired. However, the fact that cell line 14C, also obtained from a tumor pretreated with radiotherapy, did not combine relatively high Pt levels with a rather low sensitivity shows that radiotherapy-induced tolerance to cisplatin is not a general rule. Another explanation might be an increase in GSH or metallothionein, as described by Mistry *et al.* (25) for ovarian carcinomas and Yellin *et al.* (26) for HNSCC. Knowledge of the background of such aberrant behavior of the OE cell line will be important for the clinical application.

In most cell lines, the Pt content was increasing after 24 hr of continuous exposure. This finding indicates that no equilibrium had been reached and that cisplatin remained available to enter the cells. This phenomenon was cell line dependent.

In conclusion, a variable response to cisplatin was observed in a panel of HNSCC cell lines. For 7 of the 8 cell lines tested, the intrinsic sensitivity to cisplatin was correlated with the amounts of Pt accumulated in the cells during 72 hr of continuous exposure to the drug. Our results suggest that determination of the cellular Pt accumulation can be used to predict sensitivity to cisplatin.

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Chapter 3

Role of glutathione, glutathione S-transferases and multidrug resistance-related proteins in cisplatin sensitivity of head and neck cancer cell lines.

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Abstract

Resistance to chemotherapy is a major problem in the treatment of patients with head and neck squamous cell carcinoma (HNSCC). Important factors involved are drug detoxification by glutathione (GSH) and reduced drug accumulation due to active transport out of the cell by so-called 'multidrug resistance-related proteins'. We have studied a panel of 8 HNSCC cell lines showing differences in sensitivity to the anticancer drug cisplatin. Our previous studies indicated that the IC_{50} values were inversely correlated with the intracellular accumulation of platinum (Pt). In the present study, cellular GSH levels were found not to be related to the IC_{50} values. The expression levels of the enzymes glutathione-S-transferase (GST) α, μ and π, the multidrug resistance-related proteins P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and the lung resistance protein (LRP) were determined semiquantitatively by means of immunocytochemistry. The levels of the GSTs, P-gp and LRP were not found to be correlated with the IC_{50} values of the HNSCC cell lines. Surprisingly, however, an inverse correlation was found between MRP levels and IC_{50} values. The MRP expression levels were in agreement with results of the MRP functional assay, based on the transport of calcein across the cell membrane as performed for two of the cell lines. Further studies should prove whether other pump mechanisms or DNA repair are involved in the cisplatin accumulation and the subsequent HNSCC cell growth inhibition.

Introduction

Cisplatin shows activity in patients with advanced head and neck squamous cell carcinoma (HNSCC). The response of these tumours differs between patients, but a good initial response is generally seen upon cisplatin chemotherapy in 20-50% of the cases (1). Nevertheless, treatment does not lead to an increased survival as a consequence of a lack of response or its short duration.

Various mechanisms have been proposed to explain resistance to cisplatin (2). The role of glutathione (GSH) in cisplatin resistance seems to be important as cells with *in vitro* acquired resistance often show elevated levels of GSH compared with the parental cells (3, 4). Glutathione S-transferases (GST) are enzymes that catalyse the conjugation of cisplatin to GSH. The cisplatin-GSH complex has been proposed to be ejected from the cell in an ATP-dependent fashion by the glutathione S-conjugate (GS-X) export pump (4, 5)

ATP-dependent transport systems, referred to as pumps, are proposed to be responsible for resistance to multiple drugs, *i.e.* multidrug resistance (MDR) (6). Two important MDR-associated membrane-bound proteins are P-glycoprotein (P-gp), encoded by the *MDR1* gene, and the multidrug resistance-associated protein (MRP) (7, 8). P-gp and MRP actively transport a wide range of substrates across membranes into vesicles and out from the cell. A number of substrates are transported by MRP after conjugation to GSH (9). Another MDR-associated protein, recently discovered, is the lung resistance protein (LRP), possibly mediating intracellular transport (10, 11). Although cisplatin is not known to induce MDR itself, MDR-induced cells can become cross-resistant to cisplatin (12). The possible involvement of MDR in the response to platinum (Pt)-based treatments has been reported in a panel of 61 human cell lines of 8 different cancer types (13) and in patients with ovarian cancer (14). HNSCC was not included in these studies, though other studies have shown that HNSCC cells can express P-gp (15), MRP (8), as well as LRP (16).

Using a panel of 8 HNSCC cell lines that differ with respect to cisplatin sensitivity , we were able to show an inverse correlation between IC_{50} values and Pt accumulation (17). To investigate the underlying mechanism of differences in cisplatin sensitivity and Pt accumulation, we presently report on the GSH levels and the expression levels of the GST isoenzymes as well as of the MDR proteins P-gp, MRP and LRP. In addition the MRP activity was determined.

Material and Methods

Tumour cell lines

Human HNSCC cell lines UM-SCC-11B, UM-SCC-14C, UM-SCC-22A, UM-SCC-22B and UM-SCC-35 were described by Carey *et al.* (18). These cell lines were established from fresh tumour biopsies. The same holds for cell lines 92VU040T and 93VU120T (19). VU-SCC-OE was established in our laboratory from a HNSCC xenograft (17). Cells were routinely cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gibco BRL, Breda, The Netherlands) supplemented with 5% heat-inactivated fetal calf serum (Flow, Irvine, UK), 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies).

Cisplatin treatment

HNSCC cells at subconfluency were treated with cisplatin for 72 hr, washed twice with phosphate-buffered saline (PBS) and harvested by use of trypsin. The IC_{50} data of the HNSCC cells, *i.e.* the concentration of the drug causing 50% of growth inhibition compared with that of untreated control cells, have been published before (17) and were determined with the sulforhodamine B (SRB) assay (20).

GSH content in cultured human HNSCC cells

GSH was measured in untreated and cisplatin-treated HNSCC cells by high-performance liquid chromatography (HPLC) combined with precolumn derivatization with orthophtaldehyde and fluorometric detection (21). GSH levels per cell line were measured in two or three independent samples of cells cultured at subconfluency.

Immunocytochemical staining of GST isoenzymes

Expression of the GST isoenzymes α, µ and π was analysed using the immunoperoxidase staining method described by Bongers *et al.* (22). HNSCC cells were deposited on glass slides with a cytospin centrifuge, fixed with methanol for 10 min and washed with PBS. A 30-min preincubation was performed with 2% normal swine serum (Dako, Copenhagen, Denmark) diluted in PBS containing 1% bovine albumin (BSA; Sigma, St.Louis, MO), followed by incubation with rabbit antisera directed against GST-α, -µ and -π, respectively (antisera diluted 1:1 in PBS/1% BSA; NovoCastra, Newcastle upon Tyne, UK). The preparations were washed three times with PBS (5 min each), and treated for 10 min with 0.006% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, followed by three washings with PBS. The slides were then incubated for 30 min with swine anti-rabbit biotin conjugate (diluted 1:500 in PBS/1% BSA; Dako) and washed again three times with PBS. After a further incubation for 60 min with avidin-biotin complex (Vectastain ABC-kit, Vector Laboratories, Burlingame, CA), and three wash steps, antibody binding was visualized by incubation with 4 mg (v/v) 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.02% (v/v) hydrogen peroxide in PBS for 3-5 min. The slides were rinsed with tap water, counterstained with haematoxylin (Merck, Darmstadt, Germany), and finally mounted with Kaiser's glycerin gelatin (Merck). As a negative control, slides were incubated as described above except that the primary antibody was replaced by PBS/1% BSA or mouse IgG antibody. In two independent experiments all cell lines were stained simultaneously with the various antibodies.

Immunocytochemical staining of P-gp, MRP and LRP

The HNSCC cells were cultured until subconfluency and harvested onto cytocentrifuge slides, which were stored at -20°C until analysis. Immunocytochemistry was performed as described by Izquierdo *et al.* (16). In short, after thawing the cytocentrifuge preparations were acetone-fixed (10 min) before preincubation with 2% normal rabbit serum for 15 min (Dako). Then, slides were incubated for 60 min at room temperature with one of the following monoclonal antibodies (MAb): mouse MAb JSB-1 (1:100 of 10 µg/ml) against P-gp, mouse MAb MRP-m6 (1:25 of 1 µg/ml) and rat MAb MRP-r1 (1:1500 of 1 µg/ml) against MRP, rat MAb LRP-56 (1:500 of 0.5 µg/ml) and LMR-5

(1:500 of 0.5 µg/ml) both directed against LRP. These antibodies are available from Sanbio, Uden, the Netherlands. After washing with PBS for 15 min, the slides were incubated for 60 min with rabbit anti-mouse biotin (1:150; Zymed Laboratories, San Francisco, CA) or rabbit anti-rat biotin (1:100) conjugate (Dako), washed and incubated with streptavidin coupled to horseradish peroxidase (1:500; Zymed Laboratories) for 60 min. All dilutions were in PBS with 1% BSA. The washed cells were finally stained with amino-ethyl-carbazole (ICN Biochemicals, Aurora, OH) for 5 min and counterstained with haematoxylin (Merck). As a negative control, irrelevant IgG or PBS was used instead of the primary antibody. Positive controls for the expression of each of the proteins were KB-8-5 cells for P-gp (14), GLC4/ADR cells for MRP and SW-1573/2R120 cells for LRP (10, 23, 24). Immunohistochemical staining of the cell lines was performed in two independent experiments (I and II) and all slides of each experiment were stained simultaneously.

Evaluation of immunocytochemical staining

The evaluation was performed using light microscopy on coded slides. Scoring of each immunocytochemical experiment was performed blinded and independently by three observers. The number of cells that stained very strong (+++), strong (++), intermediate (+) or not (-) was expressed as a percentage of the total number of cells investigated. The semiquantitative staining index of each group was calculated as the product of this percentage and the staining intensity. The latter was estimated on a scale of 1 (+) to 3 (+++). The variation in scores between the three observers, expressed as coefficient of variation, *i.e.* the SD as percentage of the mean, was always less than 30%. Intraobserver variation of scoring was tested and was proven to be less that 20%.

Functional MRP test

The HNSCC cell lines UM-SCC-14C that showed a low sensitivity to cisplatin and UM-SCC-35, the most sensitive cell line of our panel, were analysed in two independent experiments for the presence of functional MRP as described by Feller *et al.* (25). Briefly, about 0.5 x 10^6 cells were allowed to take up calcein-acetoxymethylester (calcein-AM) by incubation in 0.5 µM of this dye for 10 min at 37°C. They were washed and subsequently incubated in fresh medium with or without the MRP-inhibitor probenecid (1.0 mM, Sigma) for 0, 10 or 60 min. The efflux was stopped by centrifugation of the cells and addition of ice-cold culture medium. In this assay, the non-fluorescent dye calcein-AM is converted by intracellular esterases to the fluorescent calcein. The calcein can be exported by active MRP, which can be prevented by the use of the MRP inhibitor. The intracellular calcein is then analysed using FACScan flow cytometry (Becton Dickinson Medical Systems, Sharon, MA). The human small-cell lung cancer cell line GLC4, which is MRP negative, and its MRP-overexpressing subline GLC4/ADR were used as controls (25).

Statistical analysis

Correlations between the various cellular parameters and the IC_{50} values of the cultured HNSCC cells were determined by Spearman's rank correlation test; the correlation coefficients (r values) and the *p* values (two-sided) were calculated. Only correlations with *p* values of 0.05 or below were considered to be significant.

Results

The efficacy of cisplatin treatment in a panel of 8 human HNSCC cell lines was compared with GSH, GST and MDR-related protein levels. As previously published, the IC_{50} values varied about 3-fold between the cell lines and showed a significant inverse correlation with the Pt accumulation in these cells, when data of cell line OE (derived from a previously irradiated patient) were omitted (Table I; 17).

Table I. Parameters determining cisplatin sensitivity in cultured HNSCC cells.

Cell line	IC_{50} value[1]	Pt accumulation[2]	GSH levels[3]
UM-SCC-35	0.9 ± 0.8	159 ± 93	10.2 ± 1.8
UM-SCC-22B	1.2 ± 0.3	149 ± 34	5.0 ± 2.3
UM-SCC-22A	1.3 ± 0.3	109 ± 9	6.3 ± 2.6
92VU040T	2.0 ± 0.5	67 ± 7	11.2 ± 3.1
UM-SCC-11B	2.2 ± 0.6	126 ± 9	7.5 ± 1.1
VU-SCC-OE	2.3 ± 0.9	566 ± 317	7.1 ± 0.7
UM-SCC-14C	2.7 ± 0.7	81 ± 10	2.0 ± 1.2
93VU120T	2.8 ± 1.0	89 ± 10	6.1 ± 0.7

[1]The sensitivity to cisplatin was determined by a cell proliferation (SRB) assay. The IC_{50} value, the concentration of the drug causing 50% growth inhibition after a 72-hr treatment, is given in μM cisplatin. These results were obtained in a previous study and were reported to be significantly correlated with Pt accumulation data when those of cell line OE were omitted (17).
[2]The total amount of Pt accumulated in the cells (expressed as pmol Pt/10^6 cells), after treatment with 10 μM of cisplatin for 72 hr, was determined with AAS in a previous study (17).
[3]Glutathione (GSH) levels (fmol/cell) were determined by HPLC according to Neuschwander-Tetri and Roll (21).

GSH levels in untreated and cisplatin-treated cells

The total levels of GSH in the 8 HNSCC cell lines varied between 2.0 fmol/cell for UM-SCC-14C and 11.2 fmol/cell for 92VU040T (Table I). No correlation was found with the IC_{50} values or the cellular Pt content. The GSH level in the VU-SCC-OE cells appeared to be within the range of the other cell lines and could, therefore, not explain the moderate sensitivity of this cell line and its high Pt content. To study possible induction of GSH by cisplatin treatment, cell lines UM-SCC-14C, VU-SCC-OE and UM-SCC-35, showing differences in IC_{50} values, were treated with 0.1 and 1.0 μM cisplatin during 5 and 24 hr. In these treated cells a small increase of GSH levels was found compared with the untreated cells (data not shown). However, this induction of GSH was slightly different among the cell lines. Therefore, these differences in cisplatin-induced GSH levels cannot be held responsible for the variation in IC_{50} values found for these cell lines.

Expression of the GST isoenzymes

In all HNSCC cell lines, the presence of the three isoforms GST-α, GST-μ and GST-π could be demonstrated by immunocytochemical staining. Over 90% of the cells of each line were positive for GST-π. The staining percentages for the other two GST isoenzymes were lower and varied considerably between the cell lines. For

GST-α the percentage of positively stained cells varied between 15% and 100%, whereas for GST-μ it varied from 3% to 100%. The calculated staining indices (see Material and Methods) differed between the cell lines and between the three isoforms of GST, but none of these correlated with the IC_{50} values (Table II), neither with the Pt accumulation in these cells after 72 hr of exposure to cisplatin, nor with the GSH levels determined in the untreated cells.

Table II. Correlation between IC_{50} values of the HNSCC cell lines and the expression levels of GST and MDR-related proteins.

Marker	Antibody designation	Experiment I		Experiment II	
		r value	*p* value	r value	*p* value
GST-α	GSTalpha	-0.02	0.95	-0.05	0.89
GST-μ	GSTmuM2	0.71	0.06	0.41	0.31
GST-π	GSTpi	0.57	0.13	0.57	0.12
P-gp	JSB-1	-0.50	0.19	-0.52	0.17
MRP	MRP-m6	-0.79	0.04	-0.71	0.05
	MRP-r1	-0.83	0.03	-0.83	0.03
LRP	LRP-56	-0.45	0.23	-0.47	0.22
	LMR-5	-0.60	0.12	-0.45	0.29

The relations between the IC_{50} values and the expression levels of the various markers, which were recognized and visualized by antibodies, were determined in two independent experiments. The Spearman's rank correlation coefficients (r values) and significancies (*p* values) are given.

Expression of MDR-related proteins

The expression level of the MDR protein P-gp, visualized by use of antibody JSB-1, was expressed in all HNSCC cell lines tested, with staining index ranging from 128 (93VU120T) to 262 (UM-SCC-35). The levels of MRP, measured with specific mouse and rat antibodies, were also different for the various HNSCC lines. The UM-SCC-14C cells appeared to be stained very weakly or not at all, indicating that MRP levels were relatively low. The data obtained with the mouse and rat anti-MRP antibodies were correlated significantly, resulting in a correlation coefficient (r value) of 0.72 (p=0.05) in the first and r=0.80 (p=0.04) in the second experiment. The staining index of LRP-56, a measure of the presence of LRP, ranged from 107 for UM-SCC-14C cells to 202 for cell line UM-SCC-35. With the LMR-5 antibody, which also recognizes LRP, similar variations in staining level were observed. The results obtained with these two antibodies recognizing LRP did significantly correlate

in the two experiments (r=0.63, p=0.02 and r=0.72, p=0.05).

To find out whether the levels of these three membrane proteins have an effect on the sensitivity of the cells to cisplatin, the relationships between these levels and the IC_{50} values were determined. A significant inverse correlation was found in the first experiment between the IC_{50} values and MRP, as indicated by the staining index of MRP-*m6* (r=-0.79, p=0.04) and of MRP-r1 (r=-0.83, p=0.03) (see Figure 1A and Table II). The second experiment (Figure 1B) confirmed this finding, showing a significant correlation of IC_{50} values with the MRP-m6 staining index (r=-0.71, p=0.05) as well as with the MRP-r1 staining index (r=-0.83, p=0.03). No correlation was found between the IC_{50} values and the P-gp or the LRP levels (Table II).

Whether or not the total amount of Pt accumulated in the HNSCC cells (with the exception of VU-SCC-OE) is correlated with the expression levels of MRP as visualized with antibody MRP-m6 is not quite clear. In the first experiment, the correlation was found not to be significant (r=0.68, p=0.09), but in the second experiment it was significant (r=0.89, p=0.03). The same holds true for the results obtained with the other MRP-recognizing antibody, showing a significant correlation between Pt accumulation levels and MRP-r1 staining results in experiment I (r=0.77, p=0.05) and no significance in experiment II (r=0.61, p=0.13).

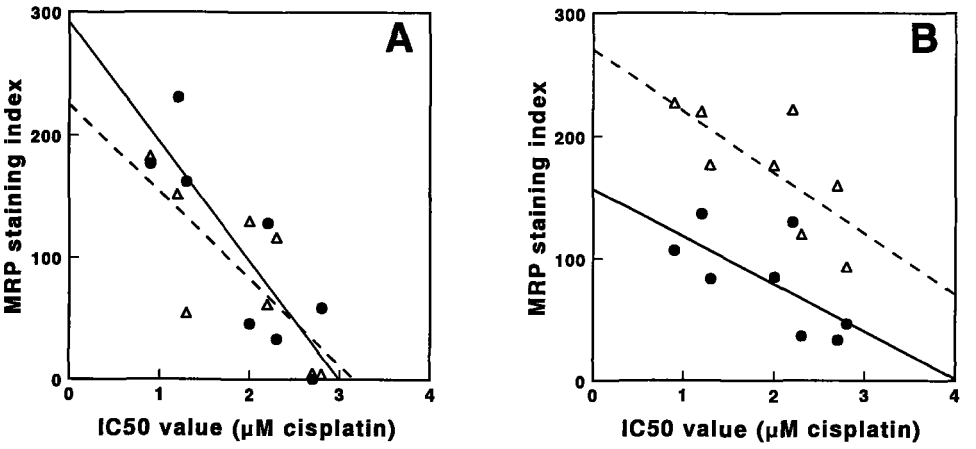

Figure 1. The relationship between the sensitivity to cisplatin, expressed as IC_{50} value, and the semiquantitative staining index of MRP using either antibody MRP-m6 (●; solid line) or MRP-r1 (Δ; dashed line) as determined in the two independent experiments I (*A*) and II (*B*).

Functional MRP-test

Cell lines UM-SCC-14C and UM-SCC-35, which differed significantly in MRP expression, were used to determine if the established differences in the levels of MRP were indicative for differences in the MRP activity in the cells. The results of the assay are given in Table III. The UM-SCC-14C cells showed hardly any activity of the MRP pump, whereas the cells of line UM-SCC-35 appeared to have functional MRP after 10-min and 60-min treatments determined with the MRP inhibitor probenecid. This is in agreement with the immunocytochemical staining results, in which the presence of MRP could not be demonstrated in UM-SCC-14C cells, whereas a relatively high expression was observed in UM-SCC-35.

Table III. Activity of MRP protein.

Cell line	Duration of calcein efflux	
	t=10 min[1]	t=60 min[2]
UM-SCC-35	1.54 ± 0.05	1.47 ± 0.21
UM-SCC-14C	1.02 ± 0.03	1.22 ± 0.15
GLC4	0.94	1.10 ± 0.03
GLC4/ADR	2.0	3.05 ± 0.39

The effect of the MRP inhibitor (1.0 mM probenecid) is expressed as the ratio of calcein accumulation in the presence of this modulator divided by that in the absence of probenecid, measured after a duration of [1]10 min or [2]60 min of calcein efflux. GLC4 was included as negative and GLC4/ADR as positive control cells. Experiments were performed in duplicate, except for the 10 min incubation experiment of GLC4 and GLC4/ADR.

Discussion

Our data indicate a minor role for GSH as a determining factor of the differences in sensitivity to cisplatin of the presently studied HNSCC cell lines. An inverse correlation between the GSH levels and cisplatin sensitivity has been reported for cell lines of various tumour types, thereby partly explaining the resistance found (3, 26). It should be noted that we studied cell lines that were not treated *in vitro* to obtain acquired resistance. Because Yellin *et al.* (27) reported that the GSH levels in HNSCC cells can be up-regulated during cisplatin treatment, the GSH levels were also determined in the cell lines UM-SCC-14C, UM-SCC-35 and VU-SCC-OE after incubation with cisplatin during various time periods. As a result, only small increases in GSH levels occurred, but this did not lead to correlations with the IC_{50} values.

In the detoxification system GSH/GST, GSTs catalyse the binding of electrophilic components to GSH. Three isoforms of GST can be distinguished in humans namely π (acidic), μ (neutral) and α (basic). Expression of GSTs and MDR-related proteins was studied by immunocytochemistry. This was known to be a reliable method because for these proteins a correlation was found with the outcome of Western blots, immunoprecipitation analyses and the determination of the corresponding mRNA levels (8, 11, 23). Comparison of the GST staining indices of our 8 HNSCC lines with the IC_{50} values of these lines revealed no correlation (see Table II), which is in agreement with the results of Yellin and colleagues (27) for a panel of 14 HNSCC lines. It cannot be excluded that the other factors in the GSH-associated detoxification system play a role in cisplatin sensitivity; this includes the enzymes glutathione peroxidase, glutathione synthetase, glutathione reductase and dipeptide gamma-glutamylcysteine (28, 29).

The importance of the MDR proteins in the efficacy of Pt-containing chemotherapy has recently been reported for leukaemia cells and colon carcinomas (30, 31). In the present study, no significant correlation was found between P-gp expression levels and the IC_{50} values, suggesting no direct involvement of P-gp in the *in vitro* response of HNSCC cells to cisplatin. A significant, but inverse correlation was found between MRP and the IC_{50} values (Table II). MRP was detected on the membranes of HNSCC cells as well as inside these cells, with the two antibodies MRP-m6 and MRP-r1. The staining results obtained with MRP-m6 were significantly correlated with those of MRP-r1, which is in line with results reported by Izquierdo *et al.* (13). It should be noted that these antibodies do not cross-react with human MDR1 and MDR3 P-gps (23). A high expression of MRP is usually determined in cell lines with acquired resistance (9, 32). It is thought that MRP is a GS-X pump (12, 32, 33), which is present on vesicles and/or the plasma membrane (8). The unexpected finding in our panel of HNSCC that the correlation of MRP with the IC_{50} data was inverse (Figure 1), and thus positive with sensitivity, and that high cellular Pt levels were associated with high MRP expression levels cannot be attributed to less active MRP because we provided evidence that the MRP was indeed active as determined by the functional MRP assay (see Table III). These data implicate that in HNSCC cells the GS-X pump activity, *i.e.* transporting GSH-conjugated cisplatin out of cells, may not be the major function of MRP. This is in agreement with results of De Vries *et al.* (34). A possible explanation for the unexpected relation of higher sensitivity in the presence of more MRP may be that endogeneous metabolites conjugated to GSH are extruded from the cell, while cisplatin is counter-transported. Another hypothesis is the regulation of endogenous (ion) channels, and possibly other transporters, by MRP as described by Loe and colleagues (12), which can lead to an increase of the influx of cisplatin into the cells and eventually into the nucleus. As a consequence, the levels of DNA-bound Pt will increase. The involvement of other as

yet undefined transport mechanisms in the sensitivity to cisplatin of the HNSCC lines under study can also not be ruled out. Possible candidates for alternative pumps are the human canalicular multispecific organic anion transporter (cMOAT), also designed as MRP2, which has been described to be overexpressed in the cisplatin-resistant human head and neck cancer KB cell line (35) and the SQM1 protein, which is present at reduced levels in HNSCC resistant to methotrexate and cisplatin (36).

High expression of the non-P-gp LRP protein in acute myeloid leukaemia and ovarian carcinoma has been associated with a poor response to chemotherapy, such as cisplatin treatment (11, 14). In our HNSCC cell lines LRP was detectable, which is in agreement with the earlier published finding that this protein is present in epithelial cells (11) and head and neck tumours (16). No correlation was found between LRP expression levels and the IC_{50} values (Table II), or with the Pt accumulation data. These results are in contrast with those found by Izquierdo and colleagues (13), who showed a predictive value of LRP for *in vitro* sensitivity to several types of drugs, among which also cisplatin, in a number of cancer types. Their study, however, did not include HNSCC. Our results in 8 HNSCC cell lines indicated that pump mechanisms other than LRP control the response of this cancer type to cisplatin. The importance of DNA damage recognition proteins in DNA repair and the nucleotide excision repair system in repairing cisplatin-DNA damage has been reviewed by Hill (37). It is clear also that other unknown factors may contribute to the differences in sensitivity to cisplatin in our HNSCC cell lines. In addition, it is obvious that intrinsic sensitivity to drugs is a very complex phenomenon, that needs further investigation.

In conclusion, an inverse correlation was found between the IC_{50} values of HNSCC cell lines, obtained after 72 hr of cisplatin treatment, and their expression level of the MDR-associated membrane-bound protein MRP. In addition, the indications for a positive relation between Pt accumulation and MRP expression levels suggest that MRP plays a role in transport of cisplatin into or inside the HNSCC cells.

Abbreviations

BSA, bovine serum albumin; calcein-AM, calcein acetoxymethylester; GSH, glutathione; GST, glutathione S-transferase; GS-X, glutathione S-conjugate export pump; HNSCC, head and neck squamous cell carcinoma; IC_{50} value, concentration of drug that inhibits cell growth to 50% of control growth; LRP, lung resistance protein; MAb, monoclonal antibody; MDR, multidrug resistance; MRP, multidrug resistance associated protein; P-gp, P-glycoprotein; Pt, platinum.

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Chapter 4

Effect of cisplatin treatment on platinum accumulation and growth inhibition in human neoplastic and normal squamous epithelial cells from the head and neck region.

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Submitted

Abstract

Aim of this study was to investigate how epithelial cells from the head and neck mucosa respond to damage, induced by cisplatin and whether this response differs between cancer and normal cells. Normal epithelial cells of eight individuals without cancer were cultured and exposed to cisplatin for 72 hr. A panel of five head and neck cancer cell lines was included for comparison. Intracellular accumulation of platinum (Pt) was determined with atomic absorption spectroscopy after exposure to 1 μM cisplatin for 1, 6, 24, 48 and 72 hr. Intracellular Pt levels reached a plateau in cancer cells after 24 hr, whereas in normal cells an increase was seen during the total incubation period. The Pt levels were significantly higher in the normal epithelial cells than in cancer cells when the exposure time was 6 hr or more; after 72 hr the difference was 20-fold. The growth inhibition was measured after 72 hr of exposure to cisplatin in a colorimetric proliferation assay. The IC_{50} values did not differ between cultures of neoplastic and normal cells; these values varied from 0.9 to 2.7 μM to 0.4 to 2.7 μM, respectively. Apoptosis was assessed on the basis of morphology after 24 hr exposure to 0.1-100 μM cisplatin and it was found that this process was not associated with Pt accumulation. At concentrations lower than 50 μM apoptosis was a very rare event. Normal cells had a higher propensity to become apoptotic than cancer cells when relatively high concentrations of 50 and 100 μM were used. The main finding of this study is that normal epithelial cells from the head and neck region have a much higher tolerance for cisplatin than their neoplastic counterparts and that this characteristic is without consequence for growth inhibition.

Introduction

The mucosa which lines the head and neck (*i.e.* the oral cavity, larynx and pharynx) forms a primary barrier between the environment and underlying tissues. The deepest layer of the mucosa is the basal cell layer, containing two types of cells. Most cells are transit-amplifying cells, *i.e.* dividing cells with a limited life-span (1). A small number of cells continuously divide and represent the stem cells, whose function is to retain the proliferative potential of the tissue. Once a cell is committed to differentiation it moves upwards through the stratum spinosum and stratum granulosum. During this movement the cells undergo the genetically controlled process of squamous differentiation, amongst others characterized by the formation of specific cytokeratins and a cornified envelope (1). Ultimately, these terminally differentiated cells shed from the tissue as dead cells.

Head and neck squamous cell carcinoma (HNSCC) is the most common form of cancer of the mucosa of the head and neck and originates from the stem cells of the basal layer. Once a mutation is present in the DNA of one of these proliferating cells, this genotype will be transmitted to the daughter cells. According to the concept of multi-step carcinogenesis, a sequence of DNA damaging events can immortalize these cells and give rise to neoplastic daughter cells (2). Therefore, protection of stem cells against DNA damage is of crucial importance. It is thought that the parabasal and superficial layers protect the basal cell layer by forming a physical barrier against genotoxic compounds (1). Once this barrier has been passed the stem cells can tolerate the genetic damage at the expense of a higher risk to become malignant. There are, however, two mechanisms to prevent the accumulation of genetic damage. First, cells can undergo growth arrest and allow DNA repair by activating cell cycle checkpoints (3). Secondly, if too much damage has accumulated, the cell can die in a programmed way through apoptosis (4). In cancer, loss of apoptotic propensity has been proposed to be an early step in oncogenesis (5).

The genotoxic compound cisplatin has a wide application as an anticancer drug, including the treatment of HNSCC (6) and therefore, it is an interesting compound to study induction of genotoxic damage. It has a simple chemical structure and enters the cell by passive diffusion. Then, it is hydrolyzed to its active form due to the low chloride concentration inside the cell and binds to proteins and nucleic acids. The intracellular concentration of platinum (Pt) may be important for the cytoxicity of the drug. In a panel of seven HNSCC cell lines we were able to demonstrate a positive correlation between the intracellular Pt concentration and the growth inhibition by cisplatin (7).

The aim of the present study was to investigate how normal head and neck epithelial cells (NHNEC) and HNSCC cells respond to DNA damage as induced with

cisplatin. Levels of intracellular platinum (Pt) were determined and related to growth inhibition and the propensity to undergo apoptosis.

Material and Methods

Culture of normal head and neck epithelial cells (NHNEC)

Epithelial cells were obtained from uvulas removed at uvulopalatopharyngoplasty for snoring and/or obstructive sleep apnoea of control persons without a cancer history. Details of how a cell population of 100% purity was cultured from this tissue have been reported previously (8). In short, the epithelial layer and connective tissue were separated by enzyme treatment and the epithelial cells were cultured in keratinocyte growth medium (KGM, Life Technologies, Paisly, Scotland) following the guidelines of the manufacturer. KGM comes as a "kit" consisting of 500 ml standard medium (low Ca^{2+}) and epidermal growth factor (human recombinant, 0.5-2.5 µg) and bovine pituitary extract (10-25 mg) as the additives. To this medium we added also gentamicin sulphate (final concentration 5 µg/ml) and amphotericin B (final concentration 0.5 µg/ml) both from Life Technologies. Cells were cultured in 6-well culture plates (Greiner, Alphen a/d Rijn, Netherlands) and were used from passage 2 to 4 for growth inhibition and apoptosis studies.

Cancer cell lines

HNSCC cell lines UM-SCC-14C (abbreviated as 14C), UM-SCC-22A (22A), UM-SCC-22B (22B) and UM-SCC-35 (35), were obtained from Dr T.E. Carey, University of Michigan, Ann Arbor, USA. The cell line VU-SCC-OE (OE) was established from a xenograft in our own laboratory. The latter was obtained after implantation of a fresh human HNSCC biopsy into a athymic nude mouse (9). Cell lines 92VU040T (040T) and 93VU120T (120T) were established at the laboratory of Human Genetics, *Vrije Universiteit*, Amsterdam, The Netherlands (10). The site, stage and histological features of the established cell lines and the gender, age and survival data of the patients from whom the tumors originated are listed elsewhere (7, 11). Cells were routinely grown at 37°C and 5% CO_2 as subconfluent monolayers in 25- or 75-cm^2 flasks (Nunclon, Nunc, Roskilde, Denmark) using Dulbecco's modified Eagle's medium [DMEM, Life Technologies, (Gibco BRL), Breda, The Netherlands], supplemented with 5% heat-inactivated fetal calf serum (FCS, Flow Laboratories, Irvine, UK) and 50 U/ml penicillin, 50 µg/ml streptomycin (pen/strep, Life Technologies).

Pt accumulation

To determine the Pt levels 10^6 cells from near confluent cultures (about 75% confluence) were exposed to 1 µM cisplatin for several time periods (1 to 72 hr). The levels of Pt in the cells were determined by atomic absorption spectroscopy (AAS) as described, previously (7).

Growth inhibition studies

To determine inhibition of cell growth in response to cisplatin exposure, the semi-automated proliferation assay with sulforhodamine B (SRB, Sigma, St Louis, MI) was used, as described previously (11). NHNEC cultures can be tested in this system under standardized conditions and cells show exponential growth from day 3 to 6 after plating. As reported previously this behavior is limited to the first five or six passages (8). NHNEC and HNSCC cells were tested with the medium that is routinely used for propagation (DMEM and KGM with supplements for the HNSCC and NHNEC cells, respectively). As for NHNEC, 3,000 cells in 150 µl culture medium were plated in the wells of 96-well plates (Greiner, Friekenhausen, Germany) and incubated at 37°C. After 72 hr in the lag-phase, 50 µl of medium containing cisplatin (Platinol; Bristol Myers Squibb, Woerden, The Netherlands) at the desired concentration was added to the wells (this time point was indicated as t=0) and the cells were further incubated for 72 hr. Wells with untreated control cells were included. When testing the HNSCC cell lines, the optimal cell density at the start was cell line dependent, however, the number

of cells at the start was chosen as such that exponential growth was ensured during the 72 hr exposure period. This varied between 1,000 and 3,000 cells per well. After 72 hr exposure the cells were fixed with 25% trichloroacetic acid and the cellular proteins stained with SRB-solution (0.4% SRB in 1% acetic acid). Absorbance was measured at 540 nm, using a micro-plate reader (Multiskan Bichromatic, Labsystem, Helsinki, Finland), after solubilization of the bound dye with 10 mM Tris (pH 10.5). The data were corrected for the amount of protein in the medium. As previously shown (12), the optical density (OD) values have a direct relationship with the number of cells. To know the OD values at t=0 a plate with untreated cells was fixed at that time point.

The sensitivities of the cell lines to cisplatin were expressed as IC_{50} values, *i.e.* the concentration of the drug causing 50% growth inhibition when compared to values of untreated cells. Cells were exposed to 0.1, 0.5, 1, 5 and 10 μM cisplatin. Growth inhibition was also assessed by comparing the cell number (based on the OD value) at the cisplatin concentration of 5 μM after 72 hr exposure with that at t=0 (start of exposure).

Experiments with cell lines were performed in triplicate. Since the availability of NHNEC was limited, the growth inhibition studies with these cells were performed once. All data of a single experiment, however, are based on three wells per drug concentration.

Measurement of apoptosis

Cells were cultured in 8-wells Lab-tek® (Chamber-slide® from Nunc, Naperville, IL) tissue culture chambers in standard cell culture medium. At 75% confluence, the medium was replaced by cisplatin containing medium at a concentration of 0.1, 0.5, 1, 5, 10 and 100 μM. Next, the medium was poured off and the cells were fixed over-night with 4% paraformaldehyde at 4°C. The slides were stored under 70% ethylalcohol until further analysis. After washing with phosphate buffered saline (pH 7.4), the cells were stained with hematoxylin and mounted with aquaperm® (Shandon, Pittsburgh, PA).

Scoring was performed on the basis of morphology (13). A cell was considered to be apoptotic when i) the cell was not swollen and not lysed and ii) the nucleus was degraded into two to six uniformly stained bodies. At least 500 viable cells were scored with a light microscope at a magnification of 250 x.

Statistical analysis

To assess a correlation we have calculated the Pearson correlation coefficient (=r) with two-tailed significance levels. Differences between groups were analyzed with the Student's t-test or the Mann-Whitney-U-test.

Results

Pt accumulation

Five cell cultures of NHNEC and five HNSCC cell lines were exposed to 1 μM cisplatin for various time periods (Table I). As shown, the intracellular Pt content increased in the course of time to a maximal value after 72 hr exposure. The concentration varied among the NHNEC cultures between 37 to 159 pmol/million cells after 72 hr exposure.

Neoplastic cells were different from NHNEC in two aspects. First, the HNSCC cells accumulated much less Pt than the NHNEC. Based on the mean values, Pt levels in NHNEC cells were 4.2, 7.0, 9.0 and 20.0 times higher at 6, 24, 48 and 72 hr, respectively. Second, the kinetics of Pt accumulation in the HNSCC cells followed

a different pattern. Already at 24 hr exposure maximal intracellular Pt concentrations could be observed (Table I).

Table I. Pt accumulation in NHNEC and HNSCC cells after exposure to 1 μM cisplatin

code	1 hr	6 hr	24 hr	48 hr	72 hr
NHNEC1	3.0	16.8	90.0	84.2	158.9
NHNEC2	0	16.7	61.2	43.2	73.9
NHNEC3	0	24.1	28.1	25.3	36.8
NHNEC4	1.7	3.5	33.9	50	59.6
NHNEC5	0	5.9	31.6	44.7	70.0
22A	0.5 ± 0.3	2.4 ± 0.3	6.8 ± 0.3	5.8 ± 0.3	4.6 ± 0.2
35	4.3	4.8	10.8	7.3	5.0
11B	0.7 ± 0.1	2.6 ± 0.3	8.6 ± 0.9	7.4 ± 0.3	5.4 ± 0.8
040	0.8 ± 0.3	1.4 ± 0.2	2.3 ± 0.9	2.5 ± 0.7	2.8 ± 0.5
120	3.6 ± 1.2	4.6 ± 1.8	5.4 ± 0.9	4.2 ± 0.5	2.2 ± 0.5

Results are expressed in pmol Pt/10^6 alive cells (mean ± SD)

Growth inhibition

Growth inhibition after exposure to cisplatin for 72 hr was analyzed for NHNEC and HNSCC cells (Table II). The IC_{50} values for the eight NHNEC cultures varied six-fold from 0.4 to 2.7 μM cisplatin. Another way of assessing the cisplatin induced growth inhibiting effect is to compare the number of cells (measured as OD) after 72 hr exposure with that at t=0 (Table II). For most cultures it was observed that this value was lower at 72 hr than at 0 hr. For two cell cultures (NHNEC7and NHNEC8) the opposite was true, indicating that for these two cell lines proliferation has outweighed cell death during the 72 hr cisplatin exposure period.

Compared to the NHNEC cultures, the five tested cancer cell lines did not differ in the response to cisplatin as judged upon the IC_{50} values. The cell number of the 5 μM exposures were always lower at 72 than at 0 hr, indicating that for all HNSCC lines cell death has outweighed proliferation. The accumulation of Pt (Table I) and the IC_{50} values (Table II) of the NHNEC cultures both determined at 72 hr appeared not to be correlated (r= -0.22, p=0.77).

The cellular growth as measured with the SRB assay of untreated NHNEC of eight persons indicated a variation in growth rates with an increase in cell number varying from 180 to 400% in 72 hr [Table II, mean 232 ± 74.8% (SD)]. The five HNSCC lines showed significantly higher growth rates (371 ± 75.6%; p=0.008,

Student's t-test). No correlation was found between these control growth rates and the parameters of growth inhibition (Table II).

Apoptosis

Apoptosis was studied to determine its relevance in the response to Pt-induced damage. Moreover, we were interested to see if a difference exists between normal and malignant cells. Cell morphology was studied after a 24 hr exposure period to cisplatin. Apoptotic NHNEC were observed at concentrations of 50 μM cisplatin or higher. The absolute level of apoptosis varied between the eight NHNEC cultures from 12 to 37% (Table II). When compared at 24 hr we did not find a correlation between Pt accumulation and the percentage of apoptotic cells at 50 and 100 μM.

Although significantly lower in number (Mann-Whitney-U-test, $p<0.001$), most HNSCC cell lines also showed apoptosis at 50 and 100 μM cisplatin (Table II). In OE, 22A and 14C apoptotic cells were present at a concentration of 10 μM. Interestingly, in the OE cell line apoptosis was found over a dose range from 0.5 to 100 μM; at 0.5 and 1 μM this was 3.5, and 4.5%, respectively and the rest of the data is shown in Table II.

Clearly necrotic cells, as characterized by an increase in cell size and the formation of intracellular vacuoles, were observed in the normal and cancer cell population. These were only detectable at the 50 and 100 μM cisplatin concentration and never exceeded 20% of the total number of cells.

Medium dependency of growth inhibition

The comparison of the growth inhibition data of NHNEC and HNSCC cells could be complicated by the fact that two different growth media were used. One major difference between the two media was the amount of protein present. With AAS we could establish that for both media the percentage of the Pt bound to proteins was similar, about 10%. Since it still could be anticipated that proteins could form a kind of buffer with a slow release of the drug, we investigated the role of proteins in two ways. First, we took advantage of the fact that the OE cell line could be tested in both media. No difference was observed with respect to the growth inhibition parameters (data not shown). Moreover, we investigated the effects of adding extra protein to the NHNEC cultures by supplementing KGM with bovine serum albumin to a final concentration of 0.05%, yielding a protein concentration that approaches the one in DMEM with 5% FCS (the medium for HNSCC cells). No difference of response could be observed for the four cell cultures tested (data not shown).

Table II. Cisplatin induced growth inhibition and apoptosis in NHNEC cultures and HNSCC cell lines.

code[1]	control cell growth[2]	IC_{50} (μM)[3]	Growth relative to start exposure[4]	apoptotic cells[5]			
			5 μM	5 μM	10 μM	50 μM	100 μM
NHNEC1	184	.7	43	0	0	29	38
NHNEC2	182	27	81	0	0	12	19
NHNEC3	180	4	67	0	0	31	18
NHNEC4	190	2.1	85	0	0	16	14
NHNEC5	270	.9	71	0	0	37	35
NHNEC6	236	.8	69	0	0	30	37
NHNEC7	217	.4	101	0	0	19	29
NHNEC8	400	1.1	166	0	0	14	20
22A	481	1.3 ± 0.3	62 ± 13	0	6.4 ± 1.3	0	0
22B	376	1.2 ± 0.3	28 ± 60	0	0	0	0.65 ± 0.2
14C	397	2.7 ± 0.7	65 ± 11	0	3.2 ± 1.2	0	15.6 ± 2.6
35	292	0.9 ± 0.8	0	0	0	10 ± 2	0
OE	309	2.3 ± 0.9	21 ± 65	8.1 ± 1.6	8.3 ± 1.6	26.3 ± 12.9	41.0 ± 11.1

Mean values are shown (± SD for the cancer cell lines), n.d..= not determined.
[1] NHNECn=code for an normal head and neck epithelial cell culture of a single non-cancer control; the others are HNSCC lines.
[2] Percentage growth of the untreated cells during the 72 hr culture period. The increase of the OD-value is shown, whereas the t=0 hr value was set at 100%
[3] The concentration that caused a growth inhibition of 50% when compared to untreated cells, as determined with the SRB-assay
[4] Indicator of growth inhibition after 72 hr exposure to cisplatin. For this purpose the OD-values are given as a percentage of the corresponding values at t=0 hr (= 100% at the time exposure started)
[5] Expressed as percentage of all nucleated cells.

Discussion

Aim of the present study was to investigate how NHNEC and HNSCC cells respond to DNA damage as induced with cisplatin. A crucial finding of this study was that despite the fact that similar IC_{50} values were measured, the levels of Pt were much higher in NHNEC than in the corresponding cancer cells. This was most extreme after the longest exposure time of 72 hr, but could already be observed after 6 hr. The high accumulation of Pt in NHNEC is not caused by the difference in the

medium the two cell types were cultured in, as discussed in the "results" section. Possible differences in the number of apoptotic cells or in the rate of cell growth are also not involved. Why do NHNEC tolerate so much more Pt than neoplastic cells, when exposed to a concentration of cisplatin that not necessarily lead to cell death? One explanation is that NHNEC have the physiological role to protect the body and serve as a non-specific "sponge" with the only intention to absorb as much toxic material as possible. The similar level of growth inhibition of normal and neoplastic cells can be explained in that view by assuming that the level of Pt-DNA adducts, hypothesized to be the decisive cytotoxic lesion (14) is similar in both cell types. In the present study we have measured the total intracellular amount of cisplatin, bound to the nucleic acid but also to proteins. The cell biological basis for the "sponge effect" may be the cytoskeleton. It has been reported that cytokeratins, as part of the cytoskeleton, can be involved in the transport and storage of cisplatin (15, 16). Previous studies from our group have established in a panel of HNSCC cell lines a correlation between the intracellular Pt content and sensitivity (7). For NHNEC we could not establish this correlation in the present study, which indicates that for this type of cells other factors are involved. It can be hypothesized that higher concentrations of glutathione or a more active DNA repair system may be present in NHNEC when compared to HNSCC. In a recent study we could show in HNSCC cell lines that intracellular glutathione levels do not correlate with growth inhibition or Pt accumulation (17), but whether this can be extrapolated to NHNEC cells is not known.

Based on the number of apoptotic cells at 24 hr, it seems that apoptosis is less prominent in the NHNEC as compared to the cancer cells. The lower propensity to become apoptotic would be in agreement with the concept that in the process of carcinogenesis the pathway to apoptosis becomes impaired (5). In the present study, however, it appears that the apoptotic route is not completely blocked. Moreover, it has to be added that the threshold concentration that induces apoptosis is found to be lower in some cell lines. In UM-SCC-14C and -35 and VU-SCC-OE apoptotic cells were seen at a lower concentration than 50 μM that had to be used for the other two cell lines and the NHNEC cultures. The notion that apoptosis is seen only in a small proportion of cancer cells even at high concentrations of cisplatin suggests that apoptosis is an infrequent event in head and neck cancer. This is in line with two histological studies (18, 19).

The present study shows a four-fold interindividual variation in Pt accumulation in NHNEC after cisplatin exposure. There is also an extensive literature on the effect of toxic compounds and gamma irradiation on the DNA of various cell types in which the effects are analyzed on the basis of chromosome aberrations (20), sister chromatid exchange (21) and DNA strand breaks (22). As for the mucosa of the head and neck variable levels of DNA-adducts have been found for a number of

carcinogens (23). A common finding that comes from those studies is that there is an interindividual variation in the response to damage. It may well be that the individuals who tolerate higher levels of DNA damage in the stem cells after challenge with environmental carcinogens are the ones that have the highest risk for cancer. At the epidemiological level much evidence has been provided that the development of HNSCC is strongly related to tobacco and alcohol exposure (24). The fast majority of people who smoke and drink extensively, however, do not develop cancer, indicating that for the development of cancer also endogenous factors related to damage processing seem to be important. In fact, some evidence has already been provided that an individually based susceptibility for HNSCC exists (25).

The differences between neoplastic and normal cells with respect to propensity to become apoptotic and the tolerance of cisplatin may also be important for the development of more tumor selective therapeutic approaches. However, before this information can be used for rational drug development, much more should be known about the mechanisms responsible for this tumor-specific effects.

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Chapter 5

Improved ^{32}P-postlabelling assay for the quantification of the major platinum-DNA adducts.

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Abstract

For the improvement of chemotherapy with platinum (Pt)-containing drugs a sensitive assay to detect the induced Pt-DNA adducts is needed. Therefore, the ^{32}P-postlabelling assay, described by Blommaert and Saris (*Nucleic Acids Res.*, 1995, 23, 1300-1306), to detect the major adducts Pt-GG and Pt-AG has substantially been improved and compared with ELISA and AAS. For the quantification of the adducts, TpT was added as an internal standard immediately after isolation of the Pt-adducts from digested DNA samples. It was found that ^{32}P-labelling of both GpG and ApG, the dinucleotides obtained after deplatination of the adducts, was equally efficient as that of TpT. To isolate the Pt-adducts on basis of a positive charge, the pH of DNA digests was adjusted to about 3 prior to separation by strong cation-exchange chromatography. For the subsequent deplatination a volume of only 12 µl of 0.2 M NaCN was used, which did not interfere with the following labelling step. The quantification of the ^{32}P-labelled dinucleotides was performed by phosphorimaging of spots after separation on TLC as well as by ^{32}P-counting of fractions collected after separation by HPLC. The method was used to determine adduct levels in *in vitro* cisplatin-treated DNA and in DNA isolated from cisplatin-treated cultured cells, tumor xenografts from cisplatin-treated mice, and from white blood cells and (tumor) tissues from cisplatin-treated patients. The results show a significant correlation with the adduct levels as determined with atomic absorption spectroscopy (high levels) or with specific antibodies (low levels). This assay appears to be useful for the determination of low levels of Pt-adducts in small DNA samples as present in clinical specimens such as blood and tumor tissue, but also in buccal mucosal cells and fine needle aspirates.

Introduction

Cis-diamminedichloroplatinum(II) (cisplatin®) is widely used for chemotherapy of a broad range of solid tumors. It is generally accepted that the antitumor activity of cisplatin and other platinum (Pt) antitumor drugs is due to the binding of these drugs to cellular DNA. Cisplatin can form mono-adducts and various types of bifunctional adducts (2). The main adduct formed is *cis*-Pt(NH_3)$_2$d(GpG) (Pt-GG), with Pt bound to two adjacent guanines. Another major intrastrand crosslink is *cis*-Pt(NH_3)$_2$d(ApG) (Pt-AG), in which the Pt is bound to adenine and an adjacent guanine. The other bifunctional adducts are *cis*-Pt(NH_3)$_2$(dG)$_2$ (G-Pt-G), the intrastrand adducts in which Pt is bound to two guanines separated by one or more other nucleotide(s) and the interstrand crosslinks on two guanines in opposite strands.

There are several methods to determine the total amount of Pt-adducts in isolated DNA (3), of which atomic absorption spectroscopy (AAS) is the most common (4,5). Also various immunochemical techniques are available using polyclonal antibodies directed against platinated DNA (6-10). None of these systems, however, is able to quantify the various DNA adducts separately. For this purpose an immunochemical method has been developed by Fichtinger-Schepman and colleagues (2,11,12), using polyclonal antibodies directed against the different types of adducts as present in digested DNA samples (ELISA method). Although this method is very sensitive, drawbacks are the rather large amounts of DNA needed, the limited availability of the rabbit antisera and the fact that these antibodies are only suitable to detect adducts induced by cisplatin and carboplatin. Also, this method is rather laborious.

Therefore, there was a need for a more generally applicable and sensitive method to determine adduct levels in small samples of platinated DNA. Such an assay was recently developed by Blommaert and Saris (1), using a ^{32}P-postlabelling technique. In this assay the DNA is digested, followed by isolation of the Pt-adducts on the basis of their positive charge, their deplatination with sodium cyanide (NaCN) and finally the labelling of the resulting dinucleotides with [γ-^{32}P]ATP. This deplatination appeared to be a crucial step to enhance the sensitivity of the assay, because the dinucleotides are labelled with a much higher efficiency than the platinated adducts themselves (13,14). Under the assay conditions published previously (1), fmol amounts of the two major intrastrand adducts Pt-GG and Pt-AG could be measured in small amounts of DNA (about 10 µg), after deplatination. The G-Pt-G and mono-adducts formed upon cisplatin treatment are not detected in this assay because they are no substrates for the labelling enzyme due to the lack of a 3'-phosphate. In the assay as described by Blommaert and Saris (1), reproducible quantitative analysis was impossible due to the absence of an internal standard. Furthermore, the recovery of the adducts was only 30%. In the present paper we

report on the improvement of the assay by optimizing the isolation of the adducts on the strong cation-exchanger. In addition, an internal standard for the labelling reaction was added and the deplatination step was modified, so that a subsequent purification could be omitted. For the quantification of the ^{32}P-labelled products thin-layer chromatography (TLC) in combination with phosphorimaging was used, as well as high performance liquid chromatography (HPLC) with ^{32}P-counting of the collected column fractions. The improved assay was found to be quite suitable to determine adduct levels in *in vitro* cisplatin-treated DNA, in DNA isolated from cisplatin-treated cultured cells, in DNA from tumor xenografts in mice given cisplatin therapy, as well as in DNA from human white blood cells and (tumor) tissues of cisplatin-treated patients.

Material and Methods

Reference compounds

The dinucleotides GpG, ApG and TpT were purchased from Sigma (St. Louis, MO) and dissolved in distilled water. The Pt-GG and Pt-AG adducts were isolated from cisplatin-treated salmon sperm DNA (2) and quantified on the basis of their Pt content by AAS. G-Pt-G was prepared by incubation of deoxyguanosine with cisplatin (2:1 ratio) for 5 hr at 50°C in the dark and purified as described by Fichtinger-Schepman and coworkers (15).

Treatment of DNA with cisplatin

Salmon sperm DNA (Worthington Biochemical Corporation, Freehold, NJ), dissolved in 10 mM sodium phosphate pH 7.2 (1 mg/ml), was incubated for 5 hr at 50°C with cisplatin (Platinol, Bristol Myers Squibb B.V., Woerden, The Netherlands) at concentrations ranging between 0 and 42.7 nM. Unbound and mono-functionally bound cisplatin were inactivated by overnight dialysis against 0.1 M NH_4HCO_3 at room temperature, followed by dialysis against water at 4°C. The amounts of the bifunctional adducts Pt-GG and Pt-AG in these DNAs were determined by competitive ELISA in the chromatographed, digested samples, as described by Fichtinger-Schepman *et al.* (12). An additional DNA sample was incubated with 3.3 µM cisplatin to obtain sufficiently high adduct levels in order to determine these also with AAS (16). Furthermore, part of this sample and untreated DNA were postincubated with 10 mM thiourea (Aldrich Chemie, Steinheim, Germany) for 1 hr at 37°C in order to see whether this procedure, introduced recently as an alternative for the NH_4HCO_3 treatment for the rapid and more reliable inactivation of unbound and mono-functionally bound cisplatin (17), could have an effect on the postlabelling results.

Treatment of cultured cells with cisplatin

The IGROV-1 human ovarian carcinoma cell line was originally described by Benard *et al.* (18). Cells were routinely cultured in RPMI 1640 medium with HEPES (pH 7.4) and phenol red, supplemented with 10% bovine calf serum, 10 mM $NaHCO_3$, 2 mM glutamine, gentamycin (45 µg/ml), penicillin (110 IU/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were frequently monitored for mycoplasm contamination.

In order to study the formation of GpG and ApG intrastrand adducts in these cells, 10^6 cells were incubated with 0 to 66.7 µM cisplatin for 2 hr at 37°C. Subsequently, medium was removed, cells were washed twice with phosphate-buffered saline (PBS) and trypsinized. After centrifugation cell pellets were stored at -20°C until DNA isolation.

Treatment of tumors with cisplatin

Human head and neck squamous cell carcinomas (HNSCC) were grown as xenografts in nude mice (19). Subsequently, these tumors were treated *ex vivo* with 1.7 mM cisplatin for 1 hr. To obtain *in vivo* treated samples, nude mice bearing human ovarian carcinomas (OVCAR1) were treated intravenously with 5 mg cisplatin/kg and sacrificed at different time periods after treatment. In addition to the tumors, liver and kidney tissues also from these mice were stored at -20°C until DNA isolation.

DNA isolation

DNA was isolated as described previously (12,17). For the cisplatin-treated IGROV-1 cells and blood samples from cisplatin-treated patients the isolation procedure with high-salt extraction was used as described by Miller *et al.* (20). When cisplatin-containing samples were postincubated in drug-free medium for several hours, the inactivation step with thiourea was not necessary because reactive mono-adducts were no longer present in platinated DNA after that period (17).

DNA digestion

Two methods were applied for the digestion of DNA to deoxynucleosides (see Scheme I, no. *1*). First, DNA was digested as described by Blommaert and Saris (1) with the exception that after the digestion only 1/100 instead of 1/10 volume of 1 M Tris-HCl pH 9.0 was added. This volume was sufficient to reach the pH optimum of calf intestinal alkaline phosphatase (Life Technologies, Breda, The Netherlands). Under these conditions a complete digestion of the major intrastrand crosslinks to Pt-GG and Pt-AG dinucleotides will be obtained (2).

A second method to digest DNA was by dissolving 10-100 µg DNA in 50 µl of 50 mM ammonium acetate (NH_4Ac, pH 5.0) to which 6 units nuclease P1 (Boehringer Mannheim, Germany) and 1 mM $ZnCl_2$ (pH 8.2) were added. After an incubation at 55°C for 2 hr, 10 µl of 1 M Tris-HCl (pH 8.2) with 10 mM $MgCl_2$ and 50 units DNAse I (Boehringer) were added, after which the incubation was prolonged for 2 hr at 37°C. Finally, 20 units alkaline phosphatase (Boehringer) were added, the pH was adjusted to 9.0 and the digestion mixture was incubated at 37°C for another 2 hr for samples containing <25 µg, and overnight for samples with more DNA. The efficacy of this digestion procedure and the amount of DNA were determined by HPLC analysis on a C_{18}-column (Inertsil ODS-80A, 150 x 4.6 mm, 5 µm) eluted with 0.1 M NH_4Ac (pH 5.5)/10% MeOH at a flow rate of 1.0 ml/min. In this system the nucleosides dC, dG, T and dA elute at 2.0, 3.1, 3.9, and 6.1 min, respectively. The exact amount of DNA could be calculated from the integrated dA peak (using ε_{260}=15,400 l/mol/cm).

Purification and concentration of cisplatin-DNA adducts

Separation of the platinated adducts from the unmodified deoxynucleosides was achieved by strong cation-exchange (SCX) chromatography, either with the strong cation-exchange column Mono-S coupled to an FPLC system (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) (Scheme I, no. 2), or by use of SCX solid-phase extraction cartridges (Lichrolut SCX, 200 mg; Merck, Darmstadt, Germany) and a vacuum manifold (Vac-Elut 10, Applied Science Group, Emmen, The Netherlands). Also GpG and ApG dinucleotides from unplatinated DNA regions, if any present, will be separated from the platinated products during this step.

1) FPLC-method. Before the purification of the platinated DNA adducts on the Mono-S column, the pH of the DNA digest was lowered to about 3. After injection of the sample, the column was eluted for 4 min with buffer A to remove the unmodified nucleosides. Then, the adducts were eluted with buffer B for 3 min at 1 ml/min, followed by washing of the column at a flow rate of 2 ml/min for 3.5 min with buffer B and 4 min with buffer A. The amount of DNA in the sample was calculated by comparison of the total peak areas of the unmodified nucleosides with those of known samples of digested salmon sperm DNA. Synthetic G-Pt-G was used as a reference to determine the elution position of the Pt-adducts. The retention time of this reference adduct was approximately 6.2 min. The adducts, present in the middle 1-ml fraction of buffer B, were collected in siliconized tubes already containing 0.5 or 0.6 pmol of TpT as internal standard and 1.2 µmol NaCN (no. *3a* in Scheme I). Then the samples were dried *in vacuo* (Scheme I, no. *3b*) The presence of NaCN in the samples

prevents digestion of the internal standard by nucleases that co-elute with the adducts from the column (data not shown).

2) SCX solid-phase extraction. On the vacuum manifold 10 samples can be processed simultaneously. Before the adduct purification, the required number of SCX cartridges were washed as follows: 2 times with 1 ml water, 2 times with 1 ml MeOH, and again 2 times with 1 ml water. The cartridges were equilibrated by rinsing twice with 1 ml of 0.05 M Tris-HCl (pH 3.0). Prior to loading, the pH of the DNA digests was also adjusted to approximately 3 by addition of 0.8 volume of 0.05 M HCl. After loading the samples, unmodified deoxynucleosides were eluted with 8 1-ml washes of 10 mM NH_4-formate buffer pH 6.0 (buffer A), followed by elution of the platinated products with 2 portions of 500 µl 0.25 M NH_4OH (buffer B). The latter two eluted fractions of 500 µl were collected in one tube containing 1.2 µmol NaCN and 0.5 or 0.6 pmol of TpT as the internal standard (no. *3a* in Scheme I). Samples were dried *in vacuo* (Scheme I, no. *3b*).

Deplatination of cisplatin-DNA-adducts

After drying, the mixture of purified Pt-adducts, reference TpT and 1.2 µmol NaCN was dissolved in 12 µl 0.1 M NaCN (final concentration 0.2 M NaCN, pH about 10) and incubated for 2 hr at 65°C in order to remove Pt from the adducts (Scheme I, no. *3c*). The samples were stored at -20°C until postlabelling.

^{32}P-Postlabelling

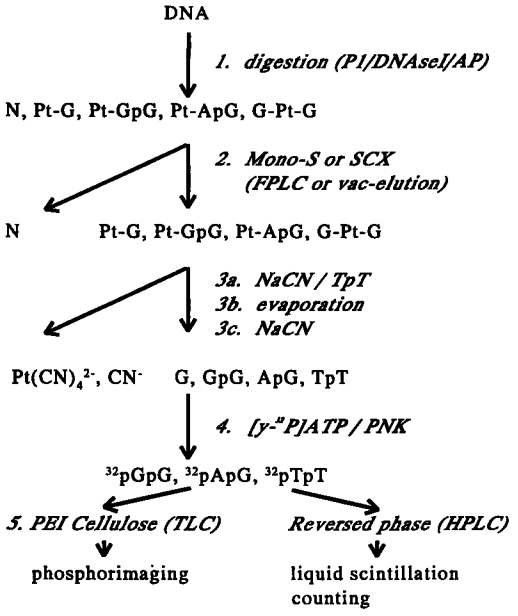
In 12 µl of the deplatinated-adduct solution containing 0.2 M NaCN, the pH was lowered with 4 µl of 3 M sodium acetate (NaAc; pH 5.4) to approximately 8.2 which is the optimum pH for the enzyme T4-polynucleotide kinase (PNK; 10 U/µl, 3'-phosphatase free; Boehringer). Because of the volatility of HCN, formed upon lowering of the pH (pKa=9.3), the following steps were performed in a hood to avoid inhalation. To each sample, 3 µl of the labelling-mixture was added followed by a 40-min incubation at 37°C. The labelling-mixture consisted of 0.5 µl [γ-^{32}P]-ATP (3000 Ci/mmol, 3.3 pmol/µl; Amersham Life Science, Amersham, UK), 2 µl 10 times concentrated kinase buffer (Boehringer) and 0.5 µl PNK (no. *4* in Scheme I).

Separation and quantification of the ^{32}P-labelled dinucleotides

The separation of the radioactively labelled products can be performed in two ways, either by TLC or HPLC (Scheme I, no *5*).

1) TLC. Polygram Cel 300 PEI TLC sheets (Macherey-Nagel, 20 [width] x 40 [height] cm, Düren, Germany) were prewashed with 50% MeOH and air-dried. After labelling, 5 µl of the mixtures were applied on the sheets and run for approximately 5 hr with 1.5 M NH_4-formate buffer (pH 4.0), after which the solvent front had reached approximately 5 cm underneath the top of the sheet. After drying of the sheets, the amount of radioactivity of each spot was quantified by use of a PhosphorImager 425 with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The dinucleotides GpG, ApG and TpT were labelled with equal efficiency (see below). Therefore, the original amounts of Pt-GG and Pt-AG adducts in the digested DNA samples could be calculated by comparing the background-corrected radioactivity of the ^{32}pGpG and ^{32}pApG spots with that of the standard TpT after labelling (*i.e.* ^{32}pTpT) according to the equation: amount of adduct = [(^{32}P content adduct) / (^{32}P content pTpT)] * amount TpT

2) HPLC. For the separation of the ^{32}P-labelled products by HPLC, the samples were diluted to 150 µl with water. Depending on the amount of adducts present, a maximum of 50 µl was injected onto the C_{18}-column. Products were separated during isocratic elution with 0.1 M KH_2PO_4 (pH 4.0)/ 2.5% MeOH. Fractions of 0.5 ml were collected and Cerenkov radiation was counted. The total amount of each compound was calculated from its total background-corrected radioactivity compared to that of the reference compound TpT, as described for the TLC method, above.



Scheme I. A schematic outline of the ^{32}P-postlabelling assay. The isolated DNA is digested into unmodified nucleosides (N) and Pt-adducts by the enzymes P1-nuclease (P1), DNAse I and alkaline phosphatase (AP) (*1*). Then, the adducts are purified by strong cation-exchange (SCX) chromatography (*2*), and collected in tubes containing NaCN and the internal standard TpT (*3a*), whereafter the samples are dried *in vacuo* (*3b*). Subsequently, after addition of NaCN to a total of 12 µl 0.2 M NaCN, the adducts are deplatinated by incubation for 2 hr at 65°C (*3c*), whereafter they are postlabelled (*4*), separated and quantified (*5*).

Results

The ^{32}P-postlabelling assay to determine the two major Pt-DNA intrastrand crosslinks Pt-GG and Pt-AG, as introduced by Blommaert and Saris (1), is a well-designed and sensitive method. However, various steps in the protocol still needed to be improved. The results of our experiments to optimize the assay and its application on clinical relevant samples are described here.

Purification of cisplatin-DNA adducts

The Pt-adducts in the DNA digests were separated from the unmodified deoxynucleosides by strong cation-exchange chromatography, by use of either the Mono-S column or the SCX-cartridge. In our hands, the Pt-adducts did not bind to

the cation-exchanger unless the pH of the DNA digest was lowered to about 3. Following removal of unmodified nucleosides 85-100% of the adducts could be recovered after elution with NH_4OH. They could be collected in 1 ml of eluent, as was verified by AAS measurements when reference Pt-GG and Pt-AG adducts were applied onto the column in a mixture with 0.5 g of digested salmon sperm DNA. The Pt-adducts were collected in tubes containing TpT as internal standard and 1.2 µmol NaCN. NaCN was added to maintain a high pH during the subsequent drying step, when NH_3 was evaporated, in order to prevent any digestion of the internal standard by traces of co-eluted nucleases (data not shown).

Deplatination of the cisplatin-DNA adducts

The final concentration of 0.2 M NaCN, contained in only 12 µl, was sufficient to obtain complete deplatination, as was concluded from the complete shifts of the UV-absorbing peaks during chromatography on a Mono-Q anion-exchange column upon deplatination of 0.5 nmol of the adducts Pt-GG and Pt-AG (not shown). The presence of such a small amount of NaCN, after adjustment of the pH, did not affect the PNK enzyme activity during the ^{32}P-labelling (see below). However, due to the presence of the cyanide, Pt-determination in the Mono-Q fractions with AAS, to double-check the deplatination, was not possible. From the shifts in the retention times it could be concluded that the deplatination of Pt-AG was faster than that of Pt-GG. A complete deplatination of Pt-AG, but not of Pt-GG, was already found when a concentration of 0.1 M NaCN was used.

^{32}P-postlabelling

The mixture of deplatinated products and NaCN could directly be used for the postlabelling. This was concluded from the equally efficient labelling of 1 pmol of ApG and GpG standards in the presence or absence of 12 µl 0.2 M NaCN. This amount of NaCN was found not to affect the reaction when 1/3 volume of 3 M NaAc pH 5.4 was added prior to the labelling in order to lower the pH to about 8.2, which is the optimum pH for the labelling enzyme PNK (data not shown).

Separation of the ^{32}P-labelled dinucleotides

This has been achieved in two different ways. First, by use of chromatography on PEI cellulose TLC sheets. Even when the whole ^{32}P-postlabelled sample (19 µl) was spotted, a good separation of the reaction products ^{32}pGpG, ^{32}pApG, ^{32}pTpT from excess of [γ-^{32}P]ATP and inorganic ^{32}P-phosphate (P_i) could be obtained as shown in Figure 1. In a second method the labelled products were separated on a C_{18}-column by HPLC (Figure 2). This method resulted in elution of ^{32}pGpG after 6 min, ^{32}pApG after 9 min, and of ^{32}pTpT after 19 min. Excess of [γ-^{32}P]ATP was eluted after 2 min.

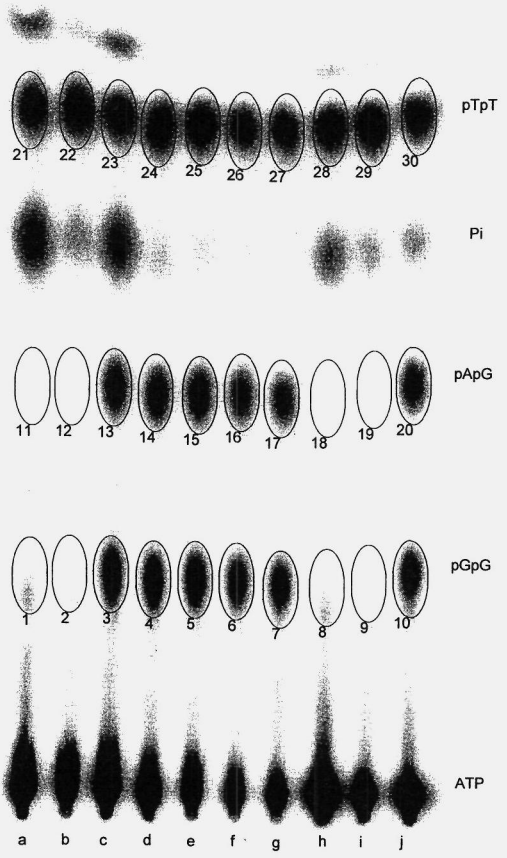


Figure 1. Phosphorimaging pattern of a TLC sheet after chromatography of ^{32}P-postlabelled samples, showing the spots of [γ-^{32}P]-ATP, ^{32}pGpG, ^{32}pApG, inorganic phosphate ($^{32}P_i$) and the internal standard ^{32}pTpT. Internal standard (500 fmol TpT) was labelled using 2 µl (lane a and h) or 0.5 µl of [γ-^{32}P]-ATP (lane b and i). Mixtures of 500 fmol of the dinucleotides ApG, GpG and TpT were labelled by use of 2 µl (lane c and j), 1 µl (lane d), 0.5 µl (lane e), 0.4 µl (lane f) or 0.2 µl of [γ-^{32}P]-ATP (lane g). The area between ^{32}pApG and ^{32}pGpG was taken as background.

Labelling efficiency of the dinucleotides ApG and GpG

To study the kinetics of the labelling reaction, various amounts (0-500 fmol) of the dinucleotide ApG were postlabelled in the presence of 500 fmol TpT and with or without 50 fmol of GpG. After quantification with the TLC method, linear dose-responses were obtained for ^{32}pApG (Figure 3A). R values of 1.0 were found for ApG alone and of 0.99 in combination with 50 fmol GpG. Similar results were found when variable amounts of GpG were postlabelled (Figure 3B), with r values of 1.0 for GpG alone and of 1.0 for the combination. These data also indicate that the presence of

other dinucleotides hardly interferes with the labelling efficiency of the dinucleotide of interest. Furthermore, the dinucleotides GpG and ApG are shown to be labelled with equal efficiency. These data were obtained after a labelling reaction time of 40 min, however, no differences were seen when the reaction was prolonged to 1 hr (data not shown). Also when the amounts of [γ-^{32}P]ATP was limited to 0.2 µl, the substrates were labelled in the same ratio (see Figure 1). When the HPLC method was used for the quantification of the adduct levels in cisplatin-treated ovarian cancer cells, also a linear dose-response was observed (see Figure 2). Comparison of the data obtained with the TLC and HPLC quantification methods indicated that both methods can be used successfully for the determination of small amounts of adducts, *i.e.* after deplatination and ^{32}P-postlabelling (data not shown).

Correlation between cisplatin-DNA adduct measurement with the postlabelling assay and the ELISA method

The results after TLC quantification of the postlabelled products were compared with those obtained with the ELISA method of the same DNA samples. For the ELISA method part of the DNA was digested to mononucleotides and 5'-phosphorylated adducts (12). The comparison was made both with *in vitro* cisplatin-treated DNA and with DNA isolated from cisplatin-treated (tumor) tissues. The correlation between the ELISA data and the results obtained after postlabelling of cisplatin-treated salmon sperm DNA, as shown in Figure 4 for the Pt-GG adduct, indicates an r value of 0.98 (p=0.00003).

In Figure 5 the correlation between the results of the two assays is given for platinated DNA samples isolated from *ex vivo* cisplatin-treated HNSCC as well as from tumor, liver and kidney samples from cisplatin-treated nude mice bearing human ovarian carcinomas. The ELISA data of the latter will be published in detail elsewhere. The corresponding r values for the Pt-AG and the Pt-GG adducts were 0.77 (p=0.045) and 0.91 (p=0.042) respectively, indicating a good correlation between these two assays.

Correlation of postlabelling results with AAS data

Results obtained with the HPLC-based postlabelling method were compared with those of AAS analysis. For this purpose, part of the DNA isolated from IGROV-1 cells that had been incubated with various concentrations of cisplatin, was analyzed by postlabelling and another part by AAS. As shown in Figure 6, highly significant correlations were observed between the total Pt-DNA content (AAS data) and the postlabelling results of the Pt-AG and Pt-GG adducts with r values of 0.99 (p=0.012) and 1.0 (p=0.003), respectively. The total adduct level measured by postlabelling, *i.e.* Pt-AG plus Pt-GG, was found to be about 85% of the overall platination level measured by AAS.

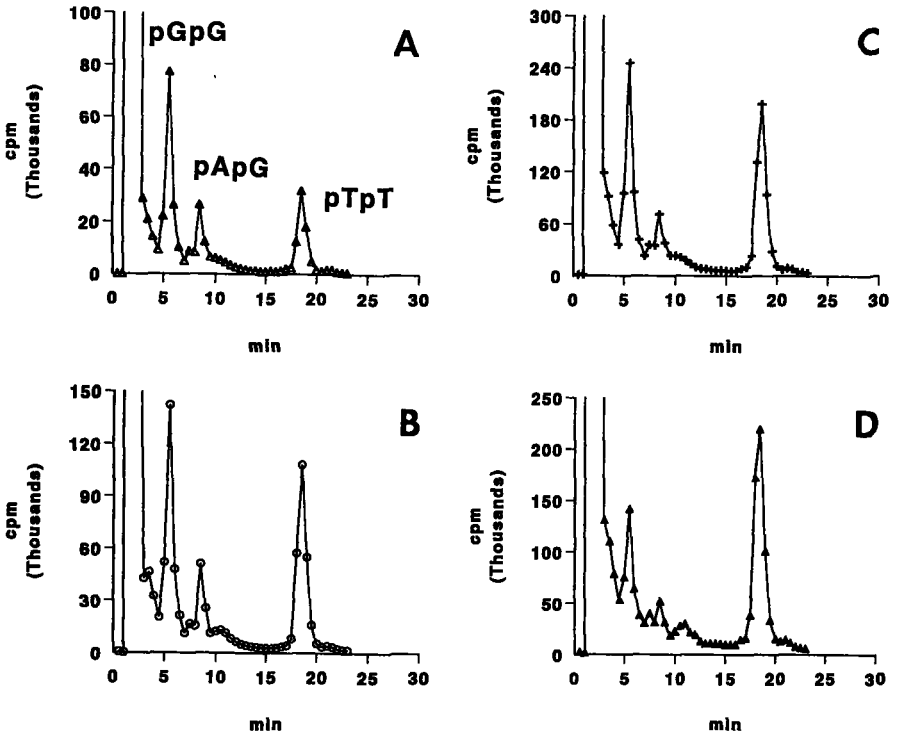


Figure 2. Representative HPLC chromatograms, showing ^{32}P-labelled pGpG, pApG and the internal standard pTpT as eluted from a C_{18}-column, using 0.1 M KH_2PO_4 (pH 4.0)/2.5 % MeOH, 1 ml/min. Fractions of 0.5 ml were collected and counted. The ^{32}pGpG and ^{32}pApG originated from the Pt-adducts in DNA isolated from human IGROV-1 ovarian cancer cells incubated with 33.3 (A), 16.67 (B), 8.3 (C) or 3.3 µM (D) cisplatin for 2 hr. The samples injected onto the column were diluted 5.5 times (A), 1.8 times (B) or not diluted (C and D) in order to obtain sufficient high and evaluable peaks.

Application of the modified postlabelling assay

The assay described here has already been applied successfully for the determination of adduct levels in white blood cells, buccal cells and tumor biopsies from cisplatin-treated cancer patients. The adduct levels in the blood samples, for example, varied between 14 and 22 adducts/10^7 nucleotides for the Pt-GG and between 5 and 8 for the Pt-AG adducts after treatment with 70 mg/m^2 cisplatin, whereas in a tumor biopt 93 Pt-GG and 30 Pt-AG adducts/10^7 nucleotides were detected. More data on patient samples will be published elsewhere. The results obtained for the blood cells were in reasonable agreement with those obtained with AAS. In some samples with adduct levels below the detection limit of AAS [about 8 adducts/10^7 nucleotides in minimally 200 µg DNA (5)] some adducts could still be detected by postlabelling [1.6 adducts/10^7 nucleotides in 10 µg DNA], which demonstrates the sensitivity of this assay.

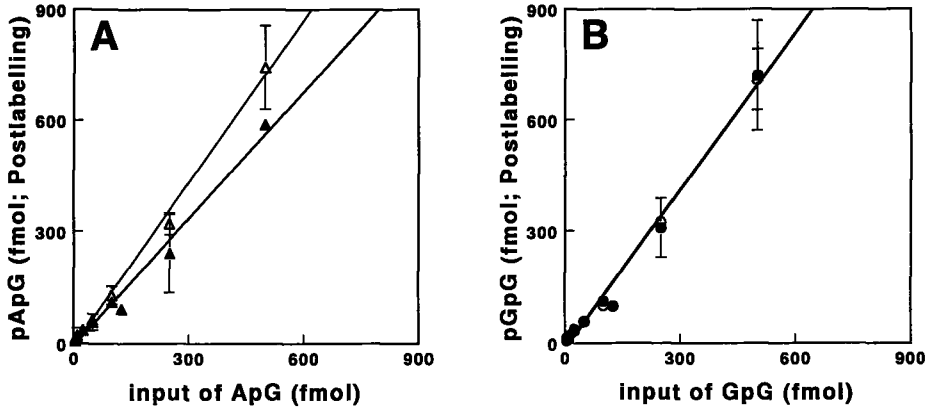


Figure 3. Relationship between the starting amounts of the dinucleotides (=input) ApG (A) and GpG (B) as determined by weight and their calculated amounts after postlabelling. The postlabelling was performed on the dinucleotides in the presence of the internal standard TpT (open symbols) as well as on mixtures to which 50 fmol of the dinucleotide GpG (A) or ApG (B) was added (closed symbols). Correlation values of 1.0 were found for ApG alone and of 0.99 in combination with 50 fmol GpG (A), of 1.0 for GpG alone and of 0.99 in combination with 50 fmol ApG (B). The dinucleotides ApG and GpG are found to be labelled with equal efficiency. Results are given as means of three independent experiments ± SD.

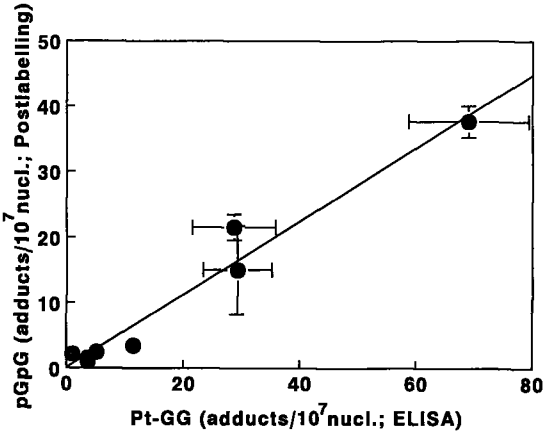


Figure 4. Correlation between the Pt-GG adduct levels as determined by the ELISA method (using 100 µg DNA) and the ^{32}P-postlabelling assay (using 10 µg DNA) in salmon sperm DNA treated *in vitro* with various concentrations of cisplatin (0 to 42.7 nM) for 5 hr at 50°C. The r value is 0.98 (p=0.00003). Means (adducts/10^7 nucleotides) of the results are given from two separate experiments (± SD).

Discussion

There are several methods to determine the cisplatin-DNA adduct levels in isolated DNA (3). With the most common of these, AAS, total adduct levels can be determined. A more sensitive method, in which the various adducts in DNA digests can be measured, was developed by Fichtinger-Schepman and coworkers (12) using competitive ELISA with antisera directed against the specific adducts. However, there is a need for an alternative method to quantify Pt-adducts, because of the limited availability of the antisera, the limitation of the use of the antibodies for only cisplatin and carboplatin adducts and the relatively large amounts of DNA that are required.

Our initial attempt to determine cisplatin-DNA adduct levels was to develop a non-radioactive assay analogous to methods described by Jain and Sharma (21), Kelman *et al.* (22) and Sharma *et al.* (23), using dansylchloride (5-dimethylaminonaphthalene 1-sulphonyl chloride) to label the phosphate groups in adducted nucleotides in order to detect the DNA damage by fluorescence. We tried to dansylate the hydroxyl groups in the deoxyribose moieties under non-aqueous conditions (pyridine) analogous to the binding of p-toluenesulphonyl chloride to carbohydrates. A major problem of this approach was the poor solubility of the nucleotides. Another approach was labelling with malondialdehyde, a method first described by Moschel and Leonard (24). Recently, the group of Shuker (25) reported the labelling and detection of various alkyl-guanines by use of phenylmalondialdehyde. In our hands, it appeared to be possible also to label nucleosides in this way, but the method was not sensitive enough to determine low adduct levels as present in biological samples.

In this paper we describe improvements and comparison with conventional methods (ELISA and AAS) of the ^{32}P-postlabelling assay for the platinated dinucleotides ApG and GpG, as described previously by Blommaert and Saris (1). The most important ameliorations are the addition of an internal standard, a more efficient isolation of the Pt-adducts and a simplification of the deplatination step. Furthermore, for the quantification of the ^{32}P-labelled products two different methods have been elaborated (see Scheme I for the outline of the whole postlabelling procedure). As the first step in this assay, (isolated) platinated DNA was digested to unmodified deoxynucleosides and platinated adducts. For the dephosphorylation by the enzyme alkaline phosphatase a pH of about 9 is required, which is obtained by addition of only 1/100 volume of 1 M Tris/HCl (pH 9.0) to the digestion mixture instead of the 1/10 volume as reported before (1). This reduced volume is very important in view of the need to decrease the pH to about 3 in order to purify the Pt-adducts on the basis of their positive charge on a cation-exchanger. The higher pH of the DNA digests during this separation step may have been the reason for the

rather low recovery of the adducts by these authors (1). The two methods of cation-exchange chromatography presented in this paper, chromatography using the Mono-S and solid-phase extraction on SCX cartridges, are equally effective. An advantage of the Mono-S column is that the amount of DNA applied on the column can be determined directly by comparing the absorbance of the unmodified nucleosides eluted from the column with that of known amounts of digested salmon sperm DNA. During the elution from the cation-exchanger, the Pt-adducts were collected in tubes already containing the dinucleotide TpT as an internal standard and NaCN. The high pH of the resulting solution (about 10) prevented digestion of the internal standard by nucleases co-eluted with the Pt-adducts. Another dinucleotide which is not supposed to be present in a platinated DNA digests and therefore a possible candidate for an internal standard is CpC. However, the ^{32}pCpC product could not be separated sufficiently from the solvent front during TLC chromatography. Optimal conditions for postlabelling were obtained when the pH of the samples after deplatination was lowered to about 8.2 by addition of NaAc (pH 5.4). Using the approach of small reaction volumes and adjustment of the pH, it appeared not to be necessary to remove the cyanide, which was still prescribed by Blommaert and Saris (1). After the labelling reaction the products were separated by use of either TLC or HPLC (see Figures 1 and 2). With both methods a good separation of ^{32}pGpG, ^{32}pApG and ^{32}pTpT was found, allowing their quantification by phosphorimaging or ^{32}P-counting. Linearity of the labelling was studied by use of reference dinucleotides ApG and GpG. The results in Figure 3 show a good correlation between the product levels after postlabelling and the starting amounts of the dinucleotides as measured by weight (input). The linearity of the method could be confirmed by analysis of DNA samples isolated from human IGROV-1 ovarian cancer cells treated with various concentrations of cisplatin (Figure 6).

The results of the postlabelling assay were compared with those of using ELISA and with data from total DNA platination levels measured with AAS. Analyses of salmon sperm DNA and of DNA isolated from (tumor) tissues, both *in vitro* treated with cisplatin, as well as of (tumor) tissues treated *in vivo* showed good and significant correlations between postlabelling (with TLC separation) and ELISA data (Figures 4 and 5) for the two adducts Pt-AG and Pt-GG. Although in the same order, the actual quantities of the adducts were different, especially for the isolated DNA samples (Figure 5). These differences, however, are not considered to be significant because in Figure 4 the ELISA data are highest and in Figure 5 the postlabelling data. At least part of the discrepancies can be explained by the variation of the ELISA results. A very good and highly significant correlation was found between the levels of the two adducts in the cellular DNA samples as determined by postlabelling (with HPLC separation) and the total platination levels of the original DNA samples measured with AAS (see Figure 6). The total amount of the two adducts measured

by the postlabelling assay, as shown in Figure 6 (dashed line), was found to be approximately 85% of the overall platination level of the DNA samples, which is in agreement with previous findings (2,26). The same holds, with a few exceptions, for the observed ratio of 3-4 between the Pt-GG and Pt-AG adducts in the cisplatin-treated samples (12,26). Combined with the TLC or HPLC separation technique, this postlabelling assay allows a very sensitive determination of adduct levels, *viz* as low as 1.6 adducts/10^7 nucleotides in a 10 µg DNA sample. When larger amounts of DNA are analyzed, even lower adduct levels can be detected. We demonstrated the presence of three adducts per 10^8 nucleotides, which indicates that the sensitivity of the assay is sufficient for measurements in clinical samples. Because of its high sensitivity, the postlabelling method described in this paper will be very suitable for determining the adduct levels in fine needle aspirates, which often contain not more than 10 µg of DNA. Also adduct levels in DNA isolated from buccal cells have already been determined with this assay. Despite the rather small number of cells that is normally obtained (about 3 x 10^5), the presence of Pt-GG could be demonstrated in the buccal mucosal cells of a patient after treatment with 70 mg cisplatin/m^2 (data not shown). In further studies we will give more attention to this application of the ^{32}P-postlabelling assay for Pt-DNA adducts.

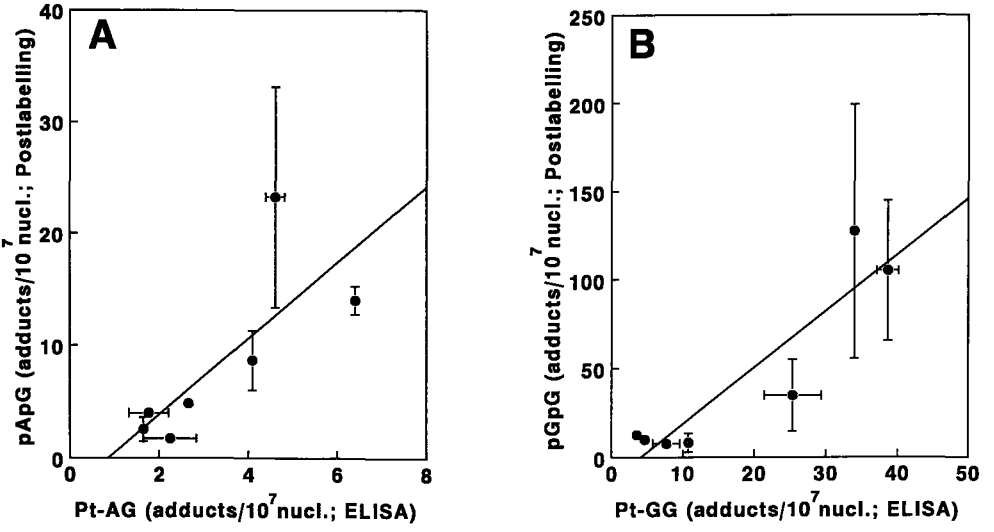


Figure 5. Correlation between Pt-AG (Panel A) and Pt-GG (Panel B) adduct levels measured with the ELISA method and with the ^{32}P-postlabelling assay (TLC separation) in DNA samples isolated from kidney, liver and xenografts treated with cisplatin either *in vivo* or *ex vivo*. Adduct levels (adducts/10^7 nucleotides) for both assays are means ± SD of data obtained from two independent experiments. The r value was 0.77 (p=0.045) for Pt-AG and 0.91 (p=0.042) for Pt-GG.

In conclusion, with the modified ^{32}P-postlabelling assay described here, the levels of the major Pt-DNA adducts (Pt-AG and Pt-GG) can be measured quantitatively in small quantities of DNA from clinical samples such as white blood cells, (tumor) tissues and buccal mucosal cells. From these results it can be expected that this assay is also applicable for fine needle aspirates. In addition to the determination of cisplatin- or carboplatin-adducts, of which the bifunctional DNA adducts are chemically identical, also adducts on two neighbouring nucleobases formed by other Pt-drugs can be quantified with this assay without further modification. The only prerequisites are that positively charged adducts can be obtained after digestion of the DNA for purification on a cation-exchanger and that the Pt can be removed upon incubation with NaCN.

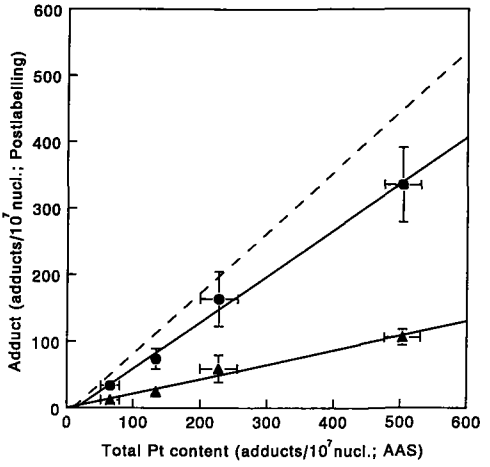


Figure 6. Correlation between AAS and ^{32}P-postlabelling data of DNA extracts from IGROV-1 ovarian cancer cells treated for 2 hr with various concentrations of cisplatin (0 to 66.7 μM). Part of the DNA was used for determination of the total Pt-content by AAS, whereas the adducts Pt-AG and Pt-GG in the other part were deplatinated, ^{32}P-postlabelled and quantified with the HPLC-based method. Circles, Pt-GG adducts; triangles, Pt-AG adducts; dashed line, total of Pt-GG and Pt-AG adducts. The r value was 0.99 (p=0.012) for Pt-AG and 1.0 (p=0.003) for Pt-GG. Data (in adducts/10^7 nucleotides) are means ± SD of results from at least three independent experiments.

Acknowledgements

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Abbreviations

AAS, atomic absorption spectroscopy; cisplatin, *cis*-diamminedichloroplatinum(II); ApG, 2'deoxyadenylyl (3'→5')-2'-deoxyguanosine; CpC, 2'deoxycytidylyl (3'→5')-2'-deoxycytidine; GpG, 2'deoxyguanylyl (3'→5')-2'-deoxyguanosine; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; G-Pt-G, *cis*-$Pt(NH_3)_2(dG)_2$; HPLC, high performance liquid chromatography; N, nucleosides; NaAc, sodium acetate; NaCN, sodium cyanide; P_i, inorganic ^{32}P-phosphate; PNK, T4-polynucleotide kinase; Pt, Platinum; Pt-AG, *cis*-$Pt(NH_3)_2d(ApG)$; Pt-GG, *cis*-$Pt(NH_3)_2d(GpG)$; SCX, strong cation-exchange; TLC, thin layer chromatography; TpT, thymidylyl (3'→5')thymidine.

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Chapter 6

Pharmacodynamics of cisplatin in human head and neck cancer: correlation between platinum content, DNA-adduct levels and drug sensitivity *in vitro* and *in vivo*.

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Brit. J. Cancer, in press

Abstract

Total platinum (Pt) contents and cisplatin-DNA adduct levels were determined *in vivo* in xenografted tumor tissues in mice and *in vitro* in cultured tumor cells of head and neck squamous cell carcinoma (HNSCC), and correlated with sensitivity to cisplatin. *In vivo*, a panel of five HNSCC tumor lines growing as xenografts in nude mice was used. *In vitro*, the panel consisted of five HNSCC cell lines, of which four had an *in vivo* equivalent. Sensitivity to cisplatin varied 3- to 7-fold among the cell lines and tumors, respectively. However, the ranking of the sensitivities of the tumor lines (*in vivo*), also after reinjection of the cultured tumor cells, did not coincide with that of the corresponding cell lines, which showed that cell culture systems are not representative for the *in vivo* situation. Both *in vitro* and *in vivo*, however, significant correlations were found between total Pt levels, measured by atomic absorption spectrophotometry (AAS), and tumor response to cisplatin therapy at all time points tested. The levels of the two major cisplatin-DNA adduct types were determined by a recently developed and improved ^{32}P-postlabelling assay at various time points after cisplatin treatment. Evidence is presented that the Pt-AG adduct, in which Pt is bound to guanine and an adjacent adenine, may be the cytotoxic lesion, since a significant correlation was found between the Pt-AG levels and the sensitivities in our panel of HNSCC, *in vitro* as well as *in vivo*. This correlation with the Pt-AG levels was established at 1 h (*in vitro*) and 3 h (*in vivo*) after the start of the cisplatin treatment, which emphasizes the importance of early sampling.

Introduction

Cisplatin is widely used for the treatment of various solid tumors. However, the response to this drug varies between tumor types, but also among tumors of a certain type. In is generally believed that the antitumor effect of cisplatin is due to direct binding of the drug to DNA, resulting in platinum-DNA (Pt-DNA) mono-adducts and various bifunctional adducts (1, 2). The main adduct formed is *cis*-$Pt(NH_3)_2d(GpG)$ (abbreviated Pt-GG), with Pt bound to two adjacent guanines. Another major intrastrand crosslink is *cis*-$Pt(NH_3)_2d(ApG)$ (Pt-AG), in which Pt is bound to guanine and an adjacent adenine. The other bifunctional adducts are *cis*-$Pt(NH_3)_2(dG)_2$ (G-Pt-G), the intrastrand adducts in which Pt is bound to two guanines separated by one or more other nucleotide(s), and the interstrand crosslinks between two guanines in opposite strands. These adducts can inhibit cell proliferation and, if not repaired or tolerated, finally lead to cell death.

In patients with the same type of tumor, a fairly large interindividual variation in DNA-adduct levels, measured in white blood cells (WBC), has been observed (3-8). These clinical investigations provided evidence that higher adduct levels correlate with a better clinical outcome. In contrast to these findings, there are also reports showing no correlation between adduct levels in WBC and the efficacy of therapy (9-11). All these clinical studies, however, were performed in patients with a variety of solid tumors and after different treatment regimens, which could be a reason for the inconsistency of the results. Furthermore, blood samples were collected at different time points, although in most studies this was done at 24 h after cisplatin administration. In addition, the methods used for adduct detection and statistical analysis (using either the highest (peak) level or the mean value over several courses) were different between the various studies.

The basic question in all these investigations, however, is whether or not Pt-DNA adduct levels in the tumors correlate with the response to the cisplatin treatment. Because it is not feasible to obtain tumor biopsies from patients for such studies, the readily obtainable WBC were used in most investigations as a substitute for the target tissue. In the present study, both the total Pt content and the Pt-GG and Pt-AG adduct levels were determined in human head and neck squamous cell carcinomas (HNSCC) grown in nude mice and as cultured cells, at various time points after the start of cisplatin treatment. Responses of the tumors to this treatment were correlated with both total Pt contents and these Pt-DNA adduct levels. Because the pharmacokinetics of cisplatin are similar in nude mice and humans (12), this study will provide more insight into factors involved in the clinical response of patients to cisplatin.

Material and Methods

Tumor lines

Human head and neck squamous cell carcinomas (HNSCC) were used in two model systems.

In vitro: Cell lines UM-SCC-14C (abbreviated as 14C), UM-SCC-22B (22B) and UM-SCC-35 (35) were established directly from the tumor biopsy of the patient (13). VU-SCC-OE (OE) and VU-SCC-RO (RO) were established from xenografts that were initiated originally by transplanting tumor fragments from patients into mice (14). Cells were cultured routinely in Dulbecco's modified Eagle's medium (Life Technologies, Inc. [Gibco BRL], Breda, The Netherlands) supplemented with 5% heat-inactivated fetal calf serum (Flow, Irvine, UK), 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies).

In vivo: Xenografts were established on female, athymic, nude-nu mice (6-8 weeks old; Harlan, Zeist, The Netherlands) either by subcutaneous injection of cultured tumor cells (14C and 22B) in suspension at both lateral sides, or by implantation of tissue fragments of the tumor from the patient (OE, RO and HN). Upon growth, the tumor lines were maintained by serial transplantation (15). Tumor volumes (mm^3) were determined twice weekly by use of a vernier calliper and calculated according to the formula: 0.5 x length x width x height. This way of measuring tumor volumes has proven to be most accurate (16). Animal experiments were performed according to the Dutch law on Animal Experiments and after approval of the appropriate ethical committees at our Institutes.

DNA index

Cultured HNSCC cells were trypsinized and harvested. Tumor tissues were dissociated by use of trypsin for 30 min at 37°C, followed by filtration through 100-µm nylon filters in order to obtain single cells. The DNA of these cells was labelled with the fluorescent DNA-intercalator propidium iodide as described earlier (14) and the DNA index was determined by flow cytometry.

Sensitivity to cisplatin

Tumor cells in culture and tumor-bearing mice were treated with cisplatin (Platinol, Bristol-Myers Squibb, Weesp, The Netherlands) in order to determine the growth response and to obtain samples for the analysis of total Pt and Pt-DNA adduct levels.

In vitro: the cisplatin-induced inhibition of cell proliferation, as a measure of the sensitivity to this drug, has been determined and published previously (14). The HNSCC cells were exposed to various concentrations of cisplatin for 72 h at 37°C and the inhibition of cell growth was determined colorimetrically with the sulforhodamin B assay (SRB). The cisplatin sensitivity was expressed as IC_{50} value, *i.e.* the concentration of the drug causing 50% growth inhibition compared to untreated control cells.

In vivo: mice bearing HNSCC tumors with a volume between 50 and 200 mm^3 were randomly divided into treatment and control groups, with 6-10 tumors per group. Cisplatin (7 mg/kg) was given intravenously, twice with a 7-day interval. The drug was administered at the maximal tolerated dose, *i.e. the dose leading to a weight loss of no more than 5-15%.* Antitumor activity was expressed as Growth Delay Factor (GDF), defined as the difference between the mean values of the time required by tumors of treated and control animals to double their volume, divided by the mean value of the tumor doubling time in control mice. A second method to quantify the therapeutic results of the cisplatin treatment was by determination of the treated/control value (T/C), *i.e.* the mean tumor volumes after treatment divided by the tumor volumes at the start of the treatment. The lowest value of this ratio at any time point during the observation period was considered as optimal T/C value. Complete responses were scored when a tumor regressed and did not show regrowth for a period of three months.

Intracellular cisplatin

The levels of total Pt were determined both in cultured cells and in xenografts of cisplatin-treated mice.

In vitro: cells were treated continuously during 1, 6, 24, 48 or 72 h with 10 μM cisplatin, whereafter the cells were washed and harvested. Part of the samples were processed to determine the cellular Pt content by atomic absorption spectroscopy (AAS) (Spectra AA-300 Zeeman AAS, Varian, Houten, The Netherlands), as described earlier (14).

In vivo: tumor-bearing mice were treated twice with cisplatin as indicated above and sacrificed at various time intervals after the second injection (3 h, 24 h, 3, 7 and 14 days). The tumors were removed, quickly frozen in liquid nitrogen, and stored at -80°C. One half of each tumor was prepared for measurement of total Pt levels by AAS according to the method of Siddik *et al.* (17). The tumor halves were weighed, lysed by addition of 0.5 ml 1 M hyamine (benzethonium hydroxide, Sigma, St.Louis, MO), and incubated overnight at 55°C. Pt was measured after addition of 4.25 ml 0.2 M HCl. Calibration standards and Quality Control samples of muscle tissue were spiked with cisplatin and treated in the same way as the samples (18).

Pt-DNA adducts

Portions of the cisplatin-treated cells and of the tumors obtained from treated mice were used for the analysis of the two DNA-adducts Pt-GG and Pt-AG. Cell pellets and tumor tissues were lysed and DNA was isolated as described by Fichtinger-Schepman *et al.* (19, 20) and stored at -20°C until further analysis. Following enzymatic digestion of the DNA, the adduct levels were determined by a novel, recently improved ^{32}P-postlabelling assay after deplatination of the purified adducts (21). The ^{32}P-labelled products were separated by thin layer chromatography and the radioactivity determined by phosphorimaging. The amounts of the two major intrastrand DNA-adducts Pt-GG and Pt-AG were calculated on the basis of the labelling of a known amount of TpT, which was included as an internal standard.

Statistical analysis

Correlations between the Pt content and DNA-adduct levels with sensitivity to cisplatin were analyzed by linear regression analysis. Only correlations with p-values of 0.05 or lower were considered to be significant. For the analysis also the area-under-the-curve (AUC) was utilized. AUC values (derived from plots of Pt content *vs* time or Pt-DNA adduct levels *vs* time) were calculated with the trapezoidal rule.

Results

In this study the sensitivity to cisplatin in a panel of HNSCC xenografts (*in vivo*) was compared to that of the corresponding cultured cells (*in vitro*) and correlated with cisplatin accumulation and DNA adduct formation. As can be seen in Table I, the cells growing as xenograft or in culture are comparable with respect to their DNA indices, with the exception of cell line RO. After several passages of RO, large cells containing more than one nucleus were observed, due to the inability of the cells to complete their cell division. It was probably for this reason that RO cells could only be kept in culture for about 12 passages. Consequently, sufficient amounts of these cells to determine the cellular Pt and the Pt-DNA adduct levels could not be obtained. Unfortunately, we were not successful in establishing a cell line from the HN xenograft and no xenografts could be established from the UM-SCC-35 cell line. The DNA content of both the tumors and the cell lines was found to be rather consistent over time and during various passages.

Sensitivity to cisplatin

The response of the tumors in the xenografted mice following cisplatin treatment are given in Table II, as well as data on the tumor doubling times in the treated group and untreated controls. The 14C and HN xenografts were not sensitive. The best response, even some complete responses were observed with RO. The intrinsic sensitivities to cisplatin of the cultured HNSCC cells were reported previously (14). They are included in Table II to allow comparison with the *in vivo* data. IC_{50} values ranged between 0.9 and 2.7 μM cisplatin.

Comparison of the ranking of the cisplatin sensitivities of the xenografts with those of the corresponding cultured cells revealed that both RO and OE differed in sensitivity when xenografted or cultured. These two tumor lines were established directly from the tumor biopsies obtained from the patient and the cell cultures were derived subsequently from the xenografts. On the other hand, the 14C and 22B tumor lines were established after injection of the cultured cells into mice. In order to determine whether the difference between *in vitro* and *in vivo* responses of the HNSCC lines was due to the difference in the way the tumor lines were established, the cultured RO and OE cells were reinjected subcutaneously into mice and, after growth to tumors, the mice were treated with cisplatin. The responses to cisplatin of these newly established tumors were comparable to those of the original xenografts (data not shown).

Table I. DNA index of HNSCC xenografts and the corresponding cultured cells.

tumor line	degree of differentiation[a]	DNA index[b]	
		xenograft	cell line
14C	poorly	3.5 ± 0.1	3.1 ± 0.2
22B	moderately	2.3 ± 0.1	2.2 ± 0.5
35	n.a.[c]	n.a.	3.0 ± 0.1
OE	moderately	3.3 ± 0.5	3.5 ± 0.3
RO	well	1.8 ± 0.2	3.7 ± 0.2
HN	poorly/moderately	3.1 ± 0.4	n.a.

[a]The xenografts were scored pathologically as described by Van Dongen *et al.* (22)
[b]DNA index was determined by flow cytometry of the tumor cells derived from xenografts or from cell cultures. The DNA content appeared to be stable during several passages. The results are mean ± SD of at least six independent measurements
[c]n.a., not available

Table II. Responses to cisplatin treatment in HNSCC xenografts and in cultured cells.

tumor line	IC_{50} value[a]	tumor doubling time (days)[b]		tumor response[b]	
		control	treated	GDF	T/C
14C	2.7 ± 0.7	9.7 ± 1.6	11.6 ± 4.5	0.3 (-)	69 (-)
22B	1.2 ± 0.3	10.2 ± 2.1	21.3 ± 6.0	1.1 (+)	34 (+)
35	0.9 ± 0.8	n.a.	n.a.	n.a.	n.a.
OE	2.3 ± 0.9	6.7 ± 1.8	12.9 ± 1.5	1.0 (+)	34 (+)
RO	2.4 ± 0.8	7.7 ± 2.0	22.8 ± 11.7	2.0 (++)	19 (++)
HN	n.a.[c]	6.1 ± 2.4	11.3 ± 5.4	0.9 (-)	56 (-)

[a]IC_{50} value (in μM), the concentration of the drug causing 50% of growth inhibition, determined in a colorimetric (SRB) assay after exposure of the cells to cisplatin for 72 h (14)
[b]Mice bearing human head and neck tumors were treated i.v. with 7 mg cisplatin/kg twice with a 7-day interval. Responses to cisplatin therapy were determined in at least 4 independent experiments and expressed as GDF and T/C. GDF < 1 or T/C > 50 corresponds to a minimal effect (-); 1 < GDF < 2 or 25 < T/C < 50 to a moderate effect (+) and GDF > 2 or T/C < 25 to a strong effect with even some complete responses (++)
[c]n.a., not available

Intracellular cisplatin

As described previously for an *in vitro* study on a panel of 7 HNSCC cell lines, most of which were also used in the present work, the cellular Pt levels correlated significantly with the IC_{50} values, after incubation of the cells with 10 μM cisplatin during 1, 6, 24 or 48 h (14). For these 7 cell lines, positive correlations were found between sensitivity and the AUC values of the Pt *vs* time curves calculated from 1 h up to each time point studied during the total cisplatin incubation period of 48 h. The r values were 0.83 (p=0.017) when AUC was calculated over 6 h, 0.78 (p=0.031) when calculated over 24 h, and 0.78 (p=0.032) over the 48-h time period. These correlations were found when data of cell line OE, derived from a patient previously treated with radiotherapy, were omitted (14). In Figure 1 the cellular Pt levels as a function of the exposure time are given only for those cell lines for which the DNA-adduct levels were also determined (see below).

To investigate whether the previously found correlations *in vitro* also exists *in vivo*, Pt levels were determined in the tumors at various time points after the second cisplatin treatment of the tumor-bearing mice. As shown in Figure 2, 14C contained the lowest and RO the highest Pt levels. Comparison of the Pt levels with tumor response (expressed either as GDF or T/C; see Table II) revealed no significant correlations at any time point measured. However, the AUC values of the Pt *vs* time curve determined over the whole period of 14 days tested, showed a positive

correlation with the GDF (r=0.95, p=0.008). When the period between 3 h and 3 days was taken as time interval for the AUC calculation, this correlation was even more significant (r=0.98, p=0.0007). Similarly, this AUC significantly correlated with the T/C values, showing an r value of 0.86 (p=0.045) when the AUC was calculated over 14 days and of 0.89 (p=0.028) when calculated over the 3-day time period. Apparently, the amount of Pt present during the first time period after administration of cisplatin is very important for the efficacy of the treatment.

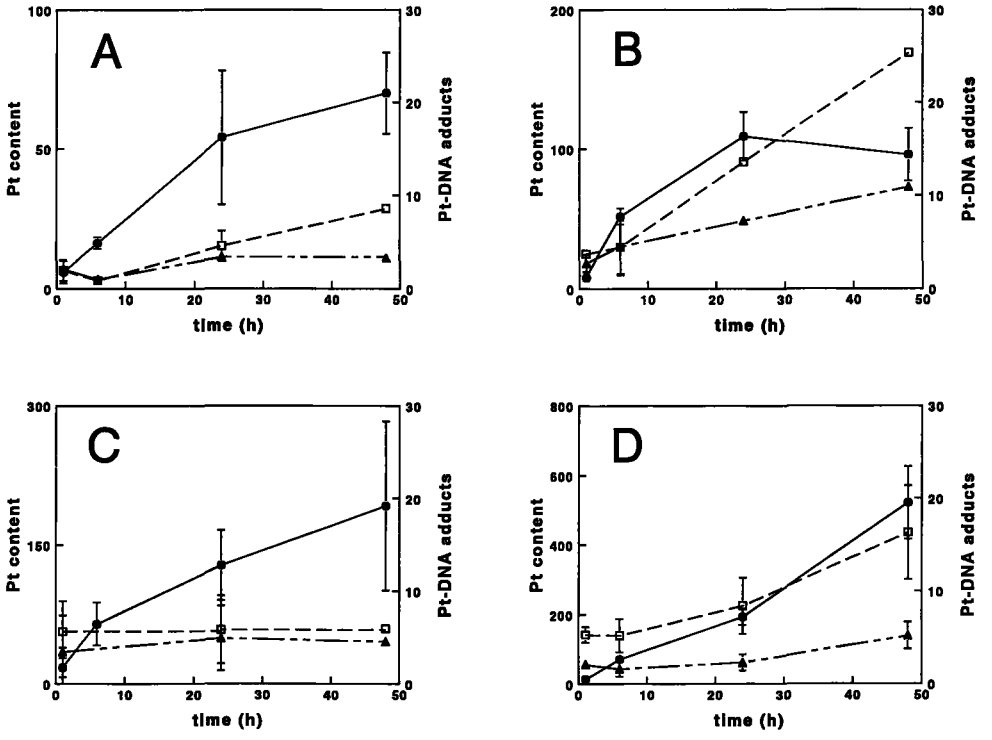

Figure 1. Cultured human HNSCC cells 14C (A), 22B (B), 35 (C) and OE (D) were treated continuously with 10 µM cisplatin for the time period indicated. Total Pt content (pmol/10^6 cells; left y-axis) was measured by AAS (●; solid line). The two major intrastrand Pt-DNA adducts (number of adducts/10^6 nucleotides; right y-axis) were measured by the ^{32}P-postlabelling assay (21); Pt-GG (□; dashed line) and Pt-AG (▲; dashed line). Results are given of two independent experiments as mean ± range of values.

Pt-DNA adducts

The kinetics of cisplatin-DNA adduct formation and removal were studied in the cultured HNSCC cells as well as in the xenografts. The levels of the major adducts (Pt-GG and Pt-AG) were determined by ^{32}P-postlabelling of digested DNA samples after deplatination of the adducts (21). The *in vitro* results are depicted in Figure 1 and show that in 3 of the 4 HNSCC cell lines the adduct levels increased with time during the continuous exposure to cisplatin. No significant correlation could be found between the IC_{50} values and either the adduct levels at the various time points, or the AUC of Pt-DNA adduct levels calculated for the various time periods. The only exception, however, was the correlation with the Pt-AG adduct levels at 1 h after the start of cisplatin exposure (r=-0,92, p=0.045).

In vivo, the Pt-GG and Pt-AG adduct levels were determined in portions of the same tumors that were used to obtain the total Pt content (Figure 2). It was found that the highest levels were reached at 3 to 24 h after the second injection with cisplatin, whereafter they decreased with time. The AUC calculated between 3 h and 14 days showed no correlation with the tumor response to cisplatin (expressed as GDF or T/C value). However, when the AUC was restricted to the period between 3 h and 3 days for both adducts, a relationship could be established. For Pt-GG the correlation with GDF was not significant (r=0.82, p=0.067), but it was for the Pt-AG adduct levels (r=0.86, p=0.042). However, such a correlation was not found when adduct data were plotted against the T/C values. When the tumor responses were compared with the two adduct levels at the various time points, a significant correlation was found only between GDF and the Pt-AG adducts at 3 h after cisplatin therapy (r=0.88, p=0.034). The Pt-AG data at 24 h did not correlate (r=0.86, p=0.096). The results suggest that sensitivity to cisplatin in HNSCC is determined by the amount of Pt-AG adduct that is present in the cell during a short time interval following cisplatin treatment.

Discussion

The relationship of responses to cisplatin treatment with total Pt content and cisplatin-DNA adduct levels was investigated in a panel of human HNSCC cultured cells and xenografts. A different ranking was observed when the responses of HNSCC to cisplatin were determined *in vitro* or *in vivo*, in particular with lines OE and RO. These discrepancies could not be ascribed to genetic changes as a result of *in vitro* culturing, because similar data were obtained when cell lines OE and RO were reinjected into mice. Also the experiments measuring the DNA index did not provide evidence for such changes in the genetic material. The finding that tumors with the original drug-response properties were obtained after injection of cultured cells into

mice was also published by Teicher *et al.* (23). This phenomenon clearly indicates that results obtained with cultured cells are not representative for the *in vivo* situation. *In vivo* apparently more important factors are involved, for example vascularisation. This difference between the *in vitro* and *in vivo* results may be eliminated when the cells are grown in a three-dimensional structure, *e.g.*, as multicellular spheroids as shown for tumor cells by Kobayashi *et al.* (24) or as postconfluent multilayered cultures as shown by Pizao *et al.* (25).

In a comparative study with human ovarian carcinoma cells, Kelland *et al.* (26) investigated the *in vitro* and *in vivo* antitumor activity of cisplatin and three other platinum agents in eight companion lines. In agreement with our results, these authors do not find a correlation between *in vitro* SRB values and *in vivo* GDF data. However, they report a significant correlation - for cisplatin only - between SRB results and tumor T/C values at 28 days, a time point which seems rather arbitrarily chosen. In our study, the lowest T/C ratio during the entire observation period was taken as optimal T/C value. Due to the rapid growth rate of the HNSCC xenografts, the 28-days time point could not be reached. It would be interesting to investigate, *e.g.*, Pt content and Pt-DNA adduct levels in the panel of ovarian carcinoma lines described by Kelland *et al.* (26), which span a considerably wider range of drug sensitivities than our HNSCC lines.

Significant correlations were found between the tumor responses to cisplatin treatment in mice and the AUC of total Pt levels present in the xenografts. Such a significant correlation was also found for the corresponding cultured cells (14). Although the ranking of the sensitivities to cisplatin of the HNSCC tumor lines and of the corresponding cell lines did not correlate, the total Pt levels in both cases did correlate with the sensitivity. Johnsson and Cavallin-Ståhl (27) showed that the Pt distribution within squamous cell carcinoma tumors was fairly homogeneous, thus cisplatin was capable to penetrate into the whole tumor. From this observation and our results, it can be concluded that the tumor (cell) response is reflected by the total Pt levels in the tumor cells.

In vitro, a relationship was established between cisplatin sensitivity of the cultured cells and the level of the major intrastrand crosslink, Pt-AG. This correlation was only found at 1 h after the start of the treatment, suggesting that the level of DNA damage immediately after cisplatin incubation is very important for the efficacy of the treatment. However, in our panel of 4 HNSCC lines no significant correlation was observed with the main adduct Pt-GG. Also in the *in vivo* study a significant correlation was found between the sensitivity of the tumor and the Pt-AG adducts. For this correlation the AUC over the 3 h to 3 days interval following the second cisplatin treatment of the mice was used, a time period during which the adduct levels reached a maximum. After 3 days these levels declined with time. This finding was consistent with results described by Poirier and coworkers (28), who reported an

increase of adduct levels in various rat tissues between 4 h and 2 days after single and multiple i.v. injection(s) of cisplatin, followed by a decrease between days 2 and 7 and stable levels until 14 days. The AUC reflects the net result of DNA-adduct formation and DNA repair. The importance of applying this AUC, derived from the Pt-DNA adduct level *vs* time plot, has been emphasized by Schellens *et al.* (8), who showed a correlation with the tumor response of this AUC in WBC of patients. This parameter, calculated over a period of 15 h following a 3-h cisplatin infusion, appeared to have a predictive value for the tumor response of patients receiving daily treatments. The fact that in the present study significant correlations were found between the Pt-AG but not the Pt-GG adduct levels and the HNSCC response, suggests that Pt-AG plays an important role in the antitumor effect in the HNSCC lines studied. This finding is in agreement with the results of Fichtinger-Schepman *et al.* (29). These authors determined the quantities of the various Pt-DNA adducts after treatment of Chinese hamster ovary cells with equitoxic doses of cisplatin and carboplatin, and provided evidence that Pt-AG may be the cytotoxic lesion. The Pt-AG adduct has also been found to be more mutagenic than Pt-GG (30, 31) and it is more effectively repaired in a cisplatin-resistant human testicular teratoma cell line (32). Los and coworkers (33) reported that the ratio of Pt-DNA adducts to total Pt levels may be informative for the response of the tumor to cisplatin therapy. They suggested that a low ratio was indicative of high cellular detoxification due to interaction with glutathione and metallothioneins. Calculation of these ratios for our panel of tumor lines revealed that RO had extremely high ratios at 3 h and 24 h after the cisplatin treatment (data not shown). This suggests that in this xenograft a relatively large amount of the cisplatin is able to bind to cellular DNA, resulting in a high level of Pt-DNA adducts, which may explain the high cisplatin sensitivity of this tumor line. In the other tumor lines, the ratio of Pt-GG adducts to the total Pt content was about 2- to 10-fold lower and in case of Pt-AG adducts 7- to 17-fold (*cf* Figure 2). Thus by calculating the ratio of Pt-AG adduct to the Pt content, the *in vivo* sensitivity to cisplatin may be predicted. However, it is very important to sample at early time points after the start of therapy. This predictive value should be tested in more extensive studies. Because the pharmacokinetics of cisplatin have been reported to be very similar in nude mice and humans (12), this ratio might also be very important for the prediction of tumor response in patients. In the present study, the *in vitro* sensitivity to cisplatin of HNSCC cells, however, was found not to be related to this ratio of Pt-DNA adducts to Pt content. This again is a discrepancy between the *in vivo* and *in vitro* situation, which suggests that cell culture systems are not reliable in predicting *in vivo* outcomes.

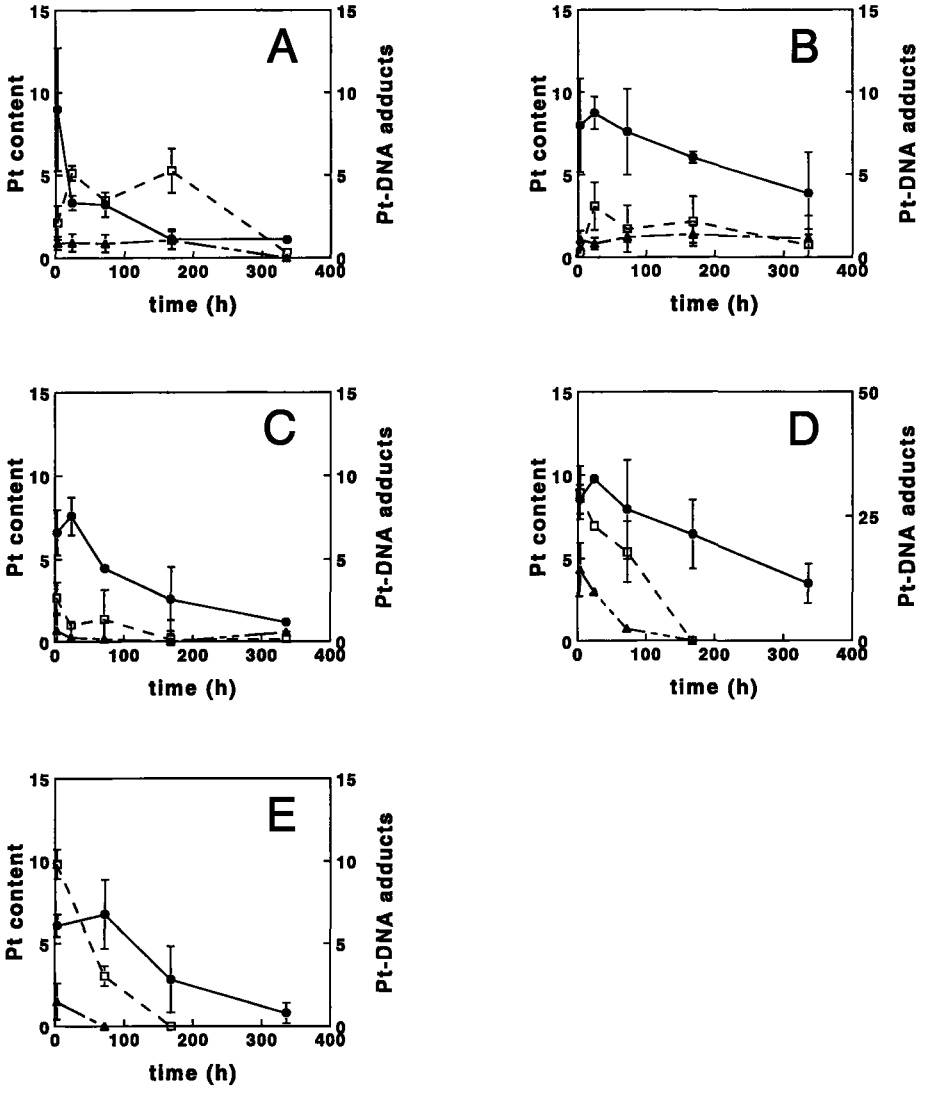

Figure 2. Mice bearing the human HNSCC tumor 14C (A), 22B (B), OE (C), RO (D) or HN (E) were treated i.v. with 7 mg cisplatin/kg, twice with a 7-day interval. Tumors were removed at various time points after the second administration of the drug (3 h, 24 h, 3, 7 and 14 days). Total Pt levels (fmol Pt/µg tissue; left y-axis) in these tumors were determined by AAS (●; solid line) and the Pt-DNA adducts (number of adducts/10^6 nucleotides; right y-axis) were measured by the ^{32}P-postlabelling assay (21); Pt-GG (□; dashed line) and Pt-AG (▲; dashed line). Results are given of three independent experiments as mean ± SD.

In conclusion, the different sensitivities to cisplatin in our panel of HNSCC lines were significantly correlated with the total Pt levels as well as with the Pt-AG adduct levels both in cultured cells (*in vitro*) and in the xenografts (*in vivo*). These data support previous evidence that Pt-AG may be the cytotoxic lesion. However, results obtained for the cultured cells are not predictive for the response to cisplatin of HNSCC tumors in nude mice.

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Chapter 7

The potential of platinum-DNA adduct determination in *ex vivo* treated human head and neck squamous cell carcinoma for the prediction of sensitivity to cisplatin chemotherapy.

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Submitted

Abstract

Background: To increase the efficacy of chemotherapy it is important to select those patients who will benefit from the treatment. Aim of this study was to determine whether the measurement of platinum (Pt)-DNA adduct levels in tumor tissue exposed *ex vivo* to cisplatin can predict the clinical response to cisplatin therapy.

Materials and Methods: First, we determined Pt-GG and Pt-AG adduct levels by use of ^{32}P-postlabelling after *ex vivo* cisplatin treatment of fragments of isolated head and neck squamous cell carcinoma (HNSCC) xenografts in nude mice. In addition, adduct levels were measured in tumor biopsies from patients with HNSCC (N=8) and testicular cancer (N=8), tumor types known to differ in their response to cisplatin treatment.

Results: It was possible to reproducibly measure DNA adduct levels in tumor fragments. After 1 h exposures from five HNSCC tumor lines to cisplatin (10 to 80 µM) the Pt-GG and Pt-AG adduct levels showed a positive correlation with the *in vivo* response to cisplatin treatment ($p< 0.05$). These levels were also correlated with the already published AUC values of the adducts after cisplatin treatment of the tumor-bearing mice ($r=0.92$; $p=0.027$ for Pt-GG and $r=0.97$; $p=0.007$ for Pt-AG). After an additional 5-h drug-free incubation period the correlation between *ex vivo* adduct levels and response was no longer significant. When exposed *ex vivo* to 80 µM cisplatin for 1 h, Pt-GG and Pt-AG adduct levels were similar in HNSCC and testicular cancer. Hardly any decrease in adduct levels was found in the testicular tumor fragments during the drug-free incubation period, which confirms the previously found lack of DNA repair in this tumor type. The adduct levels in the samples of two HNSCC patients who received cisplatin chemotherapy were in line with the hypothesis that higher adduct levels are associated with a better response.

Conclusion: Our preliminary results show that analysis of DNA adducts following *ex vivo* drug treatment is a feasible approach towards a predictive assay, which warrants further investigation.

Introduction

Cisplatin is an antitumor agent that is applied in the therapy of solid tumors, including testicular, ovarian and bladder cancer and head and neck squamous cell carcinoma (HNSCC). However, the response rates vary between tumor types. For HNSCC the complete and partial response rate is between 30 and 50%, without improvement of the long-term survival (1). For testicular cancer the application of cisplatin-containing regimens has resulted in a disease-free survival of over 90% of patients (2, 3). Because chemotherapy with cisplatin is accompanied by severe side effects (4) and because the tumor itself can be or can become resistant to cisplatin therapy, it is of interest to be able to determine the efficacy of the treatment already before the patient actually receives the drug. Unnecessary treatment could then be avoided and the dose and/or combination of the drug(s) adjusted. Such individualized drug administration could lead to improvement of response rates (5) and a better quality of life.

It is generally accepted that the antitumor activity of cisplatin results from its interaction with DNA, which leads to the formation of the so-called platinum (Pt)-DNA adducts (6, 7). A limited number of different types of adducts are formed in DNA upon reaction with cisplatin: the monoadduct Pt-G and the diadducts Pt-GG (crosslink on pGpG sequences), Pt-AG (crosslink on pApG) and G-Pt-G (crosslink on $pG[pX]_npG$ as well as interstrand crosslinks on two guanines) (7). In the white blood cells (WBC) of patients with the same tumor type treated with cisplatin, large interindividual differences in DNA-adduct levels have been observed (5, 8-12). These studies provided evidence, *e.g.*, for ovarian cancer (13) that higher adduct levels in WBC correlate with a better clinical outcome. However, in breast cancer (13) and germ cell tumors (14, 15) this correlation could not be confirmed.

A limited number of clinical studies tried to predict tumor response already prior to cisplatin treatment of the patient. Armstrong *et al.* (16) showed a correlation between adduct levels in *in vitro* cisplatin-treated bone-marrow cells and complete remissions in adult acute non-lymphocytic leukemia. Other studies have focussed on the relation between *in vivo* and *in vitro* DNA-adduct formation in WBC. Adduct levels in WBC of cisplatin-treated testicular or non-small cell lung cancer patients (*in vivo*) appeared to correlate with those observed after *in vitro* cisplatin-treatment of WBC collected from these patients prior to chemotherapy (8, 17).

No studies have been performed as yet on the tumor tissue itself to determine the possible correlation between *in vivo*- and *in vitro*-induced adduct formation and to use this to predict the clinical response. Previously, we reported on the relation between the *in vivo*-induced Pt-DNA adduct levels in human HNSCC xenografts in nude mice after cisplatin treatment (18). A significant correlation was found between the tumor response in these mice and the amount of Pt-AG adducts.

In the present study, Pt-DNA adduct levels were determined after *ex vivo* cisplatin treatment of fragmented, isolated HNSCC tumors that had been grown as xenografts in nude mice. These data were compared with the *in vivo*-induced adduct levels and with the sensitivity parameters of these HNSCC xenografts. In addition, data are shown of the DNA-adduct levels in tumors of patients after *ex vivo* cisplatin-treatment of biopsy fragments. For this purpose, two tumor types with different sensitivities to cisplatin (HNSCC and testicular carcinoma) were investigated.

Material and Methods

Collection of tumors

Human HNSCC tumor lines (HNX-14C, -22B, -RO, -OE and -HN) were maintained *in vivo* by serial subcutaneous transplantations at both lateral sides in female, athymic, nude mice (6-8 weeks old; Harlan, Zeist, The Netherlands) as described earlier (18). When the xenografts reached a volume of 100-200 mm^3, mice were sacrificed and the tumors removed. The studies were performed after approval by the ethical committee at our Institute in accordance with the Dutch law on animal experiments.

Fresh HNSCC tissues were obtained from surgical specimens of 8 patients. Testicular tumor tissues were obtained from 8 patients who underwent orchidectomy. All patients provided signed informed consent according to local regulatory requirements. The tissues were kept at 4°C in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies [Gibco-BRL], Breda, The Netherlands) during transportation (≤ 40 min) to the laboratory for further experiments.

Ex vivo cisplatin treatment

HNSCC xenografts were treated with cisplatin (Platinol, Bristol-Myers Squibb, Woerden, The Netherlands) after dissection into tumor fragments. Attempts to use single cell suspensions obtained by enzymatic disruption were not successful, as this procedure yielded too many dead cells (data not shown).

For the treatment of tumor fragments, tumors were kept on ice and cut into pieces of 3 x 3 x 3 mm^3 followed by exposure for 1 h at 37°C to various concentrations of cisplatin (0, 10, 20, 30, 40, 60 and 80 µM). The cisplatin dilutions were freshly made in DMEM supplemented with 2.5% fetal calf serum (FCS). Subsequently, the tumor fragments were washed twice with PBS and with half of the fragments the incubation was prolonged in DMEM with 5% FCS but without the drug for 0 or 5 h. After two washings with ice-cold PBS the tissue fragments were stored at -80°C. Experiments to determine Pt-DNA adducts (immunocytochemistry, ^{32}P-postlabelling) were performed at least in triplicate, *i.e.* with the tumors of three animals.

Tumors obtained from HNSCC and testicular carcinoma patients were treated with cisplatin (0 and 80 µM) according to the method described above for the tumor fragments with the exception that the testicular tumors were treated with the drug in the absence of FCS, because this influences the viability of the tumor cells (19, 20). In these experiments also a drug- and serum-free incubation period of 18 h was included. However, because of the small size of the tumor biopsies, often only single experiments were feasible and in case of HNSCC tumors, only the incubation with 80 µM cisplatin followed by the 5-h drug-free incubation was performed. The 5-h incubation period was chosen because it was expected to result in maximal DNA-adduct levels (21).

Table I. Characteristics of the human HNSCC and testicular tumors.

patient	sex[1]	age (yr)[2]	tumor type	site/pathology[3]	stage/type[4]	therapy (response)[5]
1	F	64	HNSCC	oral cavity	T4N1M0	Cisplatin (PR)
2	M	46	HNSCC	oral cavity	T4N3M1	Cisplatin (NR)
3	F	56	HNSCC	tongue	T3N0M0	surgery
4	M	62	HNSCC	trigonum retromolare	T4N0M0	surgery
5	M	52	HNSCC	oral cavity	T2N0M0	surgery
6	F	67	HNSCC	oral cavity	T3N2M0	surgery
7	M	74	HNSCC	oral cavity	T3N0M0	surgery
8	M	68	HNSCC	tongue	T3N0M0	surgery
9	M	24	Testis	non-seminoma	EC, TER, YS	surgery
10	M	39	Testis	non-seminoma	EC, TER, YS	surgery
11	M	40	Testis	seminoma		surgery
12	M	30	Testis	non-seminoma	EC, TER	surgery
13	M	43	Testis	both	EC, TER, YS	BEP (CR)
14	M	37	Testis	both	EC, TER, YS	surgery
15	M	28	Testis	both		surgery
16	M	62	Testis	seminoma		surgery

[1]F,female; M, male.
[2]The age of the patient at the time of biopsy or surgery.
[3]In case of HNSCC, the site from which the tumor was taken is indicated. In case of testicular tumor the type (seminoma or non-seminoma) is given as determined after pathological examination.
[4]TNM tumor staging of the HNSCC according to the American Joint Commission on Staging (31). For the non-seminoma testicular tumors, the tumor was EC (embryonal carcinoma), TER (teratoma) or YS (yolk sac).
[5]Therapy given after biopsy or surgery and between parentheses the response to the chemotherapy. BEP, bleomycin, etoposide and cisplatin combined therapy according to standard protocol; various cycles with weekly a cisplatin injection (70 mg/m^2); CR, complete response; NR, no response; PR, partial response.

In vivo cisplatin sensitivity

The determination of the response of the HNSCC xenografts to cisplatin therapy was described previously (22). Briefly, when the tumors reached a volume of 50-200 mm^3, the tumor-bearing nude mice were treated with cisplatin by intravenous injection, twice with a 7-day interval at the maximum tolerated dose of 7 mg/kg. The efficacy of the treatment was expressed as growth delay factor (GDF) of the tumor, defined as the difference between the median values of the time required by tumors of treated and control animals to double their volume, divided by the median value of the time required by the tumors of the control mice to double their volume.

The outcome of cisplatin therapy was available only for 3 of the 16 patients from whom biopsies (prior to treatment) could be obtained, because only these 3 patients were treated with cisplatin. The relevant characteristics of all patients are listed in Table I. Two HNSCC patients received monochemotherapy with cisplatin (four cycles of drug in a dose of 70 mg/m^2 with weekly intervals). One testicular cancer patient received a combination therapy with bleomycin, etoposide and cisplatin. Six cycles were given with 100 mg/m^2 etoposide and 20 mg/m^2 cisplatin for five days with 30 mg/m^2 bleomycin on day two.

Immunochemical staining of cisplatin-DNA adducts

Cryostat sections (8 µm thick) of untreated and cisplatin-treated tumor fragments were fixed with ice-cold (-20°C) acetone for 10 min, followed by two 1-min washings with Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.4). Then the cryostat sections were treated with Proteinase K (0.5 µg/ml; Merck, Darmstadt, Germany) for 10 min at 37°C, followed by washings with TBS for 1 min, 4 min and 1 min, respectively. The DNA was denatured by incubation with 0.07 M NaOH in TBS with 1% formaldehyde for 5 min at 37°C, followed by 2x1 min TBS. The RNA was removed by RNAse treatment [100 µg RNAse A/ml (Sigma, St Louis, MO, USA) in TBS] for 1 h at 37°C. After 3 washings in TBS the sections were preincubated for 20 min at 37°C with 2% heat-inactivated fetal calf serum (FCS, Flow, Irvine, UK) in TBS, washed with TBS and incubated for 1 h at 37°C with the first antibody ICR4 (kindly donated by Dr M Tilby, Newcastle-upon-Tyne, UK), which recognizes Pt-DNA adducts (1:1000 in TBS with 5% FCS and 0.05% Tween). The preparations were again washed in TBS (3x1 min) and incubated for 1 h at 37°C with rhodamine-labelled goat-anti-rat antibody (1:100 in TBS with 5% FCS; Kirkegaard & Perry Lab. Inc, Gaithersburg, MD, USA). The sections were then washed 3 more times and mounted with 0.4 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St.Louis, MO, USA) in Tris-glycerol [1:3], which stains all nucleated cells. The DNA adducts were visualized by fluorescence microscopy (Leitz Orthoplan, Heidelberg, Germany) or with scanning laser microscopy (Leica TCS 4D, Heidelberg, Germany).

Platinum-DNA adduct determination

For the quantitative determination of Pt-DNA adducts in *ex vivo*-treated tumor fragments the modified ^{32}P-postlabelling assay was used (23). With this newly developed method the major adducts formed upon cisplatin treatment, Pt-GG and Pt-AG, can be determined in small DNA samples with low platination levels, such as those derived from the tumor fragments. Briefly, the isolated DNA was enzymatically digested to unmodified nucleosides and Pt-DNA adducts. Then, the adducts were purified on a strong cation-exchange column and ^{32}P-postlabelled after deplatination with NaCN and addition of an internal standard (dinucleotide TpT). The labelled products were separated by thin-layer chromatography and quantified by phosphorimaging (PhosphorImager 425, Molecular Dynamics, Sunnyvale, CA, USA).

Statistics

Associations between variables were calculated by use of the Pearson correlation test. The correlation coefficients (r) of the mean values of each tumor line and two-sided significance levels were determined with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). A correlation with a *p*-value of less than 0.05 was considered to be significant.

Results

To investigate whether *ex vivo*-treatment of tumor xenograft fragments with cisplatin results in a homogeneous formation of DNA adducts across the whole fragment, the presence of adducts in such fragments was visualized by immunocytochemical staining of a section through the centre of the fragment with the monoclonal antibody ICR4, directed against Pt-DNA adducts (24). In Figure 1, an example is shown of staining of HNSCC 22B tumor fragments. This result indicates that the conditions chosen in these experiments are adequate for staining of Pt-DNA adducts in the tumor tissue, and that cisplatin can penetrate and form DNA adducts throughout the whole 3 x 3 x 3 mm^3 tumor fragment.

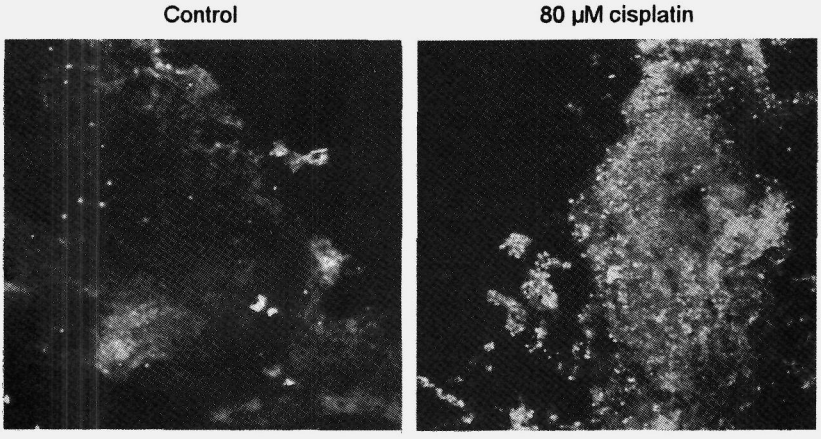

Figure 1. Immunocytochemical staining of Pt-DNA adducts in cryostat sections (whole tumor fragment) of untreated (control) and *ex vivo* cisplatin-treated (1 h, 80 µM) xenograft fragments of HNSCC 22B.

DNA adducts after ex vivo cisplatin-treatment of fragments from HNSCC xenografts

The levels of the adducts Pt-AG and Pt-GG in the *ex vivo* cisplatin-treated HNSCC tumor fragments were determined by the ^{32}P-postlabelling assay. In Figure 2, levels are shown that were obtained after a 1-h incubation with various concentrations of cisplatin with or without a 5-h additional incubation in drug-free medium. In general, the levels increased with the cisplatin concentration and were found to be higher after the 5-h drug-free incubation. As can be seen in Figure 2, the data generally fit a linear curve. We argued that a suitable way to compare the adduct levels, which takes into account the results in the whole concentration range, was to calculate the slopes of the fitted lines. The relationships between these slopes

(relative adduct levels) and the sensitivities of the tumors *in vivo* (expressed as GDF) (22) are given in Figure 3. Immediately after the 1-h exposure period, significant correlations were found for the Pt-AG adduct (r=0.92, p=0.02) and for the Pt-GG adduct (r=0.89, p=0.03). After the drug-free incubation period, the correlations were no longer significant (r=0.53, p=0.34 for Pt-AG and r=0.52, p=0.35 for Pt-GG). In general, the correlations between the Pt-GG and Pt-AG levels in the DNA samples were significant, with and without the 5-h drug-free exposure (r=0.99, p<0.001; r=0.98, p=0.003, respectively).

To establish the relevance of the *ex vivo* data for the results on adduct levels obtained after *in vivo* cisplatin treatment of the xenografts, the slopes of the lines in Figure 2 were compared with the area under the curve (AUC) of the adduct levels over the 3-h to 3-days interval in cisplatin-treated xenografted mice (18). The results given in Figure 4 show significant correlations (r=0.92, p=0.027 for Pt-GG; r=0.97, p=0.007 for Pt-AG) between the *in vivo* data and those immediately after *ex vivo* treatment (Figure 4A) but not (r=0.22, p=0.72 for Pt-GG; r=0.36, p=0.55 for Pt-AG) after the 5-h post-incubation period (Figure 4B).

DNA adducts after ex vivo cisplatin treatment of tumor fragments from patients

The Pt-GG and Pt-AG adduct levels in *ex vivo*-treated HNSCC and testicular tumor biopsies from patients were also measured by ^{32}P-postlabelling. The data after incubation of tumor fragments for 1 h with 80 µM of cisplatin followed by 5-h and 18-h drug-free incubation periods are depicted in Figure 5 and show that Pt-DNA adduct levels in the testicular tumors were comparable to those of the HNSCC tumors. In most of the eight testicular tumors the adduct levels were not decreased and even increased during the 5 to 18-h drug-free period. Because most of the HNSCC biopsies were too small to perform analyses at several time points, no such conclusions can be drawn for this tumor type.

Two of the eight HNSCC patients were treated with cisplatin as a single drug and one testicular carcinoma patient was treated with a cisplatin-containing regimen (Table I). The tumor of one of these HNSCC patients showed a partial response and the other was unresponsive. The patient with the testicular cancer showed a complete response. The *ex vivo*-treatment of the tumors from the two HNSCC patients with 80 µM cisplatin followed by a 5-h drug-free incubation resulted in Pt-GG and Pt-AG adduct levels of 27.4 and 5.1 adducts/10^6 nucleotides, respectively, for the patient showing the partial response, and of 13.2 and 2.4 adducts/10^6 nucleotides, respectively, for the non-responder (see Figure 5). Also after an 18-h drug-free incubation the levels in the more sensitive tumor were higher. The Pt-GG and Pt-AG adduct levels in the testicular carcinoma after the 5-h post-incubation were 22.9 and 10.4 adducts/10^6 nucleotides, respectively. For this patient a complete response was observed, *i.e.* he was considered free of disease.

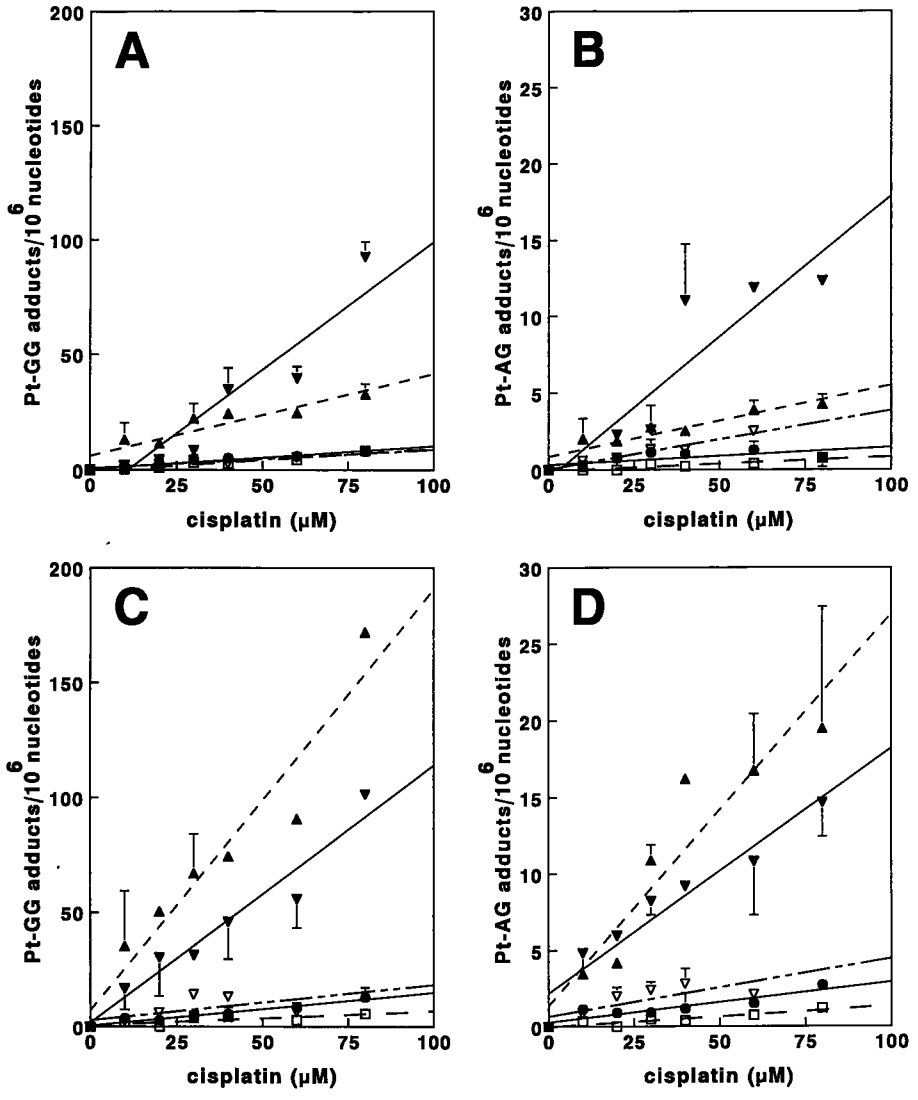

Figure 2. Tumor fragments of human HNSCC, grown as xenografts in nude mice, were treated *ex vivo* with various concentrations of cisplatin for 1 h (A and B) and for 1 h followed by a 5-h additional incubation in drug-free medium at 37°C (C and D). The amounts of Pt-GG (A, C) and Pt-AG (B, D), determined by ^{32}P-postlabelling, are given in number of adducts/10^6 nucleotides (mean ± SD). The tumor lines were studied at least in duplicate: HNX-14C (●); HNX-22B (▲); HNX-RO (▼); HNX-OE (▽); HNX-HN (□).

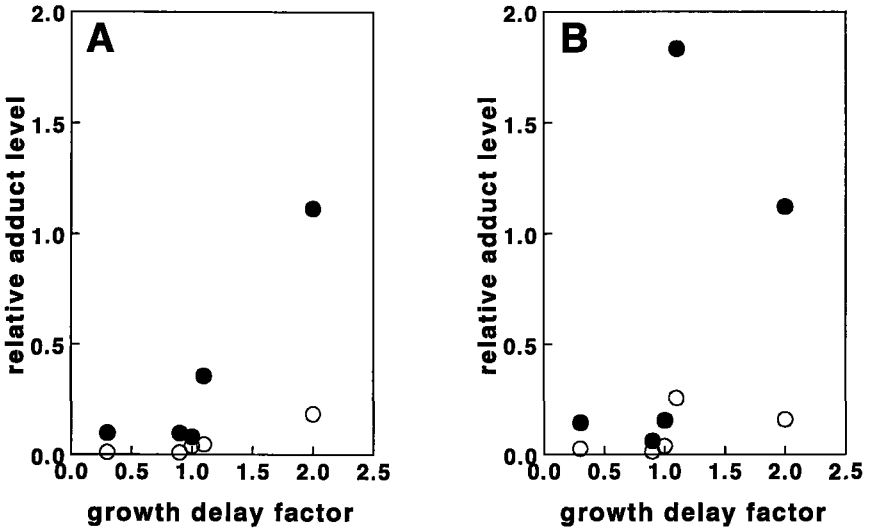

Figure 3. Relative adduct levels in DNA from the human HNSCC tumor fragments treated *ex vivo* with various concentrations of cisplatin for 1 h (A) and after the additional 5-h drug-free incubation (B) are shown as a function of the *in vivo* sensitivity of the corresponding tumors, expressed as the Growth Delay Factor. The relative adduct levels of Pt-GG (●) and Pt-AG (○) are given as slopes of the lines depicted in Figure 2 for each of the HNSCC tumor lines, thus reflecting dose-dependent accumulation.

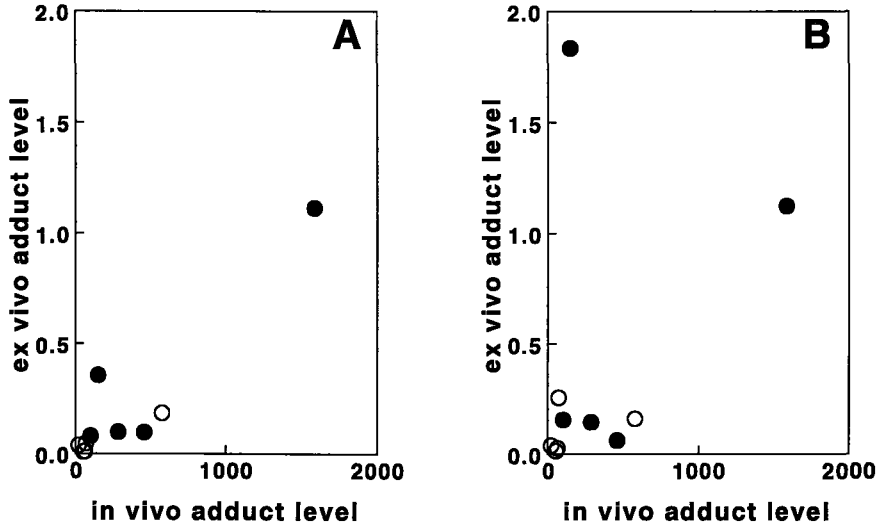

Figure 4. Comparison of the relative adduct levels in the *ex vivo*-treated HNSCC fragments immediately after 1-h treatment (A) and after an additional 5-h incubation in drug-free medium (B), expressed as the slope of the lines in Figure 2, and the AUC values over 3-h to 3-days time period of the adduct levels (number of adducts/10^6 nucleotides x h) formed *in vivo* in the HNSCC xenografts in nude mice as published elsewhere [18]. Pt-GG (●) and Pt-AG (○) adducts.

Discussion

In our studies possible methodologies were investigated and optimized to predict the efficacy of cisplatin treatment already prior to the actual chemotherapy of patients with solid tumors. Because of the conflicting results on the relation between Pt-DNA adduct levels present in WBC and the response of patients upon the cisplatin treatment (5, 8-15), we decided to determine the adduct levels in the target tissue itself, *i.e.* in the tumor. As published elsewhere (18), we found positive correlations between the response and the *in vivo* formed Pt-DNA adducts after cisplatin treatment of nude mice, in which HNSCC cells were grown as xenografts.

Since tumor biopsies are often taken from patients before chemotherapy starts, these specimens can be used in a test for predicting the possible outcome of the treatment. *A priori* culturing of these cells or explants has the disadvantage that it will take time. In addition, growth can be difficult to achieve as we experienced for human testicular tumors; after implantation in nude mice growth was only seen for 2 out of 6 tumors. Another drawback of the use cultured tumor cells for the prediction of the *in vivo* tumor response is the lack of correlation between results obtained after *in vivo* (in xenografts in mice) and *in vitro* (in the cultured cell lines) treatment of our panel of HNSCC cells (18).

In the present study the relevance of an *ex vivo* cisplatin incubation of tumor fragments for the outcome of the *in vivo* treatment was investigated in HNSCCs grown as xenografts in nude mice. Because only small biopsies will become available from patients and to ensure optimal conditions for penetration of the drug, small fragments (3 x 3 x 3 mm^3) of the HNSCC tumors were used for this purpose. As shown in Figure 1, despite the lack of blood circulation, the penetration of cisplatin into tumor fragments of this size appears to be fairly homogeneous.

The results on the Pt-GG and Pt-AG adduct levels after *ex vivo* cisplatin treatments of the HNSCCs (Figure 2) indicate that the levels increase linearly with the dose and that they are higher after a 5-h additional incubation in drug-free medium than immediately after the 1-h cisplatin treatment. This is in agreement with the findings of Fichtinger-Schepman *et al.* (21) that the conversion of cisplatin monoadducts to diadducts is complete after about 5 h.

As shown in Figure 3, immediately after the 1-h cisplatin treatment the Pt-AG and Pt-GG adduct levels were found to be correlated with the *in vivo* sensitivity of the tumor lines ($r=0.92$, $p=0.02$ for Pt-AG; $r=0.89$, $p=0.03$ for Pt-GG). However, after the 5-h additional incubation, such a correlation between sensitivity and adduct levels could not be found ($r=0.52$, $p=0.35$ for Pt-GG; $r=0.53$, $p=0.34$ for Pt-AG). The *ex vivo* levels after the 1-h exposure also correlated with the AUC values of the adducts in the tumors after *in vivo* treatment of the xenograft-bearing nude mice with $r=0.92$, $p=0.027$ for the Pt-GG and $r=0.97$, $p=0.007$ for the Pt-AG adduct (Figure 4). Such a

correlation was not found after the 5-h additional drug-free incubation period (r=0.22, p=0.72 for Pt-GG; r=0.36, p=0.55 for Pt-AG). This indicates that the overall outcome of the various processes that may be ongoing in the tumor tissue during the *ex vivo* drug-free period, *e.g.* DNA repair and cell death, is not related to the *in vivo* drug response.

In general, cisplatin-treated mammalian cells are able to remove their Pt-DNA-adducts by enzymatic DNA-repair processes (27-29). Therefore, to investigate the formation and persistence of the adducts in biopsies from patients, the *ex vivo* 1-h cisplatin-treated tumor fragments of some of the HNSCC and testicular carcinomas were also incubated for an additional period of 18 h in drug-free medium.

Testicular carcinomas are much more sensitive to cisplatin than HNSCC (1-3), however, as can be seen from Figure 5, about the same levels of the DNA adducts are formed in both tumor types. In most of the testis samples the adduct levels did not decrease during the additional drug-free incubation period, indicating that (almost) no repair of the adducts had occurred. This is in agreement with published data for cultured cell lines (28, 30-32). Unfortunately, due to the small size of the HNSCC biopsies, not enough information could be obtained for this tumor type to draw any conclusions about the persistence of the adducts.

Two of the HNSCC patients of whom biopsies were obtained, subsequently received cisplatin therapy, one showed a partial response and the other no response to the treatment. Only one of the testicular carcinoma patients was treated with cisplatin which resulted in a complete response (Table I). The adduct levels in the *ex vivo* treated biopsies of the two HNSCC patients were in line with what could be expected on the basis of the tumor response: Pt-GG and Pt-AG levels were higher in the more sensitive tumor, after 5-h as well as 18-h drug-free incubation period. Because no data are available for the responder, no comparison of the data after the 1-h cisplatin incubation is possible.

In conclusion, the bifunctional Pt-DNA adduct levels (Pt-GG, Pt-AG) present after a 1-h *ex vivo* cisplatin treatment of fragments of human HNSCC tumors xenografted in nude mice were found to correlate with the response of these tumors when the tumor-bearing mice were treated with cisplatin *in vivo*, and with the AUC levels of the adducts measured after the *in vivo* treatments. With respect to the adduct levels immediately after the 1-h *ex vivo* treatment of tumor biopsies from patients hardly any differences between testicular cancer and HNSCC were observed. The limited data on adduct levels in the tumor tissue samples of two HNSCC patients were in line with the hypothesis that higher adduct levels are associated with a better clinical response.

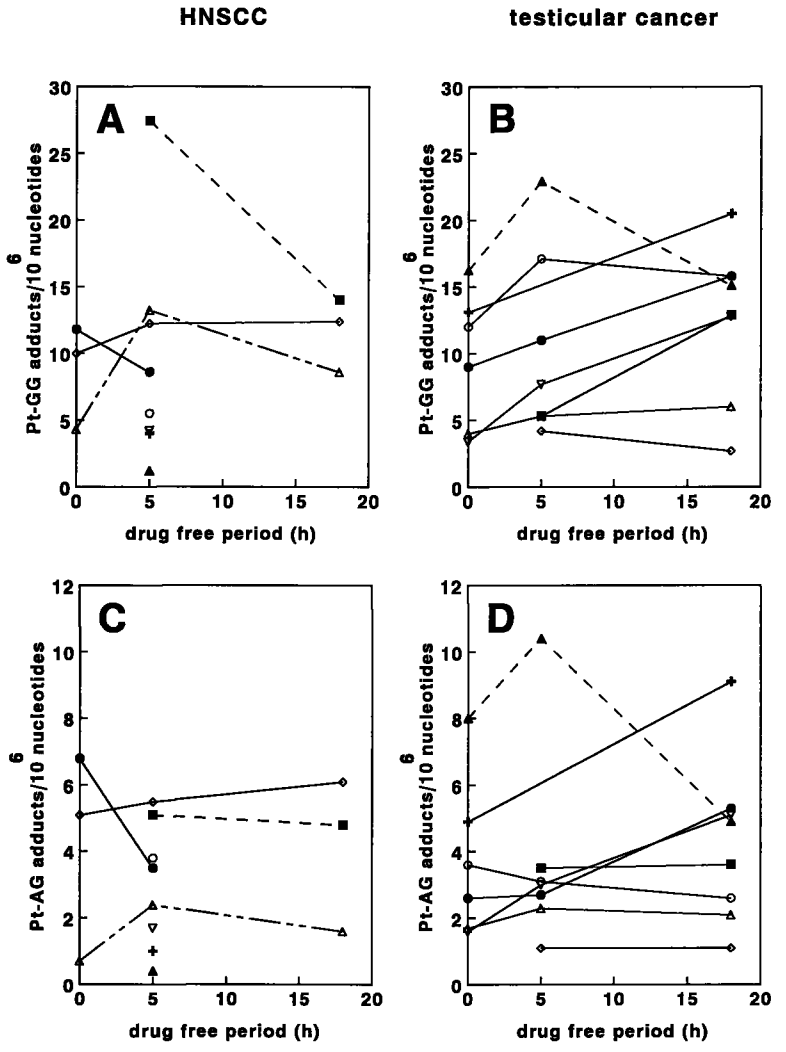

Figure 5. HNSCC and testicular tumor biopsies from untreated patients were incubated as 3 x 3 x 3 mm^3 fragments with 80 µM of cisplatin for 1 h, and subsequently incubated for 5 and/or 18 h in drug-free medium. The Pt-AG and Pt-GG adduct levels (number of adducts/10^6 nucleotides) were determined by ^{32}P-postlabelling. The data for the tumors from patients of whom the response to cisplatin chemotherapy was available, are indicated as follows: testicular cancer: ▲, complete response; HNSCC: ■, partial response and △, no response. The other patients did not receive cisplatin therapy.

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Chapter 8

Summary and Conclusions.

Summary and conclusions

The antiproliferative action of cisplatin on bacteria was accidentally discovered by Rosenberg in 1969. Further studies revealed the antineoplastic potential of this drug, which resulted in the introduction of this agent in the treatment of cancer. Since then, cisplatin and more recently its analogue carboplatin have proved to be successful chemotherapeutic agents against a broad range of solid tumors. The efficacy of the therapy with these drugs varies among the tumor types, but also among patients with the same tumor type. The latter, the so-called interindividual variation in response to drug therapy, is not well understood yet.

It was questioned why only about 40% of the patients with head and neck tumors are responding to cisplatin therapy. Possible explanations for this variation in sensitivity are intrinsic or acquired resistance to platinum (Pt) drugs. This can be due to differences in mechanisms at the cellular level such as a changed growth rate, decreased Pt accumulation, increased cisplatin inactivation (detoxification), decreased binding of Pt to DNA (Pt-DNA adduct formation) or increased DNA repair and/or tolerance of the DNA damage. From the literature it can be seen that, in general, the resistance to Pt agents is not due to only one of these mechanisms but that it is often multi-factorial.

In the present study, various factors involved in the sensitivity of tumor cells to cisplatin were investigated. They might explain the differences in clinical response to cisplatin therapy. Chapter 2 describes a panel of 10 human head and neck squamous cell carcinoma (HNSCC) cell lines, varying in sensitivity to cisplatin, which were used to find a possible correlation between the sensitivity to cisplatin and various cellular parameters: the cellular doubling time, the DNA index and the degree of differentiation. The *in vitro* sensitivity was determined with a colorimetric proliferation assay (SRB assay) and was expressed as the IC_{50} value. The cellular doubling time was calculated from data obtained with the proliferation assay, and the DNA index from the amount of DNA as determined by flow cytometry as well as by counting the number of chromosomes in metaphase spreads. In addition, cytokeratins in epithelial cells as markers for the degree of differentiation and their expression were determined by immunocytochemistry. No correlation of these parameters with the IC_{50} values could be established. However, the accumulation levels of cisplatin in the HNSCC cells, determined by atomic absorption spectroscopy (AAS), correlated significantly with the *in vitro* response; cells with higher intracellular levels of Pt showed to be more sensitive to cisplatin. This correlation was obtained when the discordant data of one cell line (VU-SCC-OE) was omitted. These OE cells showed moderate sensitivity to cisplatin, although very high intracellular levels of the

drug were found. An explanation for the tolerance to these relatively high Pt levels may be that this cell line was established from a tumor of a patient pretreated with radiotherapy. Such a treatment can change the genetic make-up of cells and may have resulted in an acquired resistance to cisplatin.

Because glutathione (GSH) plays an important role in the detoxification of cisplatin, the influence of the GSH levels and the expression levels of the enzyme glutathione S-transferase (GST), which catalyses the binding of cisplatin to GSH, were studied in the HNSCC cell lines (*in vitro*). As described in Chapter 3, the GSH levels were determined by high-performance liquid chromatography and the expression levels of the three isoenzymes of GST, α, μ and π, were measured semiquantitatively by means of immunocytochemistry. In a panel of 8 HNSCC cell lines no correlation was found between the GSH levels or the GST expression levels with the IC_{50} values. In addition, the expression levels of three proteins involved in multidrug resistance (MDR) were determined immunocytochemically: P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and lung resistance protein (LRP). Cisplatin itself is not known to induce MDR, but it is reported that MDR-induced cells often show cross-resistance to cisplatin and that MRP is able to transport a number of substrates conjugated to GSH. In the HNSCC lines both P-gp and LRP were found not to be involved in the *in vitro* sensitivity to cisplatin. Surprisingly, however, we found an inverse correlation between the expression levels of MRP and the IC_{50} values, obtained after 72 h of cisplatin incubation. High MRP levels were observed in the more sensitive cell lines and these levels were confirmed by the MRP functional assay, based on the transport of calcein across the cell membrane. This finding contradicts the previously described hypothetical function of MRP to transport cisplatin-GSH conjugates out of cells, which should result in enhanced resistance. An explanation for our finding may be that MRP, in addition to the exportation of conjugates is active in importation of cisplatin into cells. These data also suggest that other pump mechanisms and/or DNA repair are involved in the accumulation of Pt and the subsequent sensitivity to cisplatin.

In Chapters 2 and 3 the involvement of various cellular factors in the sensitivity to cisplatin was studied in a panel of HNSCC cell lines. These HNSCC cells originate from stem cells in the basal layer of the mucosa which have been mutated to cancer cells, possibly by genotoxic compounds. Therefore, protection of this basal layer against DNA damage is of crucial importance. We hypothesized that normal head and neck squamous cells (NHNEC or keratinocytes) in the upper layers protect this basal layer by forming a physical barrier and absorbing mutagenic and carcinogenic compounds. In general, after genotoxic exposure, cells that carry DNA damage can respond in one of three ways, either by growth arrest followed by DNA repair, by cell

death in a programmed way through apoptosis, or by tolerance of the DNA damage. We were interested in how these NHNEC respond to DNA damage induced with cisplatin (Chapter 4). Therefore, cell cultures were established from normal oral squamous tissues obtained from healthy individuals, who underwent surgery for snoring and/or obstructive sleep apnoea, and treated these cells with cisplatin. As for the HNSCC cells, the sensitivity to cisplatin was determined by the SRB assay and the intracellular Pt accumulation by AAS. Compared to their malignant counterparts, the NHNEC had accumulated 20-fold higher levels of Pt after 72 h of cisplatin treatment, but the sensitivity to cisplatin was similar to that of the HNSCC cell lines. These observed high levels of Pt in NHNEC are in line with our hypothesis that these cells have a protective role for the basal layer and serve as a "sponge" to absorb as much toxic material as possible. In addition, the capability of both NHNEC and HNSCC to undergo apoptosis was determined. Although in the HNSCC lines lower numbers of apoptotic cells induced by cisplatin were observed than in the NHNEC lines, no correlation was found with the intracellular Pt content. The reduced tendency of HNSCC to become apoptotic is in agreement with the theory that in the process of carcinogenesis the apoptosis pathway becomes impaired.

Because it is generally accepted that the antitumor activity of cisplatin is based on the formation of Pt-DNA adducts, it is important to investigate these adduct levels in tumor tissues. Cisplatin can form monoadducts in which Pt is bound to guanine (Pt-G), and various types of bifunctional adducts. The main bifunctional adducts are the intrastrand crosslinks Pt-GG, in which Pt is bound to adjacent guanines and Pt-AG, with Pt bound to adenine and an adjacent guanine. The other bifunctional adducts are G-Pt-G, the intrastrand adduct in which Pt is bound to two guanines separated by one or more nucleotide(s) and the interstrand crosslink on two guanines in opposite DNA strands. To determine the total amount of Pt-DNA adducts various methods can be used such as AAS and immunocytochemical techniques. However, none of these methods are sensitive enough to determine adduct levels in small samples of DNA with low platination levels. In Chapter 5 improvements of a recently developed ^{32}P-postlabelling assay are described, which enable us to measure the Pt-GG and Pt-AG adducts in small tumor specimens. The isolation of the adducts on a strong cation-exchanger was optimized in such a way that a recovery of nearly 100 % could be reached instead of the 30% published previously. Furthermore, the protocol for the subsequent deplatination of the adducts was simplified and an internal standard was included for the labelling reaction. Thus the reproducibility and the quantification were improved.

Using this modified ^{32}P-postlabelling assay the relation between the Pt-DNA adduct levels and the tumor response to cisplatin could be investigated. Several

publications provide evidence that higher adduct levels in white blood cells of tumor patients correlate with better clinical outcome. There are, however, also studies that do not confirm such a correlation. None of these studies, however, determined the adduct levels in the target tissue itself: the tumor. In Chapter 6 such a study is described. HNSCC-bearing nude mice were injected twice intravenously with cisplatin with a 7-days interval and the tumor size was measured twice weekly in order to determine the effect on tumor growth. The response to this cisplatin therapy was expressed as growth delay factor (GDF) or T/C value (the lowest tumor volume after treatment as percentage of the control tumor volume). Significant correlations were found between the *in vivo* tumor response to cisplatin and the Pt accumulation in the tumors, calculated as the area under the curve (AUC) of the Pt content versus time after the start of the cisplatin treatment. The Pt contents were determined by AAS. These results were in agreement with the significant correlation between the Pt levels and the IC_{50} values in the corresponding cell lines as described above. However, when the *in vivo* responses were compared with the *in vitro* sensitivities of the corresponding cell lines, no correlation was found, which strongly indicates that results obtained with cultured cells are not representative for the *in vivo* situation. Thus, although the ranking of the sensitivity to cisplatin between *in vitro* and *in vivo* did not correlate, in both cases the corresponding Pt contents did correlate with the sensitivity. Unfortunately, measurement of the Pt content in cisplatin-treated tumor biopsies to predict the clinical outcome of the treatment of patients cannot be used because the total amount of Pt in such small samples is below the detection limit of AAS. In such samples, however, the levels of the Pt-DNA adduct can be determined by the sensitive ^{32}P-postlabelling assay. For this reason, we focused on the determination of the Pt-DNA adducts in tumor cells after exposure to cisplatin. The studies on the correlation between Pt-DNA adduct levels and IC_{50} values indicated that *in vitro* this correlation was only significant for the Pt-AG adduct immediately after the 1-h treatment. *In vivo* significant correlations were found between the GDF and the Pt-AG adduct levels, calculated as AUC of these adducts over the period between 3 h and 3 days after the second cisplatin administration in HNSCC-bearing mice. This was not the case for the Pt-GG adduct levels or the T/C values. In the time period studied Pt-DNA adduct levels increase and after 3 days they decline with time. The fact that significant correlations were found between Pt-AG but not Pt-GG adduct levels and HNSCC response, suggests that Pt-AG adducts play an important role in the antitumor effect of cisplatin in the HNSCC lines studied.

Now that a correlation was found between *in vivo*-induced adduct levels and response of HNSCC xenografts in nude mice to cisplatin therapy, it became interesting to investigate whether Pt-DNA adduct level measurements in *ex vivo* cisplatin-treated tumors can be used to predict the sensitivity already prior to the *in*

vivo cisplatin treatment. In that case, patients can be selected who will benefit from the treatment, thereby preventing unnecessary administration of the drug. Furthermore, if possible the dose of the drug can be adjusted to increase the efficacy of the chemotherapy. In Chapter 7 a set-up of a predictive assay to individualize the treatment with cisplatin is described. The Pt-DNA adduct levels were determined by use of ^{32}P-postlabelling after *ex vivo* cisplatin treatment of fragmented (3 x 3 x 3 mm^3), isolated HNSCC tumors that had been grown as xenografts in nude mice. The data obtained were compared to the sensitivities of the HNSCC to cisplatin in the tumor-bearing mice. Immediately after the *ex vivo* cisplatin exposure period for 1 h, significant correlations were found for the Pt-AG as well as the Pt-GG adduct levels and the sensitivity of the tumors *in vivo* (expressed as GDF). The adduct levels were also determined in the tumor fragments after a prolonged incubation for 5 h in drug-free medium. Although higher adduct levels were present after this 5 h-incubation period, they did not significantly correlate with the GDF any longer. In order to establish the relevance of the adduct levels after *ex vivo* treatment, these data were also compared with the levels obtained *in vivo*, and again significant correlations were observed after the 1 h-treatment but not after the additional 5-h incubation in drug-free medium. The lack of correlation after this additional incubation period indicates that the overall outcome of the various processes that may be ongoing in the tumor tissue during the *ex vivo* drug-free period, *e.g.* DNA repair and cell death, differ from the *in vivo* drug response.

The Pt-GG and Pt-AG adduct levels in *ex vivo*-treated HNSCC (n=8) and testicular tumor biopsies (n=8) from patients were also measured by ^{32}P-postlabelling. After the 1 h exposure to cisplatin and after the additional 5 h drug-free incubation the mean adduct levels in both tumor types were similar. In most of the testicular tumors these levels did not decrease during a 5 to 18 h drug-free period, which indicates that (almost) no repair of the adducts occurred in this tumor type. Because most of the HNSCC biopsies were too small to analyse these biopsies at several time points, such conclusions could not be drawn. Actually, only two of the HNSCC and one of the testicular cancer patients of whom the biopsies were obtained, received cisplatin therapy. For HNSCC it was found that the Pt-AG and Pt-GG adduct levels were higher in the *ex vivo* treated tumor fragments of the patient with the good clinical response. This relation was also found after the additional drug-free incubation period of the samples for 5 h and even 18 h. These limited patient data are in line with our hypothesis that higher adduct levels are associated with a better clinical response.

In conclusion, several factors that might be involved in the sensitivity to cisplatin have been investigated in the studies described in this thesis. From these, only the total intracellular Pt content and Pt-DNA adduct levels correlate with the sensitivity. This was found in cultured HNSCC cells (*in vitro*) and in HNSCC-bearing mice (*in vivo*). The results suggest that the Pt-AG adduct plays an important role in the antitumor effect of cisplatin. The data shown in Chapter 7 support the hypothesis that the Pt-DNA adducts determined after *ex vivo* treatment of HNSCC fragments with cisplatin can be used to predict the *in vivo* response. Limited results in patient material were in agreement with this hypothesis, but many more biopsies have to be analysed to confirm this.

In the future, the value of the determination of both total Pt content and Pt-DNA adduct levels in tumors (*in vivo* and *ex vivo*) as factors that play a role in the response to cisplatin therapy should be studied more extensively, not only with respect to the above mentioned tumor type but also for other tumor types that are commonly treated with this drug. In addition, the preliminary study on patient biopsies using the predictive test as described in Chapter 7 warrants further investigation and the data obtained should provide an answer to the questions whether this test can predict the clinical outcome and whether this will be helpful in individualizing chemotherapy.

Samenvatting

In 1969 ontdekte Rosenberg bij toeval de groeiremmende werking van de anorganische verbinding cisplatine op bacteriën. Verder onderzoek wees uit dat cisplatine ook een antitumor-werking had en deze bevinding resulteerde in de introductie van deze stof als geneesmiddel bij de behandeling van kanker. Sindsdien hebben cisplatine en de meer recent ontwikkelde analoge verbinding carboplatine bewezen dat ze succesvolle chemotherapeutische middelen zijn tegen een scala van solide tumoren. Het effect van de therapie met deze platina (Pt)-verbindingen varieert niet alleen tussen de tumortypen, maar ook binnen één en hetzelfde tumortype. Men weet nog steeds niet waarom tumoren van eenzelfde type verschillend reageren op de behandeling, maar men vermoedt dat deze variatie in respons wordt veroorzaakt door de inter-individuele variatie, dat wil zeggen door verschillen tussen patiënten.

De vraag was waarom maar 40% van de hoofd-hals-tumorpatiënten reageert op de cisplatine therapie. Intrinsieke of verkregen resistentie tegen Pt-verbindingen zou een mogelijke verklaring kunnen zijn voor deze variatie in gevoeligheid. Resistentie ontstaat tengevolge van verschillende mechanismen op cellulair niveau zoals verandering in groeisnelheid van cellen, verminderde ophoping van Pt in de cel (Pt-accumulatie), toegenomen inactivatie van cisplatine (detoxificatie), verminderde binding van Pt aan DNA (vorming van Pt-DNA-adducten) of toegenomen DNA-schadeherstel en/of tolerantie van de DNA-schade. Uit de literatuur is bekend dat resistentie tegen Pt-verbindingen in het algemeen niet door één van die mechanismen ontstaat, maar door een combinatie hiervan, met andere woorden dat het een multifactorieel verschijnsel betreft.

Een belangrijk doel van deze studie was het bestuderen van verschillende factoren die betrokken kunnen zijn bij de gevoeligheid van tumorcellen voor cisplatine. Deze factoren zouden de verschillen in klinische respons op de cisplatinetherapie kunnen verklaren. Zoals beschreven in hoofdstuk 2 is een groep van 10 humane cellijnen van het hoofd-hals-plaveiselcelcarcinoom (HNSCC), die verschillen in gevoeligheid voor cisplatine, gebruikt om te onderzoeken of er een correlatie bestaat tussen de gevoeligheid voor cisplatine en verschillende cellulaire parameters, zoals de verdubbelingstijd, de DNA-index en de differentiatiegraad. De *in vitro*-gevoeligheid werd bepaald door middel van een proliferatietest (SRB-assay) en uitgedrukt als de IC_{50}-waarde. Dit is de concentratie van een stof waarbij 50% van de cellen geremd is in groei. De cellulaire verdubbelingstijd werd berekend aan de hand van de uit deze proliferatietest verkregen gegevens. De DNA-index werd op twee manieren bepaald, enerzijds met behulp van flowcytometrie en anderzijds door

het tellen van het aantal chromosomen in de cellen tijdens de metafase. Ook de expressie van cytokeratinen werd gemeten met behulp van flowcytometrie. Deze cytokeratinen zijn markers voor de mate van differentiatie in epitheelcellen. Er werd geen correlatie gevonden tussen al deze cellulaire parameters en de IC_{50}-waarde. Maar de accumulatie van Pt in de HNSCC-cellen, zoals gemeten met behulp van atoom-absorptie-spectrofotometrie (AAS), correleerde significant met de *in vivo*-respons. Naarmate de cellen meer Pt accumuleerden bleken ze gevoeliger voor cisplatine. Deze correlatie werd verkregen wanneer de afwijkende waarde van één cellijn (VU-SCC-OE) niet meegenomen werd in de berekeningen. De OE-cellen waren slechts matig gevoelig voor cisplatine ondanks de hoge intracellulaire Pt-concentratie. Een mogelijke verklaring voor de ongevoeligheid (tolerantie) van deze OE-cellen voor de relatief hoge Pt-concentraties zou kunnen zijn dat deze cellijn ontstaan is uit een tumor van een patiënt die voorbehandeld was met radiotherapie. Door zo'n behandeling kunnen genen in (tumor)cellen veranderen en dit zou tot de verkregen resistentie geleid kunnen hebben.

Omdat glutathione (GSH) een belangrijke rol speelt in de detoxificatie van cisplatine, is de invloed van de GSH-concentratie in de cel en de expressie van het enzym glutathione-S-transferase (GST), dat de binding van cisplatine aan GSH katalyseert, bestudeerd in de HNSCC-cellijnen (*in vitro*). Zoals beschreven in hoofdstuk 3 werden de GSH-concentraties gemeten met behulp van hoge-druk-vloeistofchromatografie (HPLC) terwijl de expressieniveaus van de drie iso-enzymen van GST, α, μ, en π, semikwantitatief bepaald werden met behulp van immunocytochemie. In de bestudeerde groep van 8 HNSCC-cellijnen werd geen correlatie gevonden tussen de GSH-concentraties of de GST-expressieniveaus en de IC_{50}-waarden.

Verder werden ook de expressieniveaus van de bij multidrugresistentie (MDR) betrokken eiwitten (proteïnen), P-glycoproteïne (P-gp), multidrugresistentie-geassocieerd-proteïne (MRP) en longresistentie-proteïne (LRP), bepaald met behulp van immunocytochemie. Het is niet bekend of cisplatine zelf MDR kan induceren, maar het is wel beschreven dat MDR-geïnduceerde cellen vaak kruisresistent zijn tegen cisplatine en dat MRP een aantal aan GSH geconjugeerd substraten uit de cel kan transporteren. Voor de HNSCC-cellijnen werd gevonden dat P-gp en LRP niet betrokken zijn bij de *in vitro*-gevoeligheid voor cisplatine. Maar voor MRP werd, tegen de verwachtingen, in een omgekeerde correlatie gevonden tussen de expressieniveaus en de IC_{50}-waarden, verkregen na 72 uur incuberen met cisplatine. De meer gevoelige cellijnen hadden hogere MRP-expressieniveaus. Dit werd bevestigd door gegevens uit de functionele MRP-test, die gebaseerd is op het transport van calceïne over de celmembraan. Deze bevindingen komen niet overeen

met de eerder beschreven hypothese dat MRP het conjugaat cisplatine-GSH uit de cel transporteert en dat dit resulteert in verhoogde resistentie. Een mogelijke verklaring voor deze bevindingen zou kunnen zijn dat MRP naast deze exportfunctie ook actief is bij het binnenbrengen van cisplatine in de cellen. Deze gegevens suggereren ook dat andere pompmechanismen en/of DNA-herstel betrokken kunnen zijn bij de accumulatie van Pt en dientengevolge bij de gevoeligheid voor cisplatine.

In de hoofdstukken 2 en 3 werd de betrokkenheid van verschillende factoren bij de gevoeligheid voor cisplatine in een groep HNSCC-cellijnen bestudeerd. Deze HNSCC-cellijnen zijn afkomstig van stamcellen in de basale laag van de mucosa, die mogelijk gemuteerd zijn tot kankercellen door genotoxische stoffen. Daarom is bescherming van de basale laag tegen DNA-schade erg belangrijk. De gebruikte hypothese is dat normale plaveiselepitheelcellen (de keratinocyten) in de bovenliggende cellagen van het hoofd-halsgebied de basale laag beschermen door als het ware een fysische barrière te vormen die mutagene en carcinogene stoffen absorbeert. In het algemeen kunnen cellen op drie manieren reageren op DNA-schade door genotoxische blootstelling: door te stoppen met groeien (proliferatiestop) gevolgd door DNA-herstel, door geprogrammeerde celdood (apoptose), of door tolerantie van de DNA-schade. Voor dit onderzoek was er interesse hoe deze keratinocyten reageren op de door cisplatine geïnduceerde DNA-schade (hoofdstuk 4). Hiertoe werden celkweken opgezet uit weefsels afkomstig van de mondholte en deze cellen werden behandeld met cisplatine. De weefsels werden verkregen van gezonde mensen die geopereerd werden vanwege snurken of die problemen hadden met hun ademhaling tijdens de slaap. Net zoals bij de HNSCC-cellijnen is de gevoeligheid voor cisplatine van deze keratinocyten bepaald met de SRB-assay en de intracellulaire Pt-accumulatie met AAS. In vergelijking met hun kwaadaardige tegenhangers hadden de keratinocyten 20 maal zoveel Pt in de cellen geaccumuleerd na een cisplatine-behandeling van 72 uur. De gevoeligheid voor cisplatine was echter vergelijkbaar met die van de HNSCC-cellijnen. De hoge Pt-concentraties in de keratinocyten passen in de hypothese dat deze cellen een beschermende rol spelen voor de basale laag. Ze dienen als het ware als een 'spons' die zoveel mogelijk toxische stoffen absorbeert. In deze studie werd ook bepaald of er apoptose optreedt in de keratinocyten en de HNSCC-cellen doordat ze met cisplatine behandeld worden. Bij de HNSCC-cellijnen werden lagere aantallen apoptotische cellen gevonden dan bij de keratinocyten, en deze gegevens correleerden niet met de intracellulaire Pt-accumulatie. De bevinding dat HNSCC-cellen minder snel apoptose ondergaan klopt met de theorie dat in het proces van carcinogenese het mechanisme van apoptose ontregeld is.

Omdat algemeen aangenomen wordt dat de antitumoractiviteit van cisplatine gebaseerd is op de vorming van Pt-DNA-adducten, is het erg belangrijk om deze adducten in tumorweefsels te kwantificeren. Cisplatine kan een monoadduct vormen, waarbij de Pt gebonden is aan guanine (Pt-G), en verschillende bifunctionele adducten. De belangrijkste bifunctionele adducten zijn het intrastrand-adduct Pt-GG, waarbij Pt is gebonden aan twee naast elkaar liggende guanines, en Pt-AG, met Pt gebonden aan een adenine en de daarnaast liggende guanine. De andere bifunctionele adducten zijn G-Pt-G, het intrastrand-adduct waarbij Pt is gebonden aan twee guanines die gescheiden zijn door een of meerdere nucleotide(n), en het interstrand-adduct met twee guanines op de tegenover elkaar liggende DNA strengen. Om de totale hoeveelheid Pt-DNA-adducten te kunnen meten zijn verschillende methoden beschikbaar, zoals AAS en immunocytochemische technieken. Deze methoden zijn echter niet gevoelig genoeg om de adducten in kleine DNA-monsters met een lage platineringsgraad te kunnen bepalen. In hoofdstuk 5 zijn de verbeteringen van een recent ontwikkelde ^{32}P-postlabelingmethode beschreven. Dit is een methode waarmee de Pt-AG- en Pt-GG-adducten in kleine tumorstukjes gemeten kunnen worden. De bestaande procedure voor isolatie van de adducten door een sterke kationen-wisselaar werd geoptimaliseerd, zodat niet de eerder gepubliceerde 30% maar bijna 100% van de adducten kon worden geïsoleerd. Het protocol voor de hierop volgende deplatineringsstap werd vereenvoudigd en een interne standaard voor de labelingsreactie werd toegevoegd om de kwantificering en de reproduceerbaarheid van de analyse te verbeteren.

Met behulp van deze gemodificeerde ^{32}P-postlabelingmethode kon de relatie tussen Pt-DNA-adduct niveaus en de tumorrespons op cisplatine bestudeerd worden. In verschillende publicaties wordt beschreven dat hogere adductniveaus in witte bloedcellen van tumorpatiënten correleren met een betere klinische respons. Maar er zijn ook studies die deze correlatie niet vinden. In deze studies zijn de adduct niveaus echter niet bepaald in de tumor zelf. Daarom zijn de adductniveaus bestudeerd in de hoofd-hals-tumoren en de resultaten zijn beschreven in hoofdstuk 6. Naakte thymusloze muizen met geïmplanteerde humane HNSCC werden intraveneus twee keer met een interval van 7 dagen geïnjecteerd met cisplatine. De tumorgrootte werd twee keer per week gemeten om zo het effect van cisplatine op de tumorgroei te kunnen bepalen. De respons op deze cisplatinetherapie werd uitgedrukt als groeivertragingsfactor (GDF) of T/C-waarde (het kleinste tumorvolume als gevolg van de behandeling, uitgedrukt als percentage van het tumorvolume in onbehandelde muizen). De correlatie tussen de *in vivo*-respons op de cisplatinebehandeling en de Pt-accumulatie in de tumoren was significant. De Pt-accumulatie werd gemeten met behulp van AAS en uitgedrukt als het oppervlak

onder de curve (AUC) van de intracellulaire Pt-concentraties versus de tijd, na de tweede toediening van cisplatine. Deze *in vivo*-resultaten kwamen overeen met de significante correlatie tussen de Pt-accumulatie en de IC_{50}-waarden in de corresponderende HNSCC-cellijnen, zoals hierboven beschreven. Wanneer de *in vivo*-respons echter werd vergeleken met de gevoeligheid *in vitro* in de corresponderende cellijnen werd er geen correlatie gevonden. Dit geeft aan dat de resultaten verkregen met gekweekte cellen niet representatief zijn voor de *in vivo*-situatie. Het meten van de Pt-concentraties na cisplatinebehandeling in tumorbiopsieën is jammer genoeg niet bruikbaar om de klinische respons van patiënten op de behandeling te voorspellen, omdat de Pt-concentraties in dit geval onder de detectielimiet van de AAS liggen. In dergelijke monsters kunnen echter wel de Pt-DNA-adducten gemeten worden met de gevoelige ^{32}P-postlabelingmethode. Daarom werd er voornamelijk geconcentreerd op het met deze methode meten van adducten in aan cisplatine blootgestelde tumorcellen. In de *in vitro*-studie naar de correlatie tussen Pt-DNA-adductniveaus en de IC_{50}-waarden werd alleen een significante correlatie gevonden voor het Pt-AG-adduct direct nadat de cellen 1 uur behandeld waren met cisplatine. *In vivo* werd ook een significante correlatie gevonden tussen de GDF en de Pt-AG-adductniveaus. Deze niveaus zijn hier uitgedrukt als de AUC van deze adducten over een periode van 3 uur tot 3 dagen nadat de tumor-dragende muizen voor de tweede keer geïnjecteerd waren met cisplatine. De correlaties bleken niet significant voor de Pt-GG-adductniveaus en/of de T/C-waarden. De Pt-DNA-adductniveaus stegen tot ongeveer 3 dagen na deze tweede cisplatinetoediening en daalden daarna weer. Het feit dat de correlatie tussen de Pt-AG-, maar niet de Pt-GG-adductniveaus en de HNSCC-respons significant was, suggereert dat in de bestudeerde HNSCC-cellijnen het adduct Pt-AG een belangrijke rol speelt in het antitumoreffect van cisplatine.

Nu er een correlatie gevonden is tussen *in vivo*-geïnduceerde adductniveaus en de respons op de cisplatinetherapie van HNSCC in naakte muizen, bleek het interessant te onderzoeken of de Pt-DNA-adductniveaus in tumoren, die *ex vivo* behandeld worden met cisplatine, de gevoeligheid voor cisplatine kunnen voorspellen, dus alvorens de eigenlijke behandeling *in vivo* zal plaatsvinden. Dan zouden patiënten geselecteerd kunnen worden die baat hebben bij de behandeling en kan eventueel de dosis van het geneesmiddel worden aangepast om zo het effect van de chemotherapie te verhogen. Ook het onnodig toedienen van het geneesmiddel zou op deze manier voorkomen kunnen worden. In hoofdstuk 7 is een methode beschreven voor het ontwikkelen van een voorspellende test om hiermee vervolgens de behandeling met cisplatine te kunnen individualiseren. De Pt-DNA-adductniveaus in de *ex vivo*-behandelde fragmenten (3 x 3 x 3 mm^3) van HNSCC-tumoren uit de muizen werden gemeten met behulp van de ^{32}P-postlabelingmethode.

De verkregen gegevens werden vergeleken met de gevoeligheid voor cisplatine van deze tumoren in de naakte muizen (*in vivo*). Direct na de *ex vivo*-incubatie met cisplatine gedurende 1 uur bleken de correlaties voor zowel Pt-AG- als Pt-GG-adductniveaus en de *in vivo*-gevoeligheid van de tumoren (uitgedrukt als de GDF) significant. De adducten werden ook gemeten in tumorfragmenten nadat de incubaties verlengd werden met 5 uur in cisplatine-vrij medium. Alhoewel de adductniveaus na deze 5-uurs incubatie hoger waren dan direct na de behandeling met cisplatine, was de correlatie met de GDF niet langer significant. Om de relevantie van de adductniveaus na de *ex vivo*-behandeling vast te kunnen stellen, werden deze adductgegevens ook vergeleken met de *in vivo* gemeten adducten. Weer werd er een significante correlatie gevonden na de 1-uurs behandeling maar niet na de 5 uur extra incubatie in cisplatine-vrij medium. Dit laatste duidt erop dat er verschillen optreden in de processen *in vivo* en *ex vivo* die plaatsvinden gedurende deze cisplatine-vrije periode, zoals DNA-herstel en celdood.

De Pt-GG- en Pt-AG-adductniveaus in *ex vivo*-behandelde biopsieën van HNSCC- (n=8) en testistumoren (n=8) van patiënten werden ook gemeten met behulp van de ^{32}P-postlabelingmethode. Direct na de 1-uurs blootstelling aan cisplatine en na de extra incubatie van 5 uur in cisplatine-vrij medium waren de gemiddelde adductniveaus in beide tumortypen vergelijkbaar. In de meeste testistumoren daalden de niveaus niet gedurende een 5 tot 18 uur cisplatine-vrije periode. Dit duidt erop dat (bijna) geen herstel van de adducten heeft plaatsgevonden in dit tumortype. Omdat de meeste HNSCC-biopsieën te klein waren om de adducten te meten na verschillende extra incubatietijden, kon er voor de HNSCC niet zo'n conclusie getrokken worden. Slechts twee van de HNSCC- en één van de testistumorpatiënten, van wie de biopsieën werden verkregen, werden ook daadwerkelijk behandeld met cisplatine. Het bleek dat de Pt-AG- en Pt-GG-adductniveaus hoger waren in de *ex vivo*-behandelde HNSCC-fragmenten van de patiënt met de betere klinische respons. Deze relatie werd ook gevonden na de extra cisplatine-vrije incubatie gedurende 5 en 18 uur. Deze resultaten verkregen met een beperkt aantal patiënten ondersteunen de hypothese dat hogere adductniveaus geassocieerd zijn met een betere klinische respons.

Concluderend kan gesteld worden dat in dit proefschrift verschillende factoren, die mogelijk betrokken zijn bij de gevoeligheid voor cisplatine, zijn bestudeerd en beschreven. Van deze factoren correleren alleen de intracellulaire Pt-accumulatie en de Pt-DNA-adductniveaus met de gevoeligheid. Dit werd vastgesteld voor zowel de gekweekte HNSCC-cellen (*in vitro*) als voor de HNSCC groeiende op muizen (*in vivo*). De resultaten suggereren dat de Pt-AG-adducten een belangrijke rol spelen in het antitumoreffect van cisplatine. De gegevens in hoofdstuk 7 ondersteunen de

hypothese dat de Pt-DNA-adducten, gemeten na *ex vivo*-behandeling van HNSCC-fragmenten met cisplatine, gebruikt kunnen worden om de *in vivo*-respons te voorspellen. De beperkte resultaten verkregen met patiëntenmateriaal kwamen overeen met deze hypothese, maar veel meer biopsieën zouden geanalyseerd moeten worden om dit te bevestigen. De hieruit verkregen resultaten zullen moeten uitwijzen wat de waarde is van deze test om de klinische respons te voorspellen en of dit nuttig is voor het individualiseren van chemotherapie.

In de toekomst zal de waarde van Pt-accumulatie en Pt-DNA-adductniveaus als factoren die een rol spelen in de respons op cisplatinetherapie verder bestudeerd moeten worden. Dit geldt niet alleen voor het in dit proefschrift beschreven tumortype, maar ook voor andere tumortypen die in de kliniek met dit geneesmiddel behandeld worden.

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Mary

Curriculum Vitae

Marij Welters werd geboren op 30 oktober 1969 te Spaubeek. In 1988 behaalde zij het VWO diploma aan de scholengemeenschap St. Michiel in Geleen. Vervolgens begon ze aan de studie Medische Biologie aan de Rijksuniversiteit in Utrecht. Het bijvak werd gevolgd bij de vakgroep Moleculaire Celbiologie onder leiding van ir. H. Ovelgonne en dr. R. van Wijk. Tijdens deze stage werd het effect van arseniet op de genexpressie van fibroblasten die in de rustfase zaten bestudeerd. Ze volgde haar hoofdvakstage bij de vakgroep Experimentele Pathologie in het Academisch Ziekenhuis te Utrecht onder leiding van dr. L.T.M. van der Ven en prof.dr. W. den Otter. Hier verrichtte zij onderzoek naar de rol van insuline-achtige groeifactoren in de groei van gezonde, goed- en kwaadaardige gladde spiercellen. Hier werd ook de literatuurstudie voor de doctoraalscriptie uitgevoerd. Dit betrof de invloed van de transformerende groeifactor beta op T-helper cellen. In oktober 1992 behaalde ze haar doctoraaldiploma medische biologie en in november van datzelfde jaar kon ze beginnen als wetenschappelijk medewerkster bij de afdeling Genetische Toxicologie van TNO te Rijswijk. Het promotie-onderzoek werd uitgevoerd onder leiding van dr.ir. A.M.J. Fichtinger-Schepman en dr. R.A. Baan. De resultaten zoals beschreven in dit proefschrift zijn verkregen in nauwe samenwerking met de afdeling Keel-, Neus- en Oorheelkunde van het ziekenhuis van de Vrije Universiteit te Amsterdam. Hier werd een gedeelte van de experimenten (o.a. het *in vivo* werk) uitgevoerd onder leiding van dr. B.J.M. Braakhuis. Sinds juli 1997 is ze werkzaam als post-doc bij de afdeling Inwendige Geneeskunde I in het Dijkzigt ziekenhuis te Rotterdam. In bloed van patiënten die een humane hartkleptransplantatie hebben ondergaan wordt de immuunrespons bestudeerd. Het onderzoek wordt uitgevoerd onder leiding van dr. L.M.B. Vaessen, prof.dr. Weimar en prof.dr. A.J.J.C. Bogers.