Clinical and preclinical pharmacokinetic and dynamic studies with cisplatin, topotecan and docetaxel

The studies presented in this thesis were carried out within the Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek)/University Hospital Rotterdam

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This thesis is dedicated to my parents,
to Shaolan and
to Beijia

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CHAPTER 1

INTRODUCTION

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1.1 CISPLATIN: INTRODUCTION

Cisplatin is one of the most potent cytotoxic drugs and is frequently applied in the treatment of various solid tumors, for example testicular, ovarian, cervical, endometrial, bladder, lung, head and neck cancer (H/N), mesothelioma and cancer of unknown primary site (CUP). The cytotoxic effect of cisplatin is most closely related to the interaction with nuclear DNA, characterized by the formation of inter- and intrastrand cross-links (adducts). The clinical application of cisplatin is limited because of the existence or development of resistance and induction of severe side effects. In the clinic wide variability exists in the response to cisplatin chemotherapy. This may be due to pharmacokinetic variability, pharmacodynamic variability or both. It is clinical practice to treat patients with a standard dose per unit of body surface area. This may result in wide variation between patients in the magnitude of exposure to the active i.e. non-protein bound cisplatin. To date, it has not clearly been established whether individualized treatment with cisplatin using predefined target levels of exposure increases the response rate and disease-free survival. In addition, in contrast to the cisplatin analogue carboplatin no simple and validated procedures are available to individualize the dose of cisplatin with the aim to reduce the pharmacokinetic variability. Several mechanisms of resistance have been characterized for cisplatin, such as increased repair of DNA-adducts, increased intracellular inactivation by thiols, reduced cellular accumulation of cisplatin by decreased uptake and increased efflux of cisplatin-glutathione complexes. These mechanisms may contribute to clinical resistance to cisplatin. Based on this, there is a strong need for combination therapy with highly active anticancer agents, which show synergistic cytotoxicity with cisplatin and non-overlapping patterns of toxicity. Topoisomerase I inhibitors are ideal candidates for this role. Irinotecan (CPT11), topotecan and 9-aminocamptothecin are presently undergoing clinical evaluation. They act on the topoisomerase I by stabilizing the topoisomerase I-DNA cleavable complex and inhibit DNA and RNA synthesis. It is of great clinical importance to characterize the interpatient variability in the dose-response relationship of cisplatin, to further elucidate mechanisms of resistance to cisplatin and mechanisms of interaction between cisplatin and topoisomerase I inhibitors *in vitro* and *in vivo*.

In addition, taxanes such as paclitaxel and docetaxel are excellent candidates for combination therapy with cisplatin. Taxanes are antimicrotubule agents, which show high activity as single agent against many solid tumor types. The toxicity profile overlaps only partly with that of cisplatin. Optimal combination chemotherapy is currently being investigated.

1.1.1 Clinical application

Cisplatin (fig.1) is one of the most widely applied anticancer agents. It is mostly used in the treatment of solid tumors especially carcinomas. Cisplatin is standard therapy in the treatment of advanced testicular and ovarian cancer (Loehrer and Einhorn, 1984). Before its incorporation in combination therapy schedules with vinblastine and bleomycine, few patients with advanced nonseminomatous testicular cancer could expect to survive beyond 1 to 2 years. Since the implementation of cisplatin in the combination chemotherapy nearly 80% of the patients achieve a complete remission (Loehrer and Einhorn, 1984). A 10-year follow-up study of patients with disseminated testicular nonseminomatous cancer revealed a cure rate of 63% in responders to cisplatin based combination chemotherapy (Stoter et al., 1989). Long-term survival data of patients with advanced epithelial ovarian cancer showed that cisplatin based combination chemotherapy (CHAP-5) increased the 10-year survival by more than 10% compared to the best treatment of the precisplatin era: Hexa-CAF (Neijt et al., 1991).

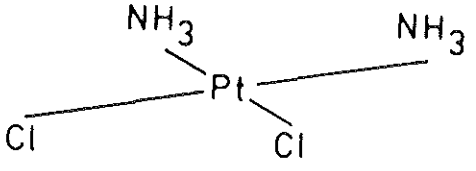

Figure 1. Chemical structure of cis-diamminedichloroplatinum (II) (cisplatin).

In addition, great improvements have been achieved in the adjuvant setting of ovarian cancer with cisplatin. Other trials revealed high response rates and increase of the disease free and/or total survival of locally advanced and metastatic bladder cancer (Stoter et al., 1987), non-small cell lung cancer (NSCLC) (Fukuoka et al., 1992; Souquet et al., 1993) and small cell lung cancer (Hansen, 1992). Favorable response rates have been documented with weekly high-dose cisplatin in NSCLC, mesothelioma (Planting et al., 1993) and H/N cancer (Planting et al., 1993a) or with cisplatin in cycles of 3 weeks (Verweij et al., 1989; Mori et al., 1992). Cisplatin is also the single most active cytotoxic agent in the treatment of recurrent or metastatic squamous cell cancer of the cervix (Alberts et al., 1991).

1.1.2 Clinical toxicology

Application of cisplatin is associated with a wide spectrum of side-effects, but most frequently encountered are renal toxicity, neurotoxicity including hearing loss and gastrointestinal toxicity. The renal toxicity is related to the dose (Blachley and Hill, 1981; Ries and Klastersky, 1986). In addition, the renal toxicity increases with the duration of therapy (Campbell et al., 1983). Age, sex and pretreatment creatinine do not predict nephrotoxicity (Campbell et al., 1983; Skinner et al., 1992). It is unclear whether dose reductions should be pursued in the case of a preexisting moderate reduction in renal function (Blachley and Hill, 1981; Campbell et al., 1983) After i.v. administration cisplatin is eliminated through the kidneys. The pathway follows glomerular filtration and active tubular secretion. The accumulation of reactive forms of cisplatin in the renal tubules leads to their disruption and loss of functions. One of the early signs is tubular loss of electrolytes such as magnesium, potassium and sodium (Blachley and Hill, 1981; Ries and Klastersky, 1986; Daugaard and Abildgaard, 1989). This can lead to severe and even life threatening symptoms. Since the mechanism of the toxicity has been recognized cisplatin is administered with active pre- and posthydration. In treatment schedules with high dose-intensity cisplatin is dissolved in hypertonic saline to further reduce the risk of renal dysfunction (Baldew et al., 1990). Employing this regimen allows administration of dosis of cisplatin as high as 200 mg/m^2 on a 3-weekly basis (Kehoe et al., 1992) or >80mg/m^2 on a weekly basis without significant loss of renal function (Planting et al., 1993). Neuropathy and ototoxicity are other significant problems associated with cisplatin therapy. These toxicities are usually cumulative with clinical signs and symptoms appearing after a total dose in excess of 300 mg/m^2 (Cersosimo, 1989; Cavaletti et al., 1991). Symptoms of gastrointestinal toxicity are nausea and vomiting (de Mulder et al., 1991). The severity of these symptoms is clearly related to the dose per course (Seynaeve et al., 1991, 1991a). It is unclear whether single exposure intensity (AUC) or total exposure intensity (cumulative AUC) are better predictors for neural and other toxicities than single or total dose intensity, because literature data are sparse.

1.2 PHARMACOKINETICS

In clinical practice cisplatin is administered intravenously in 3 to 24 h infusions. The pharmacokinetics of cisplatin are linear and the plasma concentration-time curves can be fitted best to a model with zero order rate of input and a one-exponential (Crom et al., 1981; Himmelstein et al., 1981; Corden et al., 1985) or sometimes two-exponential (Vermorken et al., 1982) decline of the curve post-infusion. The terminal half-life varies from 18 to 49 min and the volume of distribution form 47-94 l (Himmelstein et al., 1981;

Vermorken et al., 1982; Bajorin et al., 1986). The curves of the unbound cisplatin in plasma and cisplatin which is bound to plasma proteins do not run parallel (Himmelstein et al., 1981; Vermorken et al., 1982). This indicates that the protein binding is a nonreversible process or a very slowly reversible process (Hegedus et al., 1987; Yotsuyanagi et al., 1991; Melvik et al., 1992). Hence, the clearance of cisplatin is so-called restrictive and for a representative calculation of the AUC and the clearance the unbound cisplatin concentrations are to be used (Gibaldi and Perrier, 1982). Cisplatin also binds extensively to all types of tissue components (Holler et al., 1993). Cisplatin is eliminated primarily through the kidneys. During the first 4 h after cisplatin administration 17 to 22% of the dose is excreted in the urine (Bajorin et al., 1986). The remaining part of the dose is excreted very slowly due to the extremely long retention and accumulation of cisplatin in deep tissues (Tothill et al., 1992). The renal clearance of unbound cisplatin exceeds the creatinine clearance by approximately 50%, indicating active tubular secretion (Jacobs et al., 1980; Corden et al., 1985; Bajorin et al., 1986). Cisplatin is excreted to a minor extent via the bile (Caspar et al., 1979).

1.3 PHARMACODYNAMICS

1.3.1 Mechanism of action: cisplatin-DNA adduct formation and repair

The exact mechanism of action of cisplatin is still unclear. It is well established that cisplatin binding to DNA contributes to cellular toxicity (Scanlon, 1991). At least four adducts have been identified. The two major products are cis-$Pt(NH_3)_2$d(pGpG), cis-$Pt(NH_3)_2$d(pApG), both derived from intrastrand crosslinks of cisplatin on neighboring guanines and adenines. Two minor adducts are $Pt(NH_3)_3$dGMP resulting from monofunctionally bound cisplatin to guanine and cis-$Pt(NH_3)_2$d$(GMP)_2$ originating from interstrand crosslinks (ICL) on two guanine nucleotides as well as from intrastrand crosslinks on two guanines separated by one or more bases (Fichtinger-Schepman et al., 1982, 1984, 1985; Eastman 1986)(fig. 2). During the whole process of cisplatin-DNA adduct formation, first cisplatin is hydrolysed by replacement of Cl^- with H_2O which reacts to DNA to form monofunctional and subsequently bifunctional adducts (fig. 3). It is still debatable whether the major Pt-GG intrastrand crosslinks or the ICL's are responsible for the cytotoxic effect. Studies have outlined the importance of the intrastrand crosslink (Hill et al., 1994). In addition, studies using HeLa cell extracts have shown that the Pt-GG intrastrand crosslink in duplex M13 DNA is refractory to repair (Szymkowski et al., 1992). In contrast, studies in two ovarian cancer cell lines and resistant sublines have revealed significant and consistent increase in the ICL repair in the dihydrofolate reductase gene (Zhen et al., 1992). In an

earlier study using the SuSa testicular teratoma cell line, a deficiency in the removal of ICL's was observed (Hill et al., 1994a).

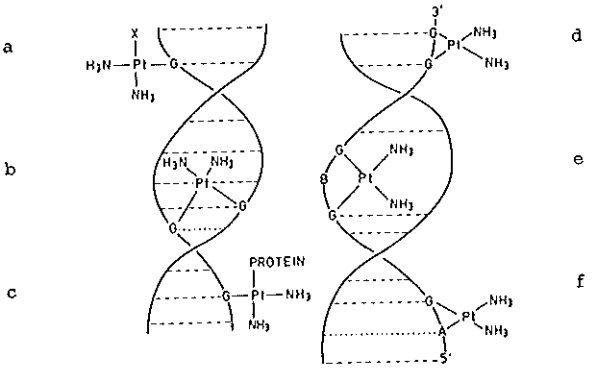

Figure 2. Schematic representation of the known cisplatin-DNA adducts, (a) cisplatin bound monofunctionally to a guanine; (b) the interstrand cross-links to two guanines; (c) the DNA-protein cross-links; (d) the GpG intrastrand cross-links; (e) the GpNpG intrastrand cross-links; and (f) the ApG intrastrand cross-links. (the figure was taken from ref. Fichtinger-Schepman et al 1985)

At the molecular level, several genes and proteins have been characterized which are involved in the repair of cisplatin DNA adducts (Schaeffer et al., 1993; Sancar, 1994; Kelland, 1994). Cellular "damage recognition proteins (DRP's)" have been characterized which bind to cisplatin induced damaged DNA. The role in the initial stage of events which lead to DNA repair has not yet fully been elucidated (Donahue et al., 1990; Chu and Chang, 1990; Chao et al., 1991; Bissett et al., 1993; Jones et al., 1991). One such proteins has been identified as the high-mobility group proteins HMG1 (Pil and Lippard, 1992). They even may delay repair of DNA. Another hypothesis is that the DRP's are factors which are important for transcription and that binding to damaged DNA reduces the amount of DRP's available for transcription, resulting in toxicity of cisplatin. It has been well established that the nucleotide excision repair (NER) pathway is involved in the repair of the cisplatin intrastrand adducts (Clugston et al., 1992; Sancar, 1994). In humans and Escherichia coli the involved excinuclease, an operational definition of repair activity, is the sole system for removing bulky DNA adducts (Sancar, 1994). The activity results from sequential and partly overlapping activities of at least 17 polypeptides. Recently, several human

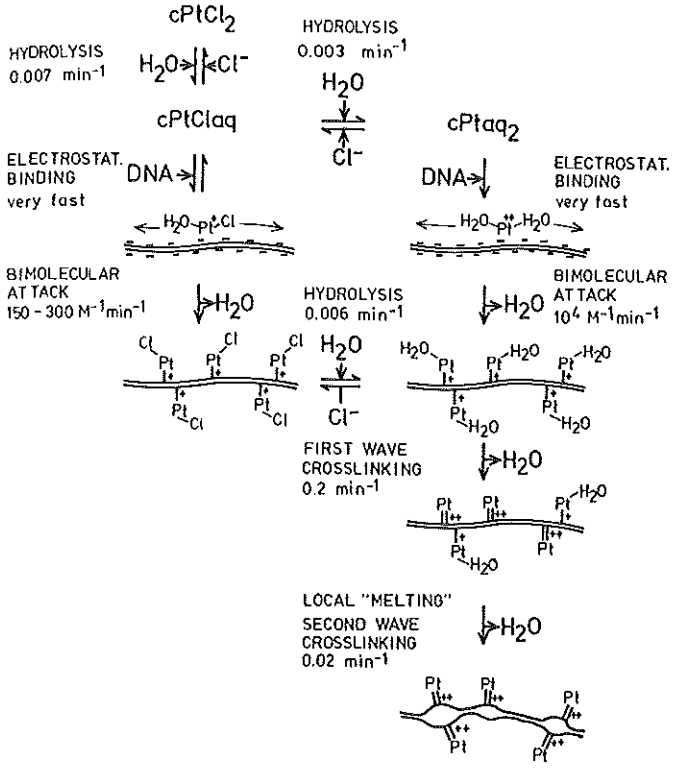

Figure 3. Kinetic mechanism of cisplatin binding to DNA in vitro. Double strand DNA is symbolized by two parallel lines. Monofunctional adducts by cisplatin are indicated by single "bonds", bifunctional adducts irrespectively of their type of crosslink by double "bonds". Symbole "-" refers to negative and "+" to positive charges. Ammine ligands are omitted from the graph. The arrows in the electrostatic complex indicate onedimensional diffusion of the platinum(II) complex along the DNA backbone. (figure was taken from ref. Holler 1994)

repair genes have been cloned. The high homology between the components of nucleotide excision repair derived from human cells and yeast cells demonstrates that the mechanism of excision repair is highly conserved between eukaryotes. Important in this development has been the characterization and cloning of Saccharomyces cerevisiae excision repair proteins, such as RAD1, 2, 3, 4, 10, 14 and 25. They are structurally and functionally homologues of the human excision repair proteins XPF, XPG, XPD, XPC, XPA, ERCC1 and XPB (Prakash et al., 1993), some of which are subunits of the general transcription factor TFIIH. After recognition, a nuclease subunit binds to the DNA, and after incision a subset of the excinuclease unit remains bound to the DNA. Catalytic turnover of the enzyme is facilitated with proliferating cell nuclear antigen (PCNA). The excinuclease subunit is replaced by repair synthesis proteins and subsequently gap filling and ligation take place. Recently, the human excision repair gene ERCC-1, part of NER, was shown to be more involved in the repair of ICL's than of intrastrand adducts (Larminat and Bohr, 1994). ERCC-1 is a determinant for resistance in CHO cells (Lee et al., 1993). The ERCC-1 gene encodes a protein of 297 amino acids of which the first 214 amino acids show a high degree of homology with the sequence of the yeast RAD10, which is required for both NER and mitotic recombination (van Duin et al., 1986).

Transcription and repair are closely coupled processes (Selby and Sancar, 1990; Drapkin et al., 1994; Feaver et al., 1993). Some repair proteins participate in initiation or elongation steps of transcription and thus couple repair to transcription (Bootsma and Hoeijmakers, 1993; Hanawalt, 1994; Hanawalt et al., 1994a; Sancar, 1994; Donahue et al., 1994). In addition, there is preferential repair of the transcribed strands of expressed genes (Bohr et al., 1985; Mellon et al., 1987; Mellon and Hanawalt, 1989; Hanawalt and Mellon, 1993). Furthermore, gene products implicated in transcription-coupled repair are defective in three human hereditary disorders (xeroderma pigmentosum XP, Cockayne's syndrome and trichthiodystrophy) (Venema et al., 1990; Hanawalt, 1994). In XPC cells only the transcribed strands of active genes are repaired whereas in Cockayne B cells there is no preferental repair of active genes. Recent studies (Verhage et al., 1996) in *S. cerevisiae* revealed that there exist indeed two subpathways for nucleotide excision repair: one pathway (TCR) is coupled to transcription whilst the other is a global system (GGR) that can repair the whole genome. TCR is more efficient than GGR resulting in preferential repair of transcribed strands. The GGR system is dependent of the presence of the *RAD7* and *RAD16* gene products (Verhage et al., 1994). *rad7* and *rad16* mutants have the same repair phenotype as cells derived from XPC patients. The TCR system is dependent on transcription and is

stimulated by the *RAD26* gene product. *RAD26* is the yeast homologue of the human ERCC/CS-B gene (van Gool et al., 1994).

1.3.2 Assays for measurement of cisplatin-DNA adducts

After exposure to cisplatin DNA adducts can be determined quantitatively using a number of different procedures. One of the assays measures total Pt bound to DNA with atomic spectroscopy (AAS). This method has frequently been used in *in vitro* and *in vivo* experiments (Reed et al., 1988; Ma et al., 1994, 1995). Studies were directed to validate this procedure (chapters 2 and 7 and Ma et al., 1994, 1995) and reproducible. The limitations of AAS are that the assay does not discriminate between the individual adducts or monofunctional and bifunctional bound adducts. In addition, *in vitro* cisplatin concentrations have to be used exceeding the IC90 value in the cell culture system. This significantly limits the interpretation of the results. An elegant but extremely laborious ELISA method has been developed which can identify the (bifunctional) individual adducts (Fichtinger-Schepman et al., 1987, 1989, 1990). The P^{32}-postlabeling assay is a promising alternative to the ELISA (Försti and Hemminki, 1994; Blommaert and Saris, 1995, Maliepaard et al., 1996). It quantitates the 2 major intrastrand adducts (Pt-GG and Pt-AG) at very low concentrations (at the fmol range) and has a higher sensitivity than AAS. The P^{32}-postlabeling was shown to be 2 to 10 fold more sensitive than the ELISA (Blommaert and Saris, 1995). Another great advantage is that the assay can also be used for the quantitation of DNA adducts originating from other platinum analogues, such as carboplatin or lobaplatin and oxaliplatin. ICL's can be quantitated with an ethidium bromide or an alkaline elution method (de Jong et al., 1986; Ali-Osman et al., 1993).

Comparison of the previously described ELISA and AAS methods for the quantitation of adduct levels reveals striking differences. The adduct levels in WBC of patients measured with the ELISA method of Reed et al. (1986, 1990) are substantially lower (amol/μgDNA-range) than when the adduct levels are measured with the AAS method (fmol/μgDNA-range) of Reed et al. (1988 and 1993). This methodological difference has not fully been explained (Poirier et al., 1993). The adduct levels measured with AAS showed very high interpatient variation (>100-fold) in patients with ovarian cancer who were treated with cisplatin in cycles of 3 weeks (Parker et al., 1991). In several studies, using ELISA or AAS, the range of adduct levels in the responders and nonresponders was almost completely overlapping (Reed et al., 1988a, 1990, 1993; Parker et al., 1995). The ELISA method developed by Fichtinger-Schepman showed adduct levels in the same range as the AAS method of Reed et al. (1988) and the validated AAS method developed by Ma et al. (1994), which is presented in chapters 2, 3 and 7. A new WBC sample preparation

method (Ma et al., 1995) demonstrated that DNA-adduct levels shortly post-infusion and DNA-repair in WBC by the method of Fichtinger-Schepman et al. (1987, 1990) are overestimated. The range of the adduct levels determined with the new method shows only 5-fold interpatient variation (chapter 2). The mean adduct level was about 5-fold lower than the mean level reported by Reed (1993) in the patients who were treated with combination of cisplatin and carboplatin and it was in accordance with the range of data in the ELISA of Fichtinger-Schepman et al., (1987) although, as outlined, the adduct levels in the latter method shortly post-cisplatin infusion are higher. Another indirect method for the measurement of the dynamics of cisplatin is the measurement of adducts in buccal smears (Blommaert et al., 1994). These data correlated with the adduct levels in WBC which were measured in the same patients. Recently, another promising method for quantitation of DNA-adducts using an antibody technique has been described (Meijer et al., 1995) This observation supports the concept that adduct formation in WBC parallels adduct formation in other tissues which most likely includes tumor tissue.

1.3.3 Relationship between adduct kinetics and cytotoxicity

The level of DNA adduct formation and the rate of repair are correlated to the cytotoxicity of cisplatin (Eastman and Schulte, 1988; Johnson et al., 1994a). Differential repair of platinum DNA adducts has been observed in human bladder and testicular cell lines which are resistant and sensitive to cisplatin respectively (Bedford et al., 1988). The sensitive testicular cancer cell lines were deficient in the repair of intrastrand crosslinks as well as ICL's. Resistant sublines derived from cisplatin sensitive human ovarian cancer cell lines showed significantly higher levels of total DNA platination levels as well as ICL's at equitoxic concentrations indicating that increased DNA-damage tolerance may also be a component of the resistance mechanism (Johnson et al., 1994 and 1994a). The analysis of cell death induced by the presence of cisplatin DNA adducts reveals DNA fragmentation and subsequent processes consistent with induction of programmed cell death or apoptosis (Chu, 1994).

1.3.4 Relationship between adduct formation and antitumor response

Due to lack of tissue samples and several technical difficulties only a small number of patient tissues have been analyzed for cisplatin DNA-adduct formation. In a series of studies an ELISA and an AAS method were used to correlate cisplatin-DNA adducts in WBC with the antitumor response (Reed et al., 1986, 1988a, 1990, 1993; Fichtinger-Schepman et al., 1987, 1990; Parker et al., 1991). In these studies significantly higher adduct levels post cisplatin infusion were found in patients who showed a partial or complete response than in nonresponding patients (stable or progressive disease). With the ELISA

method the pattern of individual adducts was determined whereas with the AAS method the total number of adducts was quantitated. It is important to realize that the relative occurrence of the four adducts obtained from DNA of the blood of testicular cancer patients and a sample from a testicular tumor of a patient was strikingly similar (Fichtinger-Schepman et al., 1990). In addition, the relative distribution is also very similar to the distribution of the adducts in various normal tissues and in a tumor in rats (Fichtinger-Schepman et al., 1989) and similar to the adducts induced in cultured human cells such as bladder and testicular cell lines (Bedford et al., 1988), normal and repair deficient human fibroblasts (Dijt et al., 1988) and multiple tissues of cancer patients (Poirier et al., 1992, 1993). From these results it can be postulated that measurement of the total number of adducts in WBC instead of the pattern of individual adducts does not reduce the discriminative power of the method if clinical antitumor response is correlated to DNA-adduct formation in WBC. No data are available on a relationship between the profile of the individual adducts in WBC and tumor tissue in patients and the antitumor response.

1.3.5 Mechanisms of cisplatin resistance

Resistance to cisplatin is multi-factorial. It includes reduced accumulation of platinum, increased platinum-DNA repair, and increased cellular detoxification systems by GSH and metallothioneins (Andrews et al., 1991; Kelland et al., 1992a, 1992b; Schellens et al., 1995; Eastman et al., 1988; Hosper et al, 1988). It is believed that the cell's ability to repair either Pt-GG or Pt-AG adducts contributes to cisplatin resistance (Fichtinger-Schepman et al., 1985; Plooy et al., 1985; Hospers et al., 1990; Eastman et al., 1988; Calsou et al., 1992). There appears to be a relationship between repair of cisplatin-DNA damage and cytotoxicity *in vitro* (Scanlon et al., 1989 and 1991) and *in vivo* (Kashani-Sabet et al., 1990). Several enzymes involved in the process of DNA-repair have been found to be involved in the development or existance of cisplatin resistance (Timmer-Bosscha et al., 1992).

As outlined, gene selective removal of adducts has been demonstrated, which may contribute to a resistant phenotype (Jones et al., 1991). Several other biochemical pathways of resistance have been described *in vitro*, however the contribution to development or existance of resistance *in vivo* has not yet been clearly established. These mechanisms relate to reduced cellular uptake of cisplatin (Scanlon et al., 1989 and 1991; Morikage et al., 1993), increased intracellular inactivation by glutathione (GSH) and/or metallothioneins (Hospers et al., 1988; Kelland et al., 1994; Andrews, 1994), or disruption of signal transduction pathways which may contribute to the evolution of drug resistant clones (Scanlon et al., 1991). Possibly, also

increased efflux of cisplatin-glutathione complexes through the multidrug related protein (MRP) pump contributes to resistance (Ishikawa et al., 1994; Müller et al., 1994). In addition, replicative bypass of platinum-DNA adducts may also be associated with cisplatin resistance (Mamenta et al., 1994). Presently, third generation platinum II and IV analogues are under clinical investigation. Preclinical tumor models have revealed high cytotoxic activity in cell systems with documented cisplatin resistance and circumvention of outlined resistance mechanisms (Kelland et al., 1992a)

1.3.6 Circumvention of cisplatin resistance

As the mechanisms of cisplatin resistance become more and more elucidated, rational strategies emerge to exploit or circumvent the phenotypic properties of cisplatin resistant cells (Scanlon et al., 1991). Biochemical modulators have been identified that modulate cisplatin resistance by inhibition of DNA-repair, increase the reduced cisplatin cellular accumulation, depletion of cellular GSH, and interference with the signal transduction or through schedule-dependent cytotoxic combinations (Timmer-Bosscha et al., 1992; Morikake et al., 1993). Increase in the dose-intensity is another clinically important approach to prevent development of resistance (Ozols, 1989). The latter approach is based on the established relationship between concentration and cell kill *in vitro* and dose-intensity and antitumor response *in vivo* (Ozols, 1989; Rosso et al., 1991; Kaye et al., 1992; Dittrich et al., 1993). One of the schedule dependent modulators of cytotoxicity is etoposide (VP16), an inhibitor of topoisomerase II. This is one of the nuclear enzymes involved in the repair of cisplatin induced DNA-damage (Eder et al., 1990; Tepper and Studzinsky, 1992; Ali-Osman et al., 1993). In several clinical studies cisplatin and etoposide have been shown to act synergistically (Hainsworth et al., 1992; O'Dwyer et al., 1985; Bonomi, 1986; Evans et al., 1986; Henwood and Brogden, 1990; Murphy et al., 1992), without significant mutual pharmacokinetic interactions (Minami et al., 1991). In addition, topoisomerase II inhibition by VP16 and novobiocin *in vitro* resulted in more than additive cytotoxicity when combined with cisplatin (Eder et al., 1990; de Jong et al., 1993). Inhibition of topoisomerase II *in vitro* impaired cisplatin DNA adduct repair in a glioblastoma cell line (Ali-Osman et al., 1993). Inhibition of topoisomerase II activity *in vitro* with a selective topoisomerase II inhibitor novobiocin resulted in reduction of cisplatin induced DNA-repair and synergistic cytotoxicity (Eder et al., 1990).

1.4 INTERPATIENT VARIATION IN PHARMACOKINETICS AND ADDUCT FORMATION OF CISPLATIN

It is not sufficiently elucidated which clinical, biochemical and pharmacologic parameters contribute to the observed interpatient variation in drug disposition and DNA adduct kinetics. After administration of the same dose or narrow dose-range three- to four-fold variation has been observed in the terminal half-life, AUC and volume of distribution of unbound cisplatin (Corden et al., 1985; Himmelstein et al., 1981; Vermorken et al., 1982, 1986). Previous limited studies have suggested that the AUC of unbound cisplatin correlates with the creatinine clearance and serum albumin although the latter correlation coefficient was relatively low (0.49, $p=0.002$) (Vermorken et al, 1986; Tanabe et al., 1991). These results were obtained with single linear regression analysis. Our results indicate significant but also relatively low correlations between the dose and the AUC and between the creatinine clearance and the AUC (Schellens et al., 1996a). The renal clearance of cisplatin which is 50-200 ml/min contributes for only 20-30% to the plasma clearance of unbound cisplatin which is of the order of 250-600 ml/min (Vermorken et al., 1986). Hence unbound cisplatin disappears mainly through other pathways such as binding to plasma and tissue components. In addition, no data are available to describe the inter- and intrapatient variability in DNA adduct formation in terms of variation in clinical, biochemical and pharmacological parameters. Considering the almost lacking population kinetic data of cisplatin (Thomson and Whiting, 1992) there is a strong need for characterization and quantitation of sources of variability in prospective studies.

1.5 CLINICAL PHARMACOKINETICS AND DYNAMICS OF THE CISPLATIN ANALOGUE CARBOPLATIN

Carboplatin is a cisplatin analogue which is frequently applied in the treatment of solid tumors, for example testicular, ovarian cancer and small cell lung cancer (Childs et al., 1992; Jodrell et al., 1992; Posthumus et al., 1992). The cytotoxic effect is also mediated through DNA adduct formation (Hengstler et al., 1992). Relationships between pharmacokinetics (AUC) and dynamics (thrombocytopenia) have allowed the development of equations for rational dosage individualization (van der Vijgh, 1991). Simple formulae have been established based on renal function to individualize the dose of carboplatin and achieve a predefined target AUC (Egorin et al., 1985, 1992; Calvert et al., 1989; Newell et al., 1993). There was evidence for an AUC-response relationship in a study in good prognosis metastatic nonseminomatous testis tumors (Childs et al., 1992). There was also a significant relationship between the AUC and the toxicity in patients with

ovarian cancer (Jodrell et al., 1992; Reyno et al., 1993). For carboplatin a pharmacologic based dosing allows a better prediction of toxicity to normal tissues (Jodrell et al., 1992; Hande, 1993). In addition, there appeared to exist an AUC-antitumor response relationship (Childs et al., 1992). No randomized study results are available to compare the fixed dose regimen with the dose-individualized approach, which is proposed for cisplatin. The outlined pharmacokinetic-dynamic relationship of carboplatin greatly encourages the development and prospective evaluation of a pharmacologic-based dosing method for cisplatin.

1.6 TOPOISOMERASE (TOPO) I INHIBITORS: CLINICAL APPLICATION

A number of topo I inhibitors are presently undergoing clinical testing: irinotecan (CPT11), topotecan, 9-amino-20(S)-campthothecin and GI-147211 (fig.4) (Tanizama et al., 1994). Phase II clinical studies yielded favourable response rates of ± 25% in colorectal cancer, 32% in nonsmall cell lung

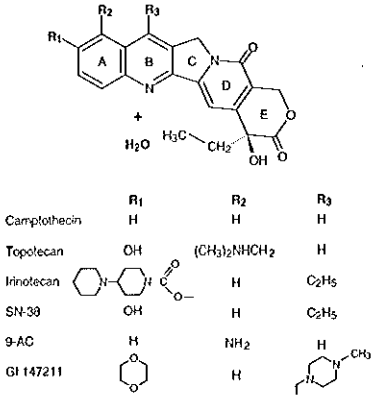

Figure 4. Chemical structure of topoisomerase I inhibitors.

cancer, 30% in non-Hodgkin lymphoma and 25% in acute leukaemia. The dose limiting toxicities are neutropenia and diarrhea. Other side effects are thrombocytopenia and anaemia, alopecia, nausea and vomiting and fatigue (Schellens et al., 1996b). Using the recommended dose for phase II testing of 60 mg/m^2 of irinotecan on days 1, 8 and 15 and 80 mg/m^2 of cisplatin on day 1 every 4 weeks, Masuda et al. (1992) reported a high response rate of 54% combined with acceptable toxicities in advanced non-small cell lung cancer.

Phase II trials with topotecan are ongoing and favourable response rates have been observed in ovarian cancer and small cell lung cancer. Preliminary evaluation of the activity of topotecan revealed responses in head and neck cancer, in soft tissue sarcoma and in glioma (Schellens et al., 1996 for review). The bioavailability of topotecan after oral administration of 32 ± 11.5% illustrates significant exposure to the drug (Schellens et al., 1996b). Repeated oral administration would enable chronic treatment and reduce the inconvenience of intravenous administration. Topoisomerase I inhibitors are of great clinical interest because of their unique mechanism of action, high activity in preclinical tumor models and high expression of the enzyme in various human tumor types (van der Zee et al., 1994; Slichenmeyer et al., 1993; Potmesil et al., 1994; McLeod et al., 1994).

1.7 PHARMACODYNAMICS OF TOPO I INHIBITORS

1.7.1 Mechanism of action

DNA-topoisomerases are essential nuclear enzymes that are involved in multiple nuclear functions such as chromosomal recombination, DNA synthesis and repair, transcription and chromatic assembly (Pommier et al., 1985; Wang et al., 1985; Liu, 1989; Gedik et al., 1990). Topo I produces single strand breaks by forming a covalent intermediate between topo I and DNA, the so-called cleavable complex (Liu, 1989; Liu et al., 1993). Topo I poisons reversibly inhibit topo I activity by stabilizing the cleavable complex. This results in collision of the replication fork and finally double strand breaks and cell death (Tsao et al., 1993). In addition to reversible fragmentation of DNA and inhibition of DNA and RNA synthesis, topo I inhibitors induce a number of cellular responses such as S-phase-specific cytotoxicity, G2-phase cell cycle arrest, cellular differentiation and sister-chromatid exchange (Tsao et al., 1993; Potmesil, 1994). Camptothecin analogues undergo a pH dependent reversible hydrolysis yielding the lactone ring-opened hydroxy acid form. At physiologic pH the hydroxy acid form, which is pharmacologically inactive, predominates. Preclinical testing revealed impressive cytotoxicity against a large number of experimental tumor models (Kingsbury et al., 1991). *In vitro* studies have also depicted that the cytotoxicity is greatly increased by prolonged exposure to the drug (Burris et al., 1992).

In yeast, three topo enzymes have been characterized encoded by the genes *TOP1*, *TOP2* and *TOP3* (Kim and Wang, 1992). The strain *Saccharomyces (S.) cerevisia* and many others have been used as a model to study the cytotoxic activity of the classical topo I poison camptothecin (Bjornsti et al., 1989, 1994). Studies with yeast DNA topo mutants indicate that topo I is normally required to relax transcriptionally induced supercoils (Brill and

Sternglanz, 1988). In *S. cerevisiae* a general transcription repression and reduction of mRNA's was observed in the stationary cell phase which was dependent on topo I (Choder, 1991). Overall, yeast was found to be a excellent model to study the topo I enzyme and pharmacodynamics of the topo I inhibitor camptothecin (Bjornsti, 1994; Nitiss and Wang, 1988).

1.7.2 Mechanism of resistance

Unfortunately, existence or development of drug resistance to these agents is a matter of clinical importance. The etiology seems to be multifactorial, involving decreased conversion of irinotecan to SN38 in resistant tumor cell lines (Kanzawa et al., 1990), reduced topo I content, or catalytic activity as well as decreased formation of cleavable complex were noted in some of those resistant cell lines (Sorensen et al., 1995; Fujimori et al., 1995). Also, presence of a mutant form of the enzyme or rearrangement of the gene of this enzyme have been reported (Sugimoto et al., 1990; Madelaine et al., 1993;). Reduced accumulation of topotecan, due to Pgp overexpression, has been reported to yield resistance to topotecan as well (Hendricks et al., 1992). In addition, resistance may involve increased ability of cells to repair DNA-breaks (Beck and Danks, 1991; Chen and Liu, 1994). The implications of these mechanisms have to be considered in ongoing and future (clinical) studies. In yeast, single amino acid mutants of *S. cerevisiae* have been developed which were significantly resistant to camptothecin (Knab et al., 1993).

1.7.3 Rationale for the combination of cisplatin and topo I inhibitors

Preclinical studies have revealed strong synergy between topo I inhibitors and cisplatin (Kano et al., 1992; Masumoto et al., 1995; Chou et al., 1994; Schellens et al., 1995a). Of note, the combination was synergistic in a cisplatin resistant human ovarian cancer cell line (Schellens et al., 1995a). Topo I is highly expressed in tumors of the colon, cervix (McLeod et al., 1994), ovaries (van der Zee et al., 1994) and prostate (Husain et al., 1994). In the clinic, topo I inhibitors are potent anticancer agents with a pattern of toxicity which does not overlap with cisplatin. In addition, it is of great clinical interest to determine the effect of combinations of a topo I inhibitor and new third generation platinum compounds in cisplatin resistance.

1.8 TAXOID DRUGS: PACLITAXEL AND DOCETAXEL

1.8.1 Mechanism and clinical application

Paclitaxel (fig.5) is a plant-derived antitumor agent which was extracted in the later 1960's from the bark of pacific yew and has shown high activity in a

variety of solid tumors (Spencer et al. 1994; Rowinsky 1991, 1991a). Docetaxel is another novel compound with the taxene ring (fig.5) which has been found to be extremely potent against different types of mouse and human cancer

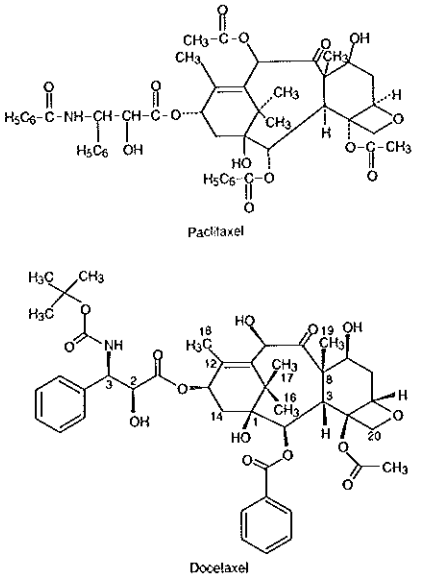

Figure 5. Chemical structure of paclitaxel and docetaxel.

models *in vitro* and *in vivo* (Verweij et al., 1994). Clinical studies with docetaxel have revealed high activity against a great number of solid tumors (Schellens et al., 1996b for review). The mechanism of action of paclitaxel and docetaxel is associated with preferential and reversible binding to tubulin subunits in the microtubule system (Schiff et al.,1980; Parness and Horwitz, 1981; Manfredi and Horwitz, 1984). The binding of taxoids enhances polymerization of the tubulin into stable microtubules and inhibits microtubule depolymerisation, thereby inducing the formation of stable microtubule bundles (Rowinsky and Donehower, 1991; Horwitz, 1992). This disruption of the normal equilibrium finally leads to cell death. In preclinical studies the activity of docetaxel was about 2.5-fold higher than that of paclitaxel (Kelland and Abel, 1992; Bissery et al., 1991).

In the phase I/II trials, both paclitaxel and docetaxel show promising activity in the treatment of ovarian cancer, breast cancer and NSCLC (Schellens et al.,1996). The toxicity profiles show many similarities and include early and short time-lasting neutropenia and thrombocytopenia. The non-

hematologic toxicity consist for example of nausea and/vomiting, diarrhoea and mucositis. Docetaxel is associated with fluid retention.

1.8.2 Rational for the combination of cisplatin and docetaxel

Cisplatin and docetaxel have documented high activity against a number of solid tumors. As outlined, the mechanism of action of the two drugs is different, cisplatin acts through direct induction of DNA-damage, whereas docetaxel inhibits the tubulin/microtubule system. The toxicity pattern of cisplatin and docetaxel is only partly overlapping. Cisplatin mainly induces neurotoxicity and renal toxicity, whereas the dose-limiting toxicity of docetaxel is myelosuppression and fluid retention. These characteristics form a strong basis for evaluation of the feasibility of combination chemotherapy. For investigation of the optimal combination two sequences of administration should be evaluated: docetaxel followed by cisplatin and the reverse sequence. Pharmacokinetic-dynamic analysis should reveal mechanisms of any sequence related influence on the dose-effect relationship of the two drugs. A sequence dependent effect on the toxicity has been described for the combination of cisplatin and paclitaxel. Cisplatin given prior to paclitaxel was associated with increased myelosuppression, which may be related to a reduction in clearance of paclitaxel by cisplatin (Rowinsky et al., 1991a).

1.9 SCOPE OF THE INVESTIGATIONS

1.9.1 General objectives

The aim of the studies was to investigate concentration-effect relationships of cisplatin in clinical and preclinical models when given as single agent and in combination with topoisomerase I inhibitors or taxanes. Cisplatin was applied with or without daily low-dose etoposide in a large clinical pharmacologic study to characterize concentration-effect relationships of cisplatin when given on a weekly dose-intensive schedule. The pharmacodynamics were determined as well as the formation of DNA-adducts in white blood cells, a surrogate pharmacodynamic end-point. Furthermore, two sequences of administration of the combination of cisplatin and docetaxel were investigated in a large phase I/II study in patients with various types of solid tumors. The pharmacokinetics of both compounds were investigated as well as relationships between pharmacokinetics and toxicity.

1.9.2 Analytical procedures

For any pharmacokinetic and pharmacodynamic study it is a prerequisite to measure concentrations of active drug and/or metabolites and pharmacodynamic end-points. For cisplatin, an AAS method for the measurement of total and unbound platinum and cisplatin induced DNA-adducts was validated as well as a method for optimal WBC sample preparation.

1.9.3 Studies using topoisomerase I inhibitors and taxanes

Topo I inhibitors and taxanes are promising novel anticancer agents which are currently investigated in patients as single agent and in combination with other anticancer agents. In this thesis mechanisms of interaction between cisplatin and the topo I inhibitors topotecan and SN38 (active metabolite of irinotecan) and between cisplatin and the taxanes paclitaxel and docetaxel have been investigated *in vitro*.

Furthermore, experiments have been directed to unravel mechanism of resistance of sublines of the human ovarian cancer cell line IGROV-1 to cisplatin and topotecan.

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Clinical Studies

CHAPTER 2

CURRENT SAMPLE HANDLING METHODS FOR MEASUREMENT OF PLATINUM-DNA ADDUCTS IN LEUCOCYTES IN MAN LEAD TO DISCREPANT RESULTS IN DNA ADDUCT LEVELS AND DNA REPAIR

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SUMMARY

DNA-adduct levels were measured with atomic spectroscopy in white blood cells (WBCs) of patients with solid tumours who were treated with six weekly courses of cisplatin. In 21 patients (I) the WBCs were collected after thawing frozen whole blood samples according to a previously described method. In 32 other patients (II) WBC were collected immediately after blood sample collection. The two methods for WBC collection were also compared *in vitro*. The maximal DNA-adduct levels *in vivo* after the first course in I were 2.48 ± 1.14 and in II 1.28 ± 0.40 Pg of platinum per μg of DNA ($p < 0.0001$). The DNA-'repair' in the first course (DNA-adduct level at the end of the infusion minus 15 h post infusion) was in I 40 ± 29% and in II 18 ± 29% ($p = 0.009$). These differences were consistent in all measured courses. *In vitro*, the DNA-adduct levels in the freshly prepared WBC were significantly lower at 0, 1 and 4, but not 24 h, after start of the incubation with cisplatin than in the WBC collected after freezing and thawing the blood sample. The same experiment with carboplatin *in vitro* resulted also in significantly lower adducts in freshly isolated WBCs. The higher DNA-adduct levels and DNA-'repair' in I are caused by remaining unbound cisplatin in the sample tubes, which can form DNA-adducts *ex-vivo*. The same results *in vivo* can be anticipated when carboplatin is used.

INTRODUCTION

cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most potent anticancer agents with substantial clinical activity against testicular cancer, ovarian cancer, head and neck (H/N) cancer and other tumours (Loehrer & Einhorn, 1984; Reed *et al.*, 1987). The antitumour activity of cisplatin correlates with its interaction with DNA, resulting in a limited number of inter- and intrastrand crosslinks ("adducts") (Fichtinger-Schepman *et al.*, 1990). In previous studies wide interpatient variation was established in the DNA-adduct kinetics in white blood cells WBCs, which was monitored with different enzyme-linked immunosorbent assays (ELISAs) as well as with atomic spectroscopy (AAS) (Reed *et al.*, 1987 and 1988 and 1990; Fichtinger-Schepman *et al.*, 1987 and 1989 and 1989a; Parker *et al.*, 1991). The DNA adduct levels in WBCs correlated to the clinical response in testicular, ovarian and other cancer patients who were treated with cisplatin, however substantial overlap in DNA adduct levels was observed between responding and non-responding patients (Reed *et al.*, 1987 and 1988 and 1993). It is important to realize that quantitation of the DNA adduct kinetics with different published ELISA and AAS methods (Fichtinger-Schepman *et al.*, 1987 and 1990; Reed *et al.*, 1987 and 1987a and 1988 and 1988a; Parker *et al.*, 1991; Poirier *et al.*, 1993) and different sample preparation techniques (Reed *et al.*, 1987; Fichtinger-

Schepman *et al.*, 1987 and 1990) resulted in >10-fold differences in the DNA-adduct levels and shape of the DNA adduct-time curve in patients treated with the same dose range of cisplatin. Hence cross-validation of the published methods is very much needed.

We monitored the DNA adduct kinetics in WBC of patients who were treated with six weekly courses of cisplatin. In the first group of patients we applied the protocol for sample preparation which is described by Fichtinger-Schepman *et al.* (1987 and 1990) and which is also applied in other ongoing studies. During the study we modified the sample preparation procedure, because we suspected that application of the previously published method resulted in overestimation of the DNA adduct levels shortly post-infusion and of the DNA-repair. We describe the two procedures and the influence on the quantitation of the DNA adduct levels *in vivo*. We measured the DNA adduct-time curve from WBCs in patients to determine the time point at which the DNA adduct level reaches its maximal value. Furthermore, we simulated the two sample preparation procedures *in vitro* using cisplatin as well as carboplatin as model substrates, to clearly identify the mechanism behind the difference of the two assays.

PATIENT SELECTION, MATERIALS AND METHODS

Patient selection and treatment schedule

All patients gave informed consent according to local regulatory requirements. Eligibility for the clinical study required a pathologically confirmed mesothelioma, melanoma, non-small-cell lung cancer, colon cancer, adenocarcinoma of unknown primary or H/N cancer. The performance status had to be ≤1 on the WHO scale and life expectancy ≥3 months. Each patient had a complete medical history taken and underwent physical examination, complete blood count and platelet count and serum tests. All patients had adequate renal and liver function, i.e. serum creatinine < 120 μmol/l (1.4 mg/dl) and total bilirubin < 25 μmol/l (1.5 mg/dl), WBC > 4.0×10^9/l and platelet count >100×10^9/l. Additional pretreatment investigations included an audiogram and a neurologic examination.

H/N cancer patients received six weekly courses of 80 mg/m^2 cisplatin. Patients with colon cancer received 75 mg/m^2 cisplatin in combination with oral VP16 (50 mg, day 1-15 and 29-43) and all other patients 70 mg/m^2 cisplatin in 6 weekly courses on day 1, 8, 15, 29, 36 and 43 in combination with oral VP16. Cisplatin was dissolved in a 3% sodium chloride solution and administered as a 3 hour infusion with pre- and posthydration.

Blood sample collection

In all patients 20 ml heparinised blood samples were drawn during the first, third and sixth course: before cisplatin infusion (baseline), after the end of the infusion and 15 hours after the end of the infusion. In the first group of 21 patients (I) the second blood sample was taken after the end of the infusion, whereas in the second group of 32 patients (II) the sample was taken at 60 min after the end of the infusion.

The whole blood samples collected in group I were frozen immediately at -80°C until analysis. The whole blood samples which were drawn in group II were centrifuged immediately for collection of the WBCs.

In 7 of the 32 patients of group II more blood samples were collected for measurement of the DNA-adduct levels. The sampling times in these patients were at base line and at 0, 1, 2, 5 and 15 hours after the end of the 3-hour infusion of cisplatin.

Chemicals

Platin (Pt) standard solution (500 p.p.m.) and ammonium chloride were obtained from Baker (Deventer, The Netherlands), DNAse I (EC 3.1.21.1) and RNAse (EC 3.1.27.5) from Sigma (St. Louis MO, USA), proteinase K and cesium chloride from Merck (Darmstadt, Germany), sodium dodecyl sulphate (SDS) from Brunschwig (Amsterdam, The Netherlands); all other chemicals from Baker and were of analytical grade or higher.

Instruments

A flameless Perkin-Elmer Model 3030B atomic spectrometer was equipped with an AS60 autosampler and HGA600 controller system (Uberlingen, Germany). The UV spectrophotometer was a Beckman DU62 (Fullerton CA, USA).

Determination of total and unbound platinum in plasma and DNA-adducts in WBCs

Total and unbound platinum and DNA adduct levels were analyzed by AAS according to the method of Reed *et al.* (1988b) with modifications which have previously been described (Ma *et al.*, 1994). The DNA was quantitated by UV spectrophotometry at 260 nm (Kirby *et al.*, 1968).

Collection of WBC from freshly drawn whole blood samples

Immediately after collection of a volume of 20 ml of whole blood from a patient, the sample was centrifuged for 5 minutes at 4°C and 1500 g. The buffy coat of WBC was taken with a glass pipet and transferred to a 50 ml plastic tube (Greiner, type 210261, Alphen a/d Rijn, The Netherlands). Ice-cold phosphate buffered saline (PBS) was added to a final volume of 10 ml.

Remaining red blood cells were lysed by addition of 30 ml of a buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, 1mM EDTA). The mixture was shaken gently and put on ice-water for 10 minutes afterwards. After centrifugation (5 min at 4°C and 1500 g) the WBC were collected and washed with PBS and a Tris-EDTA buffer (10 mM Tris, 2 mM EDTA, pH 7.35). The WBC were resuspended in 9 ml of the Tris-EDTA buffer and stored at -80°C. The DNA was isolated and analyzed within 2 months. The DNA was isolated according to a previously published method (Miller *et al.*, 1988), with minor modifications. Briefly: after thawing the sample 0.9 ml of ammonium bicarbonate (1.1 M) was added. The cell lysates were digested overnight at 42°C with 0.45 ml of 20% SDS and 0.6 ml of a 10 mg/ml proteinase K solution. After the digestion was complete, 3 ml of saturated NaCl (6 M) was added to each tube and shaken vigorously for 15 sec, followed by centrifugation at 2500 g for 15 min. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 50 ml polypropylene tube. Exactly two volumes of room temperature absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a glass pipet, washed twice with 70% ethanol and subsequently dissolved in a Tris-EDTA buffer (10 mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.35). Before quantitating the DNA, it was allowed to dissolve overnight at room temperature.

Collection of WBC from frozen whole blood samples

This method is described by Fichtinger-Schepman *et al.* (1987). Briefly: two portions of 10 ml of frozen whole blood are thawed and a volume of 30 ml of the lysis buffer is added to each of the portions. The rest of the procedure is the same as outlined above.

In vitro simulation of the 2 sample preparation methods

A volume of 120 ml of whole blood was taken from 4 healthy volunteers. The whole blood was incubated with cisplatin (approximately 10 μg/ml) at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After 0 (i.e. no incubation after addition of cisplatin), 1, 4 and 24 hours after start of the incubation 30 ml of blood was taken and divided into two portions. In one portion the WBCs were collected and frozen immediately as described above. The second whole-blood portion was frozen immediately at -80°C. On a second occasion a volume of 30 ml of whole blood was collected from the same volunters and incubated with cisplatin (same concentration of 10 μg/ml). After 0 hours of incubation the sample was divided into two portions: one for immediate collection of WBCs before storage at -80°C and the other for immediate storage at -80°C. These samples were stored at -80°C for only

20 min thawed and processed immediately afterwards. All other samples were stored at -80°C for two weeks. Subsequently, the WBCs were collected as outlined above. The DNA adduct levels of these samples were measured on the same day.

In another experiment a volume of 120 ml was collected from the four volunteers for the incubation experiment with carboplatin. The incubation concentration was 200 μg/ml. The incubation conditions were the same as in the experiment with cisplatin. The storage of duplicate samples for only 20 min at -80°C was not repeated with carboplatin.

To study the influence of addition of RNAse on the DNA yield and DNA-adduct level a separate experiment was carried out. A volume of 20 ml of heparinised whole blood was drawn from four other subjects. The WBCs were collected immediately and the DNA isolated as outlined. The DNA was dissolved in a volume of 2 ml of a Tris-EDTA buffer (10 mmol Tris, 2 mmol EDTA, pH 7.35) and divided into 2 equal portions. RNAse, which was heated before use for 5 min at 80°C, was added to one of the portions to a final concentration of 100 μg/ml and incubated for 2 h at 37°C. The other portion served as control. After 2 hours the DNA was isolated again as outlined by addition of 0.33 ml of saturated NaCl (6 M) and subsequently precipitated with absolute ethanol. The DNA content and the DNA adduct levels were compared with the control samples. Each experiment was carried out using four samples in duplicate.

Statistical analysis

The unpaired student t-test was used to describe differences between the DNA adduct levels *in vivo* in patient groups I and II. The paired student t-test was used to describe differences between the DNA adduct levels *in vitro*. The paired student t-test was also used to describe differences between the DNA adduct levels *in vivo* measured at the end of the infusion of cisplatin and at 1 hour post-infusion in the outlined group of seven patients.

RESULTS

Patients and treatment

The 21 patients of group I received in the first course a dose of 1665 mg/m^2 (mean ± s.d. = 74.0 ± 4.6 mg/m^2) and a total dose of 2610 mg cisplatin (141 ± 16 mg) and the 32 patients of group II 2355 mg/m^2 (73.6 ± 4.8 mg/m^2) and a total dose of 4318 mg (135 ± 21 mg) (I and II not significantly different). Seven patients in group I and ten in group II had H/N cancer and received 80 mg/m^2 cisplatin. Three patients of group I and 1 of group II received 75 mg/m^2 cisplatin. All others received 70 mg/m^2 cisplatin. No

significant differences in age and gender distribution or renal function and plasma protein level were observed between the two groups of patients.

DNA adduct levels in the two groups of patients

The DNA-adduct levels at the three time points during the three observed courses are given in Figure 1. The maximal adduct level (Amax) after the end of the infusion is significantly higher in group I than in group II. The Amax values of the first course were: in group I 2.48 ± 1.14 and in group II 1.28 ± 0.40 pgPt/μgDNA ($p < 0.0001$). In addition, the coefficient of variation of Amax in group I of 46 % was much higher than of Amax in group II, which was 32 %. This was consistent in all measured courses. The base line levels of all courses were not significantly different between group I and II. The levels at 15 hours post infusion were higher in group I during the first ($p = 0.04$) and sixth course ($p = 0.05$) and not significantly different during the third course ($p > 0.3$) (Table 1). In group I the decrease in DNA adduct level between the end of the infusion and 15 hours post-infusion of 40 ± 29 % was significantly greater than in group II, which was 18 ± 29 % ($p = 0.009$). This was consistent in all measured courses (Table 1).

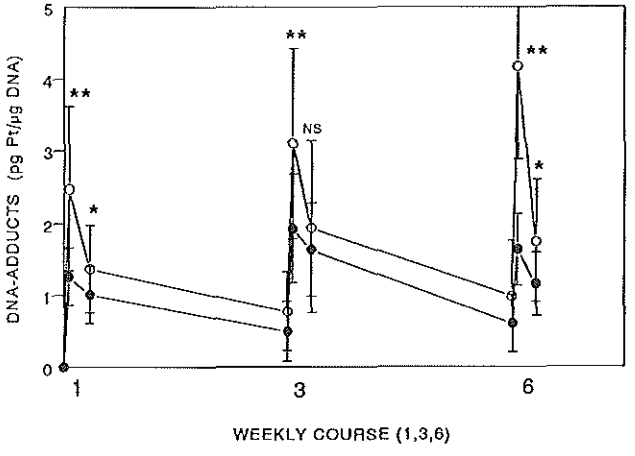

Figure 1. DNA adduct levels in WBC during course 1, 3 and 6 in two groups of patients treated with weekly cisplatin. ○ WBCs were collected after thawing frozen whole blood samples, ● WBC were collected immediately after collection of whole blood samples of the patients. Time points were baseline, end of 3-h infusion (group I) respectively 1 h post infusion (group II) and 15 h post infusion.
**$p < 0.0001$, *$p \leq 0.05$, NS = not significant

DNA adduct-time curve in seven of the patients of group II

The results of measurement of the DNA adduct-time curves are shown in Figure 2. The DNA adduct level at the end of the infusion was 1.01 ± 0.29

Table 1. DNA-adduct levels in WBC of patients treated with 6 weekly courses of cisplatin.

Course	DNA - adduct level (pg Pt/μg DNA)									DNA-'repair'		
	Baseline			A max			A15			%		
	I	II	p	I	II	p	I	II	p	I	II	p
1	0	0	-	2.48±1.14	1.28±0.40	<0.0001	1.37±0.61	1.05±0.48	0.04	40±29	18±29	0.009
3	0.78±0.55	0.52±0.42	NS	3.11±1.32	1.84±0.75	0.002	1.95±1.19	1.64±0.65	NS	39±28	16±22	0.02
6	0.99±0.78	0.61±0.40	NS	4.18±1.29	1.64±0.50	<0.0001	1.75±0.85	1.16±0.44	0.05	60±24	37±25	0.02

The DNA adduct-time points were: baseline, 0 minutes (group I) or 60 minutes (group II) post infusion (A max) and 15 hrs post infusion (A15). Group I consisted of 21 patients and the DNA adduct levels were determined in WBCs which were collected after thawing a frozen whole blood sample. Group II consisted of 32 patients and the WBCs were collected immediately after blood sample collection. NS = not significant. DNA 'repair' (%) is defined as the relative difference between Amax and A15 ([Amax-A15]x100/Amax).

pgPt/μgDNA (n=7) and at 1 h post infusion 1.40 ± 0.25 pgPt/μgDNA (n=7, p=0.013). In all patients the DNA adduct level at 1 hour post infusion was higher than at the end of the infusion.

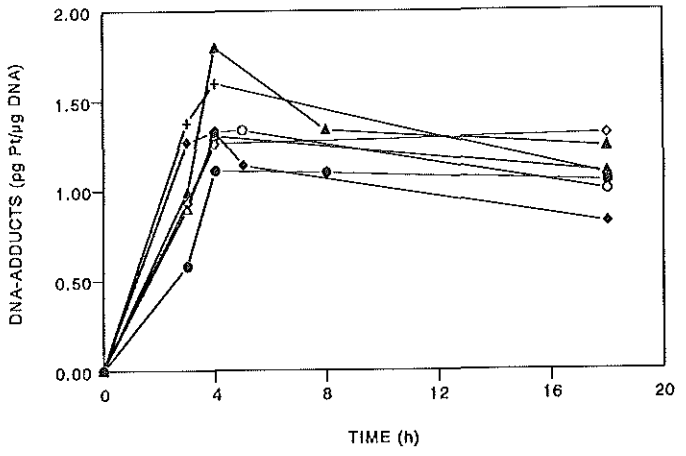

Figure 2. DNA adduct-time curves obtained in seven patients of group II, treated with a dose of cisplatin of 70-80 mg/m^2. WBC were isolated immediately after collection of the whole blood sample.

Difference in DNA adduct levels in vitro in WBCs in the two sample preparation methods

Application of the two sample preparation methods resulted in significantly different levels of DNA adducts after 0, 1 and 4 h of incubation with cisplatin (Figure 3). The DNA adduct levels in the WBCs which were collected after thawing frozen whole blood samples were higher than in the WBCs which were collected immediately after blood sample collection. The DNA adduct level at 0 h after start of the incubation was 494% higher (p<0.0001), at 1 h 110% higher (p<0.0001), at 4 h 19% (p=0.02) and at 24 h only 6% (not significant). The DNA adduct levels in the samples which were stored at -80°C for only 20 minutes were not significantly different from the samples which were stored for 2 weeks at -80°C.

After incubation with carboplatin, the DNA adduct levels in WBCs collected after thawing the frozen whole blood sample were significantly higher at 0, 1, 4 and 24 h of incubation compared with the freshly collected WBC (Figure 4). At 0 hour the DNA adduct level was 335 % higher (p=0.001), at 1 h 168 % (p=0.002), at 4 h 121 % (p=0.008) and at 24 h 11 % (p=0.03) higher than in the freshly isolated WBCs.

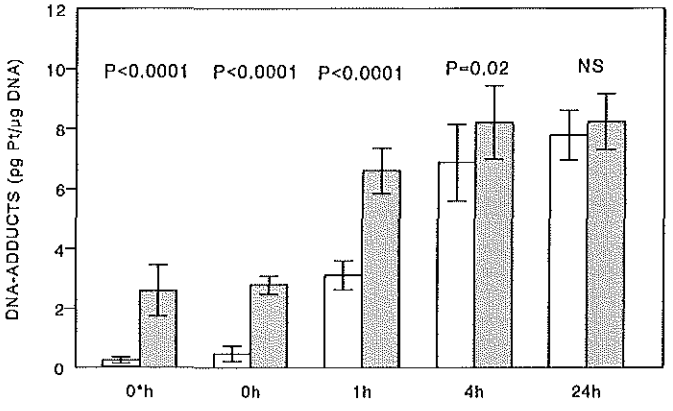

Figure 3. DNA adduct levels in WBC from four healthy subjects after 0*, 0, 1, 4 and 24 hr incubation of whole blood with cisplatin *in vitro*. Open bar: WBCs were isolated immediately after the incubation period. Closed bar: the whole blood samples were frozen immediately post incubation and the WBCs and DNA were collected after thawing the samples. NS=not significant.
0 hr* = samples stored at -80°C for only 20 min, all other samples were stored for 2 weeks at -80°C.

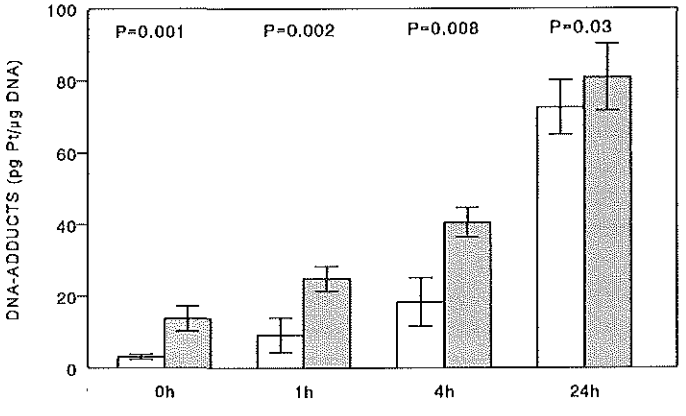

Figure 4. DNA adduct levels in WBCs from four healthy subjects after 0, 1, 4 and 24 h incubation of whole blood with carboplatin *in vitro*. Open bar: WBCs were collected immediately after the incubation period. Closed bar: the whole blood samples were frozen immediately post incubation and the WBC were collected after thawing the samples.

DNA content and purity of the two sample preparation methods

The DNA content of the samples of the freshly prepared WBCs was 186.8 ± 29.9 μg/10 ml of whole blood. The DNA content of the samples of the WBCs which were collected after thawing the frozen sample was 159.2 ± 56.9 μg/10 ml whole blood (not significantly different). The UV ratio 260/280 nm was consistently 1.9-2.0 in both methods. The DNA content of the four samples in duplicate which were treated with RNAse was 214 ± 29 and in the control samples 219 ± 29 μg/10 ml whole blood (not significant). The DNA-adduct levels were 7.28 ± 1.10 after treatment with RNAse and 7.30 ± 0.66 pgPt/μgDNA in the control samples (not significantly different).

Concentration-time profiles of total and unbound platinum in vitro

The concentration-time profiles of total and unbound platinum in the *in vitro* experiment after incubation of cisplatin and carboplatin are shown in Figure 5 and 6. The concentration of total platinum in both experiments is almost constant. The concentration of unbound platinum after incubation of cisplatin decreases with a half-life of 1.0 h. After 1 h the concentration of unbound platinum in the incubation tube was 6.13 ± 0.37 μg/ml, after 4 h 0.75 ± 0.08 and after 24 h 0.48 ± 0.07 μg/ml. After incubation of carboplatin the unbound platinum concentration decreased much more slowly. The half-life *in vitro* was approximately 24 h. After 24 h of incubation the concentration of unbound carboplatin was 54% of the starting level.

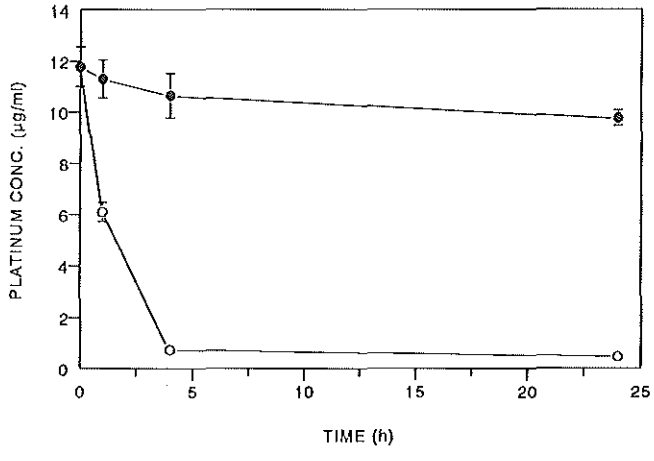

Figure 5. Concentration-time curve of total (●) and unbound (○) platinum *in vitro* in plasma of whole blood incubated with cisplatin at 37°C (mean ± s.d., N=4).

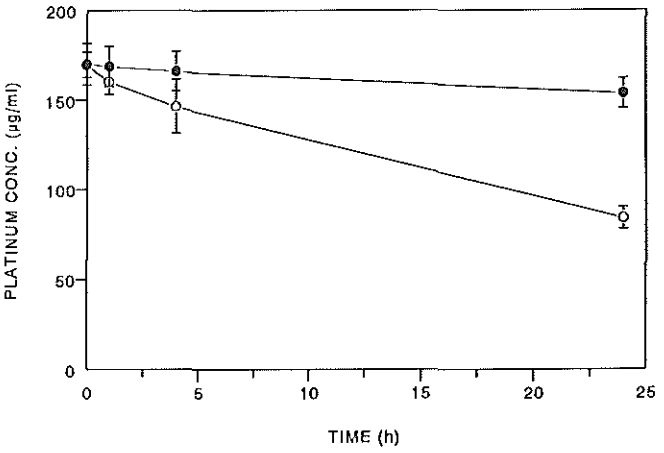

Figure 6. Concentration-time curve of total (●) and unbound (○) platinum *in vitro* in plasma of whole blood incubated with carboplatin at 37°C (mean ± s.d., N=4).

DISCUSSION

The cisplatin-DNA adduct formation and repair has been measured in clinical and preclinical studies in order to correlate the kinetics of DNA adduct formation and repair to the tumour response (Reed *et al.*, 1987 and 1988 and 1990 and 1993; Fichtinger-Schepman *et al.*, 1989 and 1990; Parker *et al.*, 1991; Poirier *et al.*, 1993). In clinical trials the DNA adduct kinetics have been measured in WBC because of the limited access to tumour tissue. The mean level of DNA-adduct formation in responding patients was significantly higher than in non-responding patients, although the range of the data showed an almost complete overlap (Reed *et al.*, 1987 and 1988 and 1993). In a recently published study in patients with germ cell tumours however, no significant positive correlation was observed between DNA adduct formation in WBCs and favourable clinical response (Motzer *et al.*, 1994). Studies on the removal of DNA adducts illustrate that the level decreases more than 40% in the first few hours post infusion, which was interpreted as DNA-repair (Fichtinger-Schepman *et al.*, 1987).

We monitored the kinetics of formation and repair of DNA adducts in WBCs of patients with solid tumours who were treated with six weekly courses of high-dose cisplatin of 70-80 mg/m^2. In the first 21 patients the DNA adduct levels were measured in WBCs, which were collected after thawing frozen whole blood samples drawn at the end of the infusion of cisplatin according to the method described (Fichtinger-Schepman *et al.*, 1987 and 1990). The method of WBCs collection was modified, because we suspected an overestimation of the DNA adduct levels shortly post infusion and an

overestimation of the DNA repair. In addition, we wanted to determine the time point after infusion of cisplatin at which the DNA adduct level in WBC is at its maximum value. In the modified procedure WBCs were isolated immediately after collection of the blood sample of the patient. The differences in DNA adduct levels between the two methods are illustrated in Figure 1. The DNA adduct levels immediately post infusion in the patients denoted as group I are significantly higher than in the patients of group II. The variability in the DNA levels in group I was also higher than in group II, which is illustrated by the higher coefficients of variation.

There was a difference in sampling time of nearly 60 min between the groups I and II. Measurement of the DNA adduct levels at 1 h post infusion in seven patients of group II revealed that the levels were 40% higher than at the end of the infusion. Hence the difference in the sampling time cannot explain the observed difference. In addition, it seems to be favourable to measure DNA adduct levels in WBCs at 1 h post infusion to obtain the best estimate of the maximal DNA adduct level. The half-life of unbound cisplatin *in vivo* is approximately 30 min (Vermorken *et al.*, 1982), which indicates that during the first hour post infusion WBCs are still exposed to significant concentrations of active cisplatin. This appears to be the most reasonable explanation for the increased DNA adduct levels at 1 h post infusion compared to the level at the end of the infusion.

The difference between the DNA adduct levels in group I and II can be explained by the presence of unbound cisplatin in the blood sample tube, which can form DNA-adducts *ex-vivo*. As a consequence of the short half-life of unbound cisplatin, the concentration of unbound cisplatin is almost zero 15 h post infusion. If WBC are isolated immediately after collection of the blood sample, then the redistribution of cisplatin and binding to the DNA in WBCs *ex-vivo* can be prevented. The sharp decline of the DNA adduct level between the end of the infusion and some time the next morning (approximately 15 h later) has been previously interpreted as DNA repair (Fichtinger-Schepman *et al.*, 1987 and 1990). The DNA repair in group II during the first course was on average more than 50 % lower than in the patients of group I (Table 1).

The differences between the DNA-adduct levels at 15 h post infusion in group I and II were much smaller. It was significantly higher during the first course, not significant during the third and marginally significant during the sixth course. This is in line with the almost undetectable concentration of unbound cisplatin in patients at this time point. It cannot be excluded that unbalanced patient selection and variation in the dosage of cisplatin did contribute to the observed differences between group I and II. The mean and s.d. of the administered dose of cisplatin in the two treatment groups

were not significantly different, however the pharmacokinetics of unbound cisplatin may have been different. Variation in the pharmacokinetics may result in differences in the DNA adduct levels between the patient groups, because in a previous study a linear relationship was observed between the exposure to unbound cisplatin and the level of the DNA adduct formation in WBCs (Ma *et al.*, 1994a).

The two methods of WBC collection were simulated *in vitro* to further exclude the contribution of differences in the sampling and pharmacokinetics of cisplatin. The DNA adduct level in WBCs of whole blood incubated *in vitro* with cisplatin was 494% higher after 0 h of incubation and 110% after 1 h of incubation (Figure 3). After 4 h the difference was reduced to 19%, whereas after 24 h the difference was only 6% and not significant any more. The concentration-time curve of cisplatin *in vitro* (Figure 5) illustrates that at 0 and 1 h after start of the incubation high levels of unbound cisplatin are present in the incubation tube, whereas the concentration is much lower at 4 and 24 h after start of the incubation. This experiment illustrates that in WBC DNA adduct formation with remaining unbound cisplatin in the blood sample collection tube will take place. Even immediate storage of the sample tube at -80°C did not prevent DNA adduct formation *ex-vivo*. The combination of high DNA adduct levels shortly post infusion and low levels 15 h later may therefore erroneously be interpreted as high level of DNA repair. The DNA adduct levels in the WBCs stored only for 20 minutes at -80°C were not different from the levels after storage of the WBCs for 2 weeks at -80°C. This indicates that formation of DNA adducts proceeds during thawing and work-up of the sample and not during the storage at -80◦C. We speculate that the high level of DNA adduct formation during the thawing and work-up, in the presence of unbound cisplatin, is caused by an increase in the permeability in the cell membranes of the WBCs. This may result in increased intracellular concentrations of cisplatin and DNA adduct formation.

No significant differences were found in the DNA content of the samples obtained with the two different sample preparation methods. The UV ratio indicated no protein contamination in both methods. The results of the experiment in which RNAse was added before quantitation of DNA and DNA-adduct levels illustrate that RNA contamination is likely to be negligible.

Carboplatin was used as a second substrate, because DNA-adduct formation in WBC has also been studied extensively in patients treated with carboplatin. In addition, assessment of the same relationship between DNA-adduct formation *ex-vivo* and the presence of unbound carboplatin in the sample tubes confirms the mechanism of the described phenomenon. The DNA-adduct levels *in vitro* after incubation with carboplatin are significantly

higher even after 24 h of incubation. The disappearance half-life of carboplatin *in vitro*, however, is much longer than of cisplatin, and after 24 h the concentration of unbound carboplatin is still 54% of the starting level. This explains the significant difference in the DNA adducts levels between the two sample handling methods at this late time point.

WBC collection from frozen whole blood samples is applied in ongoing clinical studies, for example in one carried out within the framework of the World Health Organization. The aim of these studies is to establish the relationship between DNA adduct kinetics and the tumour response in patients with solid tumours. It should be realised that the WBC collection method in these studies will result in overestimation of DNA adduct levels and DNA 'repair'.

In addition, WBCs should be collected immediately after the blood sample has been drawn to further reduce variation in the DNA adduct measurement. Standardization of this procedure is recommended. It is of interest to reveal any contribution of method variation to the inter-patient variability in DNA adduct levels in WBC. It may reduce the observed overlap in DNA adduct levels between responders and non-responders to cisplatin chemotherapy. Also after administration of carboplatin WBCs should be isolated immediately if the blood sample is drawn during the time period that unbound carboplatin in plasma can be anticipated.

We have quantitated DNA adduct levels in WBCs from 43 patients with various types of solid tumours treated with cisplatin at a dose of 70-80 mg/m^2. Preliminary results indicate that the coefficient of variation of the DNA adduct levels at 1 hour post infusion is only 27 % with a less than 6-fold range (Ma *et al.*, 1994b). In our opinion, use of the outlined optimised method reduces the contribution of assay variation to the inter-patient variability in DNA adduct levels in WBC.

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CHAPTER 3

COMPARISON OF ETHANOL PLASMA PROTEIN PRECIPITATION WITH PLASMA ULTRAFILTRATION AND TRICHLOROACETIC ACID PROTEIN PRECIPITATION FOR THE MEASUREMENT OF UNBOUND PLATINUM CONCENTRATIONS

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ABSTRACT

Sample preparation for measurement of nonprotein bound platinum was evaluated by precipitation of plasma proteins with cold ethanol. The method was compared with the routinely used plasma ultrafiltration and with trichloroacetic acid (TCA) protein precipitation. After incubation of human plasma samples with cisplatin or carboplatin unbound platinum concentrations were determined applying Amicon Diaflo ultrafiltration membranes and Millipore ultrafree-MC filters. For protein precipitation 1 ml of cold (-20°C) pure ethanol was added to 0.5 ml of human plasma and the supernatant was collected after 2 hours, or 0.5 ml of cold 20% TCA was added to 0.5 ml of plasma (22). Platinum was analyzed by atomic absorption spectrophotometry (AAS). There were no significant differences between the ethanol and ultrafiltration methods in the unbound platinum concentration. The protein content in the supernatant (1.00 ± 0.20 %) was slightly higher than in the Amicon (0.58 ± 0.05 %) and Millipore (0.55 ± 0.04 %) ultrafiltrates. On average, the TCA and ethanol method seemed to be equally appropriate. The ethanol precipitation method is concluded to be simple, convenient, reproducible and has negligible costs.

INTRODUCTION

The quantitation of the unbound, i.e. nonprotein bound, plasma concentration of platinum is important for determination of the pharmacokinetics of the platinum drugs cisplatin and carboplatin. Only the unbound concentration of the drug is likely to be pharmacologically active. After intravenous administration cisplatin and carboplatin become tightly bound to plasma proteins and other tissue components. Previous studies have revealed that the binding to plasma proteins is covalent and almost irreversible (3, 4). Due to its high reactivity the binding of cisplatin to plasma proteins is very rapid, whereas the rate of binding of carboplatin is much lower (9, 10, 23, 24). Plasma ultrafiltration for the measurement of unbound platinum applying ultrafiltration tubes is routinely used for this assay (2, 6, 7, 9, 11, 16, 17, 23). Mostly a cut off level of the filtration membranes of 30.000 D is used. It has previously been suggested that precipitation of plasma proteins with cold ethanol may be a good alternative to the ultrafiltration of plasma for measurement of unbound platinum (18). In addition, trichloroacetic acid (TCA) has been used to measure unbound platinum (8, 13, 21, 22, 25). Here we report experiments in which the ethanol precipitation method is validated and compared with the results obtained with 2 ultrafiltration systems and the TCA precipitation method.

MATERIAL AND METHODS

Cisplatin was obtained from Lederle (Wolfratshausen, Germany) and carboplatin from Pharmachemie (Haarlem, The Netherlands). Amicon Diaflo ultrafiltration membranes (cut of level 30 000 D) and MAPS-1 Starter Kit were obtained from Amicon (Beverly MA, USA) and Millipore filters (ultrafree-MC millipore cut of level 30 000 D) from Millipore (Bedford MA, USA). The AAS was a Perkin-Elmer 3030B (Uberlingen, Germany). Triton X-100 was obtained from Baker (Deventer, The Netherlands), Cesium Chloride (CsCl) and absolute ethanol (analytical grade, Merck product 983, 99.8%) from Merck (Darmstadt, Germany). All other chemicals were obtained from Baker (Deventer, The Netherlands) and were of analytical grade.

Whole blood samples of 20 ml were obtained from 4 healthy volunteers and plasma was collected. In addition, plasma samples were used from 11 patients with solid tumors who were treated with cisplatin in an ongoing phase II and pharmacologic study for which approval was obtained from the local ethical committee.

Of the 4 volunteers a volume of 10 ml of plasma was incubated with 5 μg/ml of cisplatin or 50 μg/ml of carboplatin at 37°C for 24 hours. Immediately, 1, 2, 4 and 24 hours after addition of the platinum drug, 3 plasma aliquots were taken and used for ultrafiltration at 4°C and 1000 g for 30 minutes, or for addition of 2 volumes of cold (-20°C) ethanol (99.8% absolute). The latter sample was mixed on a whirl mixer for 10 seconds and subsequently stored at -20°C for 4 hours. The sample was then centrifuged at 4°C and 4000 g for 10 minutes and the supernatant was carefully transferred to a clean Eppendorf 2 ml tube and stored at -20°C until analysis. Total and unbound platinum were analyzed after dilution (total platinum with 0.2% triton X-100 and 0.06% CsCl and unbound platinum with deionized H_2O). The protein contents of the plasma samples prior to treatment and of the samples obtained after ultrafiltration and protein precipitation was determined by the Lowry method (14). Also, TCA precipitation of plasma protein was compared with the ethanol method as well as with ultrafiltration. At a second occasion, a volume of 20 ml of whole blood was collected of the same volunteers and used for incubation of plasma with cisplatin and carboplatin. The time periods of incubation were 1 and 24 hours. An equal volume of cold 20% TCA was added to 0.5 ml of plasma and the mixture was shaken on a whirl mixer and put on ice for 10 minutes, as described by Siddik et al. (22). The other assays were carried out as described. All assays were carried out in triplicate or more.

The time period of storage at -20°C of the ethanol treated sample prior to collection of the supernatant was varied to study the influence of storage on the remaining protein content and unbound platinum recovery in the

supernatant. Four 5 ml plasma samples were treated with cisplatin as outlined and stored at -20°C. Aliquots were taken at 0, 1, 2, 4 and 16 (overnight) hours after storage and treated as described.

Unbound platinum concentrations were also determined in the plasma of 11 patients who were treated with a dose of 70 mg/m^2 of cisplatin administered at a weekly schedule and by a 3-hour infusion. A volume of 4 ml of whole blood was taken at 0.5 and 3 hours after the end of the cisplatin infusion and immediately processed according to the outlined ultrafiltration and ethanol precipitation methods.

The two-sided student t-test was used to evaluate differences between the treatment methods.

RESULTS

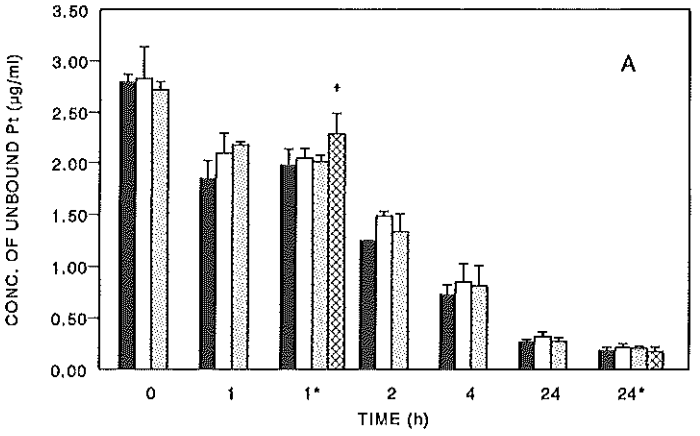
The concentration of unbound platinum in the Amicon ultrafiltrate immediately after addition of cisplatin to plasma was 2.71 ± 0.08 μg/ml, in the Millipore ultrafiltrate 2.83 ± 0.31 μg/ml and in the supernatant after ethanol treatment 2.79 ± 0.08 μg/ml (N=3; not significant (NS)). After carboplatin addition, the concentrations were 26.51 ± 1.24 (Amicon), 27.64 ± 1.02 (Millipore) and 27.53 ± 1.4 (ethanol) (NS). The variation coefficient obtained with the ethanol method was lower or of the same order as with the ultrafiltration methods (figure 1 A and B). The concentrations of total platinum in the plasma samples were 3.07 ± 0.12 μg/ml after cisplatin and 25.14 ± 0.75 μg/ml after carboplatin. The recovery of unbound platinum in the ultrafiltrates and supernatant after ethanol precipitation is given in table 1.

The protein content of the samples after ultrafiltration or ethanol precipitation is given in table 1. The protein amount in the supernatant after ethanol protein precipitation is slightly but significantly higher than in the ultrafiltrates.

Immediate collection of supernatant after addition of cold ethanol to plasma and vortexing resulted in a higher amount of remaining protein (2%) and higher platinum recovery (5-10%). After 2 hours of storage at -20°C the protein amount decreased to 1%, which did not further decrease upon longer storage.

A total of 4 Millipore filters was needed to obtain the same volume of ultrafiltrate as with one Amicon unit. With one Millipore filter a volume of 10 μl ultrafiltrate could be obtained.

Analysis of the Amicon and Millipore ultrafiltrates resulted in much higher background signals on the AAS than of the supernatant after ethanol protein precipitation.



+ p<0.05 compared to the 3 other methods

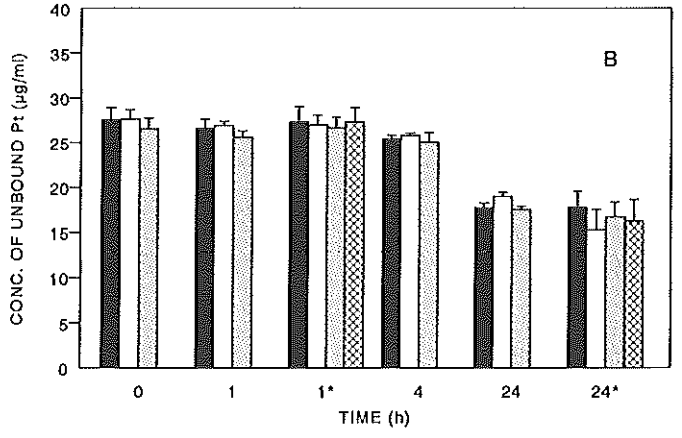


Figure 1. A: Unbound platinum concentration in plasma supernatant ▩ (cold ethanol protein precipitation) and in plasma ultrafiltrates using Millipore II and Amicon ⁞⁞⁞ ultrafiltrates. Plasma samples were incubated with 5 µg/ml of cisplatin *in vitro* and analyzed at different time-points after start of the incubation.
At a second occasion the *in vitro* incubation experiments were repeated using plasma of the same volunteers, which was incubated for 1 hour (1*) and 24 hours (24*) with cisplatin. The TCA method ▦ was compared with the other 3 methods.

B: Unbound platinum concentration in plasma supernatant ▩ (cold ethanol protein precipitation) and in plasma ultrafiltrates using Millipore II and Amicon ⁞⁞⁞ ultrafiltrates. Plasma samples were incubated with 50 µg/ml of carboplatin *in vitro* and analyzed at different time-points after start of the incubation.
At a second occasion the *in vitro* incubation experiments were repeated using plasma of the same volunteers, which was incubated for 1 hour (1*) and 24 hours (24*) with carboplatin. The TCA method ▦ was compared with the other 3 methods.

TABLE 1. COMPARISON OF THREE PLASMA PRE-TREATMENT METHODS FOR MEASUREMENT OF UNBOUND PLATINUM.

METHOD	UNBOUND Pt RECOVERY (%) (CDDP)	UNBOUND Pt RECOVERY (%) (CBDCA)	REMAINING PROTEIN (%)
MILLIPORE FILTER	92±10	110±1	0.58±0.05
ETHANOL PRECIPITATION	91±6	109±4	1.00±0.206*
AMICON FILTER	88±3	105±2	0.55±0.04

CDDP = cisplatin, CBDCA = carboplatin
#: MEAN±SD (n>3)
*: $p<0.05$ compared to Millipore and Amicon

The concentrations of unbound platinum after incubation with cisplatin for 1 hour were slightly (12%), but significantly higher after TCA protein precipitation (figure 1 A). At 24 hours after incubation there were no significant differences between any of the 4 methods. After incubation with carboplatin no significant differences in the unbound platinum concentrations were observed (figure 1B).

The concentrations of unbound platinum in the patient samples at 0.5 hour after the end of the cisplatin infusion were 568 ± 182 (ethanol), 561 ± 231 (Millipore) and 511 ± 198 ng/ml (Amicon) (N=11; NS). At 3 hours post-infusion the concentrations were 69 ± 17 (ethanol), 74 ± 19 (Millipore) and 68 ± 17 ng/ml (Amicon) (N=11; NS).

DISCUSSION

Measurement of the unbound platinum concentration after treatment with cisplatin and carboplatin is essential for the assessment of the pharmacokinetics of these drugs (1, 15, 19, 23). Previous studies have revealed significant relationships between the area under the platinum plasma concentration-time curve (AUC) and likelihood of tumor response and toxicity (1, 5, 12, 15, 19, 20). Results of the present study illustrate that treatment of plasma with cold ethanol to eliminate proteins is adequate to prepare the sample for measurement of nonprotein bound platinum. Comparison with the routinely used ultrafiltration methods reveals that the recovery of unbound platinum is not significantly different from the Amicon and Millipore ultrafiltrates. The % of remaining protein in the supernatant after ethanol protein precipitation is slightly higher than in the ultrafiltrates. The magnitude of the difference seems to be unimportant, because the recovery

is not different from the ultrafiltration method. The concentration of unbound platinum in the samples of patients who were treated with cisplatin also showed no difference between the 3 methods. The recovery of platinum after addition of cisplatin *in vitro* is slightly lower 100% (table 1). The most plausible explanation is that during sample work up the binding process of cisplatin to plasma proteins continues. The recovery of carboplatin was slightly higher than 100% in all methods.

The TCA method resulted in a slightly higher unbound platinum concentration after incubation of plasma for 1 hour with cisplatin. This was similar to the result obtained if plasma supernatant was immediately collection in the ethanol precipitation method. This could be explained by partly reversible binding of cisplatin or incomplete precipitation of proteins. Another option is that during the longer sample work up with the other 3 methods cisplatin plasma protein binding continues thereby underestimating the unbound concentration. No significant differences were obtained with carboplatin, however the percentage bound is much lower that of cisplatin. The result is in contrast with previously published data (22). However, in that study another species was evaluated and the binding kinetics of carboplatin were much faster than in the present study. On average, the TCA and the ethanol method seem to be equally appropriate.

The optimal time period of storage of the ethanol treated sample at -20°C appears to be 2 hours. After that time period no further decrease of the remaining protein amount could be observed.

The yield of ultrafiltrate with the Millipore filters was only one quarter of the amount obtained with the Amicon filters.

The background signal on the AAS was lowest with the ethanol method indicating that the sample is relatively clean, compared to the ultrafiltrates. This may be beneficial in case of very low plasma concentrations.

Cold ethanol precipitation of plasma proteins is a simple, practical, reproducible and extremely inexpensive method to prepare samples for measurement of unbound platinum.

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CHAPTER 4

RELATIONSHIP BETWEEN THE EXPOSURE TO CISPLATIN, DNA-ADDUCT FORMATION IN LEUCOCYTES AND TUMOUR RESPONSE IN PATIENTS WITH SOLID TUMOURS

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SUMMARY

The study was designed to investigate possible relationships between tumour response and exposure to cisplatin (area under the curve of unbound cisplatin in plasma: AUC) and DNA-adduct formation in leucocytes (WBC) in patients with solid tumours. Patients were treated with 6 weekly courses of cisplatin at a dose of 70 or 80 mg/m^2. The AUC was determined during the first course and DNA-adduct levels in WBC during all courses at base-line, 1 hour (Amax) and 15 hours after a 3-hour infusion of cisplatin. The area under the DNA-adduct-time curve (AUA) was calculated. The tumour response was determined after 6 courses. Forty-five evaluable patients received 237 courses of cisplatin. Sixteen patients with head and neck cancer received a dose of 80 mg/m^2 and 29 with various other tumour types 70 mg/m^2 plus daily 50 mg oral etoposide. There were 20 responders (partial and complete) and 25 nonresponders (stable and progressive disease). The AUC was highly variable (mean $\pm$ SD = 2.48 $\pm$ 0.51 μg.hr/ml; range 1.10 - 3.82) and was closely correlated with the AUA ($R=0.78$, $p<0.0001$) and Amax ($R=0.73$, $p<0.0001$). The AUC, AUA and Amax were significantly higher in responders than in nonresponders in the total population ($p<0.0001$) and in the 2 subgroups treated at 70 or 80 mg/m^2. In logistic regression analysis AUC, AUA and Amax were important predictors of response.

The magnitude of exposure to cisplatin is, through DNA-adduct formation the major determinant of the response rate in this population. Hence, individualized dosing of cisplatin using AUC or DNA-adducts should lead to increased response rates.

INTRODUCTION

Cisplatin is considered the most active drug in testicular and ovarian cancer (Loehrer, 1984, Motzer, et al., 1988, Ozols, et al, 1988, Kaye, et al, 1992, Bajorin, et al, 1993, Levin, et al, 1993, Stoter, et al, 1995) and it has considerable activity against several other solid tumours (Alberts, et al, 1991, Clover, et al, 1987, Stoter, et al, 1987, Hansen, 1992, Slotman, et al, 1992, Hainsworth, 1993, Krarup, 1991, Planting, et al, 1993a, Paccagnella, et al, 1994, Roth, et al, 1994, Planting, et al, 1994). The clinical application of cisplatin is limited however by the existence or development of resistance and the induction of severe side effects (Loehrer, 1984, Eastman, 1988, Daugaard, 1989, Ozols, 1989, Cavaletti, et al, 1992, Siegal, 1990).

It is common practice to dose cisplatin per m^2 body surface area. However, this strategy results in wide interpatient differences in the magnitude of exposure to cisplatin, i.e. the area under the concentration-time curve (AUC) in plasma or tissues (Himmelstein, et al, 1981, Reece, et al,

1987, Reece, et al, 1989). Importantly, several clinical studies in ovarian and testicular cancer clearly established significant relationships between dose, dose-intensity and total delivered dose on the one hand and tumour response rate and side effects on the other (Ozols, et al, 1988, Kaye, et al, 1992, Levin, et al, 1993, Ozols, 1989, Bruckner, et al, 1981, Samson, et al, 1984, Levin, 1987, Markman, 1993). Interpatient differences in the dose-response and dose-toxicity relationship can be explained by interpatient differences in the dose-AUC relationship, by pharmacodynamic variability, or by both.

For the cisplatin analogue carboplatin, retrospective analyses in ovarian and testicular cancer revealed significant relationships between the AUC and the likelihood of a tumour response (Jodrell, et al, 1992, Childs, et al, 1992). Earlier studies revealed that the AUC was predictive of the dose-limiting thrombocytopenia (Calvert, et al, 1982, Egorin, et al, 1984). This, combined with the close correlation between renal function and the AUC of carboplatin has lead to the clinical application of practical methods to individualize carboplatin treatment (Childs, et al, 1992, Egorin, et al, 1985, Calvert, et al, 1989).

The cytotoxicity of cisplatin is most closely correlated with its covalent binding to nuclear DNA, so called crosslinks or adducts (Eastman, 1986, Reed, et al, 1986, Reed, et al, 1987, Fichtinger-Schepman, et al, 1987). For practical reasons DNA-adducts have been frequently quantitated in WBC (Reed, et al, 1986, Reed, et al, 1987, Fichtinger-Schepman, et al, 1987, Reed, et al, 1993, Parker, et al, 1991, Hengstler, et al, 1992, Motzer, et al, 1994). Clinical studies with cisplatin and carboplatin in various types of solid tumours revealed significantly higher DNA-adduct levels in WBC and buccal cells in responders than in nonresponders (Reed, et al, 1986, Reed, et al, 1993, Parker, et al, 1991, Hengstler, et al, 1992, Reed, et al, 1988, Reed, et al, 1990, Gill, et al, 1991, Blommaert, et al, 1993). DNA-adduct levels in tumour tissue were correlated with the levels in healthy tissues (Poirier, et al, 1992). Of note, no significant relationships have been established between the AUC of cisplatin and the DNA-adduct formation (Reed, et al, 1988).

We hypothesized that the likelihood of a tumour response in potentially sensitive tumours and interindividual variation in the formation of DNA-adducts in WBC are dominated by interpatient differences in the magnitude of exposure to active, i.e. nonprotein bound, cisplatin. We tested this hypothesis prospectively in a patient population with various types of solid tumours with potential sensitivity for cisplatin.

METHODS

Selection of patients and treatment schedule

All patients gave informed consent according to local regulatory requirements. Eligibility for the study required a pathologically confirmed cancer not curable by surgery, radiotherapy or chemotherapy and with potential sensitivity for cisplatin, such as head and neck cancer (H/N), mesothelioma, non-small cell lung cancer (NSCLC), melanoma, cervix cancer and adenocarcinoma of unknown primary site (ACUP).

The performance status had to be ≤ 2 on the WHO scale (WHO Handbook, 1979), life expectancy ≥ 3 months and the age between 18 and 75 years. No previous chemotherapy with cisplatin or carboplatin was allowed and no radiotherapy for at least 4 weeks prior to entry in the study. Lesions had to be measurable according to WHO criteria (WHO Handbook, 1979). Each patient had a complete medical history and physical and neurologic examination, complete blood count, and determination of serum chemistries including albumin, total protein, electrolytes, urea (BUN), creatinine and complete liver function tests. The creatinine clearance was determined prior to each administration of cisplatin using the serum creatinine and 24-hour urinary creatinine excretion.

Neurologic evaluation was carried out as described previously (Goldberg, 1979, van der Hoop, et al, 1990) prior to entry in the study, at 2 weeks and at 3 and 6 months after the end of the cisplatin therapy. Briefly: the severity of neuropathy was evaluated by a questionnaire of neurologic symptoms, by performing a sensory neurologic examination and by measurement of the vibration perception threshold (VPT).

All patients had to have adequate renal and liver function, i.e. serum creatinine ≤ 1.4 mg/dl (120 μmol/l) or clearance ≥60 ml/min and serum bilirubin ≤ 1.5 mg/dl (25 μmol/l), WBC ≥ 3.0×10^9/l and platelet count ≥ 100×10^9/l. The tumour response was scored after 6 courses as complete (CR) or partial response (PR), stable disease (SD) or progressive disease (PD). CR and PR were grouped as responders and SD and PD as nonresponders. The response was determined earlier during treatment if there was any indication of early progressive disease. Toxicity was scored according to the common toxicity criteria (Common Toxicity Criteria). Complete blood count, serum chemistries, urinalysis and determination of the creatinine clearance were repeated weekly.

H/N cancer was treated with weekly courses of cisplatin at a dose of 80 mg/m^2 on days 1, 8, 15, 22, 29 and 36 according to a previously established schedule (Planting, et al, 1993). The treatment was used as an induction regimen, preceding surgery and/or radiotherapy. All other tumour types

were treated with weekly cisplatin at a dose of 70 mg/m^2 on days 1, 8, 15, 29, 36 and 43 plus 50 mg of oral etoposide from day 1-15 and 29-43 according to a previously established schedule (Planting, et al, 1994, Planting, et al, 1991). In the latter group, in case of a response etoposide was to be continued thereafter for up to 4 cycles at an oral dose of 50 mg/m^2 from day 1-21 every 4 weeks. Cisplatin was dissolved in 250 ml of 3% sodium chloride and administered as a 3-hour infusion with standard pre- and posthydration.

Pharmacologic studies

Sample collection

During the first course heparinized blood samples were collected at 0, 1, 2, 3, 4, 6, 8 and 18 hours after start of the infusion. The samples were of 4 ml each except at 0, 4 and 18 hours which were of 16 ml each. During all subsequent courses samples of 16 ml were collected at 0, 4 and 18 hours after start of the infusion with cisplatin. During the first course all urine was collected up to 24 hours after start of the infusion with cisplatin.

Analysis of cisplatin in plasma and DNA-adduct levels in WBC

Total and nonprotein bound cisplatin and the total DNA-adduct levels of cisplatin were determined with atomic spectroscopy (AAS) according to the method of Reed et al. (Reed, et al, 1988), with modifications (Ma, et al, 1995).

Data analysis

The area under the plasma concentration-time curve (AUC μg.hr/ml) of unbound cisplatin (measured with AAS as platinum (Pt)) was determined with extended least squares regression analysis (Sheiner, 1985). Plasma clearance (CL) of unbound cisplatin was calculated by dose/AUC (ml/min). The terminal half-life of unbound cisplatin was calculated by ln2/k (min), where k is the rate constant of the terminal phase. The renal clearance of cisplatin was calculated by multiplying the fraction of the dose of cisplatin excreted in the urine by the CL of unbound cisplatin. The DNA-adduct level 1 hour post-infusion was denoted Amax (Ma, et al, 1995) and expressed as picogram of platinum/μg DNA (pgPt/μgDNA). The area under the DNA-adduct-time curve (AUA, pgPt.hr/μgDNA) was calculated up to 15 hours post-infusion with the trapezoidal method, using the 3 DNA-adduct-time points (figure 1). The Siphar software package was used for pharmacologic calculations (version 4.0, SIMED, Creteil, Cedex, France).

Statistical analysis

Linear regression analysis and Pearson correlation analysis were used to quantitate the relationship between AUC and AUA and AUC and Amax. The

Pearson correlation coefficient was used for calculation of the correlation between the creatinine clearance and the renal and plasma clearance of cisplatin. The unpaired two-sided student t-test was used to test for differences between responders and nonresponders in Amax, AUA, and AUC. In addition, this test was used to assess any significant differences in AUC, plasma clearance and terminal half-life of unbound cisplatin and AUA and Amax between the 2 subgroups who were treated with 70 or 80 mg/m^2.

Logistic regression analysis (Hosmer, 1989) was applied to establish the relationship between AUC, as well as AUA and Amax and the likelihood of a response. The equation can be written as:

Likelihood of a response = $\{1 + \exp\{-(A + B*X)\}\}^{-1}$

where: the dependent parameter is the likelihood of a tumour response, A and B are coefficients and X is the independent parameter (AUC, AUA or Amax). Goodness-of-fit of each logistic model was assessed with the Hosmer-Lemeshow test (Hosmer, 1980).

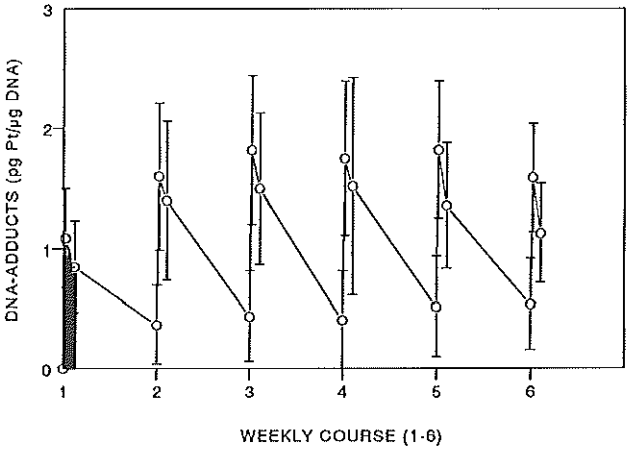

Figure 1. DNA-adduct-time curve of cisplatin during 6 weekly courses of 70-80 mg/m^2 in 45 patients (mean ± SD). The DNA-adduct-time-points (o) per course were: base line, 1 hour and 15 hours post-infusion. Shaded area = area under the DNA-adduct-time-curve (AUA), calculated using the 3 time points and the trapezoidal method.

The Pearson and Spearman rank correlation coefficient and chi-square test were applied to test for relationships between myelosuppresion, renal and neurotoxicity and AUC, AUA and Amax. For statistical analysis of neurotoxicity the maximal sum score post-treatment of the neurologic questionnaire and sensory examination were used, as well as the logarithm of the maximal VPT post-treatment (log VPT).

Multiple linear regression analysis was used to test for differences in the AUC-AUA relationship (i.e. exposure to cisplatin and DNA-adduct formation) between the 2 subgroups treated with 70 or 80 mg/m^2 of cisplatin. The statistical analysis was carried out by application of Stata (version 3.1, Statistics/Data Analysis, Computing Resourse Center, Santa Monica, CA, USA).

RESULTS

Population demographics

Patient demographic characteristics are shown in table 1. A total of 50 eligible patients were entered in the study. Eight patients had previously received radiotherapy and 2 patients chemotherapy. One received isolated regional limb perfusion with melphalan for melanoma 4 years prior to entry into the study and one had systemic cyclophosphamide for adenocarcinoma of unknown primary site 5 years prior to entry. Five patients were not evaluable for tumour response. Three of the nonevaluable patients developed renal toxicity (2 patients grade 1 and one grade 3, after 1, 2 and 1 course, respectively) preventing further treatment. One patient stopped because of grade 3 gastrointestinal toxicity after 4 courses and one patient refused further treatment after 2 courses. Data of these 5 patients were included in the evaluation of renal toxicity. The 45 patients who were evaluable for response received a total of 237 courses. All patients received at least 1 course and were followed for at least 3 months. The mean number of courses per patient was 5.3 (88 % of planned). The dose-intensity in the subgroup treated at 70 mg/m^2 cisplatin plus VP16 was 53.5 mg/m^2/week (89 % of planned) and in the subgroup treated at 80 mg/m^2 cisplatin as single agent 71.2 mg/m^2/week (89 % of planned). Overall there were 20 responders (44%; 2 CR and 18 PR) and 25 nonresponders (56%; 16 SD and 9 PD). In the subgroup with cisplatin and VP16 there were 10 responders (34%; all PR) and 19 nonresponders (66%; 12 SD and 7 PD).

Pharmacokinetics, DNA-adduct formation and tumour response

The AUC of unbound cisplatin showed substantial interpatient variability (table 2). The AUC varied from 1.1 - 3.82 μg.hr/ml and the coefficient of variation (%CV) was 21%. The plasma clearance of unbound cisplatin was 635 $\pm$ 217 ml/min (range 312-1477) and the half-life 38 $\pm$ 10 min (range 23-72). The volume of distribution of unbound cisplatin was 34 $\pm$ 13 liters (range 15-86). The renal clearance quantitated using the first 24-hour urine portion was 167 $\pm$ 71 ml/min (range 102-338). The correlation coefficient between the

Table 1. Patient characteristics

Characteristics	cisplatin 70 mg/m^2 + VP 16	cisplatin 80 mg/m^2	All
Total entered			
male	24	16	40
female	6	4	10
Median age, yrs (range)	61 (39-70)	53 (44-73)	59 (39-73)
Median performance score (range)	1 (0-2)	1 (1-2)	1 (0-2)
Prior therapy			
chemotherapy	1	0	1
radiation therapy	5	3	8
chemotherapy and radiation	1	0	1
no prior therapy	23	17	40
Diagnosis			
head and neck		20	20
mesothelioma	12		12
NSCLC	10		10
ACUP	6		6
cervix	1		1
melanoma	1		1

Performance score according to WHO criteria
NSCLC = non-small cell lung cancer
ACUP = adenocarcinoma of unknown primary site

creatinine clearance and renal clearance of unbound cisplatin was 0.70 ($p<0.01$, $n=20$), between creatinine clearance and plasma clearance of unbound cisplatin 0.46 ($p<0.01$, $n=20$) and between renal and plasma clearance of unbound cisplatin 0.92 ($p<0.0001$, $n=50$).

Also the AUA and Amax varied considerably (table 2). The %CV of the first course AUA was 25% and of the Amax 27%. The variability of the AUA and Amax during the subsequent courses was of the same order as during the first course.

There was a highly significant correlation between the AUC and the AUA and Amax. The correlation coefficient was 0.78 ($p<0.0001$, $n=44$) between AUC and AUA (figure 2) and 0.73 ($p<0.0001$) between AUC and Amax. Of note, there was no significant correlation between the absolute dose given and the AUC ($R=0.1$, $p=0.53$). No significant correlations were observed between the kinetics of total, i.e. bound plus unbound cisplatin and DNA-adduct formation (AUA and Amax).

Table 2. Pharmacologic parameters of cisplatin in 45 patients treated with 6 weekly courses of 70 mg/m^2 + oral VP16 daily 50 mg or 80 mg/m^2 of cisplatin as single agent.

Patients	Response	N	AUC [μg.hr/ml]	Amax 1st [pgPt/μgDNA]	Amax 1-6 [pgPt/μgDNA]	AUA 1st [pgPt.hr/μgDNA]	AUA 1-6 [pgPt.hr/μgDNA]
cisplatin + VP16	yes	10	2.64 ± 0.40 2.16 - 3.22	1.21 ± 0.28 0.84 - 1.61	1.55 ± 0.28 1.19 - 1.90	20.0 ± 3.7 15.3 - 25.6	23.5 ± 4.6 16.1 - 28.0
	no	19	2.08 ± 0.51 1.10 - 3.16	0.78 ± 0.19 0.34 - 1.15	1.32 ± 0.24 0.69 - 2.30	13.0 ± 2.9 8.0 - 19.5	18.8 ± 3.1 10.8 - 33.1
	p		0.006	$< 10^{-4}$	0.03	$< 10^{-4}$	0.003
cisplatin	yes	10	3.09 ± 0.53 2.30 - 3.82	1.58 ± 0.26 1.10 - 1.81	1.95 ± 0.28 1.60 - 2.67	25.3 ± 4.2 17.2 - 32.4	30.4 ± 3.8 24.9 - 37.9
	no	6	2.47 ± 0.45 2.16 - 2.88	0.92 ± 0.22 0.58 - 1.15	1.49 ± 0.43 1.07 - 1.52	16.6 ± 4.5 10.3 - 21.3	24.3 ± 6.1 14.5 - 26.5
	p		0.03	0.0001	0.02	0.001	0.03
ALL	yes	20	2.86 ± 0.51 2.16 - 3.82	1.38 ± 0.36 0.64 - 1.81	1.75 ± 0.34 1.24 - 2.67	22.6 ± 5.1 11.5 - 32.1	27.0 ± 5.4 16.1 - 37.9
	no	25	2.17 ± 0.51 1.10 - 3.16	0.81 ± 0.21 0.30 - 1.15	1.36 ± 0.30 0.69 - 2.30	13.7 ± 3.8 7.4 - 21.3	20.1 ± 4.5 10.8 - 33.1
	p		$<10^{-4}$	$< 10^{-7}$	0.0001	$< 10^{-7}$	$< 10^{-4}$

Mean ± SD and range are given.

AUC = Area under unbound cisplatin plasma concentration-time curve
Amax = cisplatin-DNA-adduct level in WBC 1 hour after 3-hour-infusion
AUA = Area under cisplatin-DNA-adduct-time curve in WBC [0-18hrs]

1st = 1st course of cisplatin
1-6 = mean of all courses
Response = CR+PR; No response = SD+PD

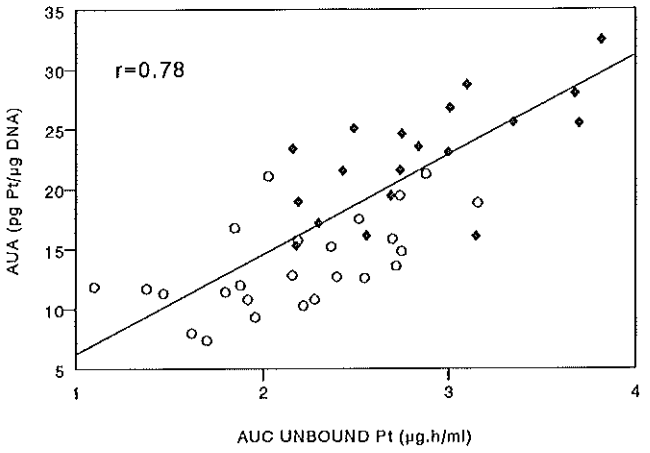

Figure 2. Relationship between the magnitude of exposure to unbound cisplatin (AUC) and AUA during the first course of cisplatin (R=0.78, p<0.0001). ♦ response, ○ non-response

The AUC, AUA and Amax were significantly higher in responders than in nonresponders (table 2, figure 3). This was evident in the total population as well as in the 2 subgroups treated with cisplatin at a dose of 70 mg/m^2 (various tumour types) and 80 mg/m^2 (H/N). In addition, the mean value of the AUA and Amax of all administered courses was also significantly higher in responders than in nonresponders. The AUC in the subgroup treated at 80 mg/m^2 of cisplatin (2.85 ± 0.55 µg.hr/ml) was significantly higher than in the subgroup treated at 70 mg/m^2 (2.28 ± 0.54 µg.hr/ml, p=0.002). The AUA and Amax were also significantly higher in the 80 mg/m^2 subgroup as a results of the dose difference. VP16 did not appear to influence the pharmacokinetics of cisplatin, as there were no statistically significant differences between the 2 treatment groups in plasma clearance, renal clearance or terminal half-life of cisplatin. In addition, VP16 did not affect the DNA-adduct formation significantly, as reflected by the slope of the linear regression relationship between AUC and AUA, which was not significantly different between the two subgroups treated with and without VP16 (p>0.2). An influence of VP16 on the DNA-adduct formation could not be tested directly, because the 2 subgroups did receive a different dose of cisplatin.

Logistic regression analysis

Logistic regression analysis revealed highly significant sigmoid relationships between AUC, AUA and Amax, and the likelihood of a response. This was evident in the total population and in the subgroup treated with 70 mg/m^2 of cisplatin (p value of coefficients A and B < 0.01 for the total popula-

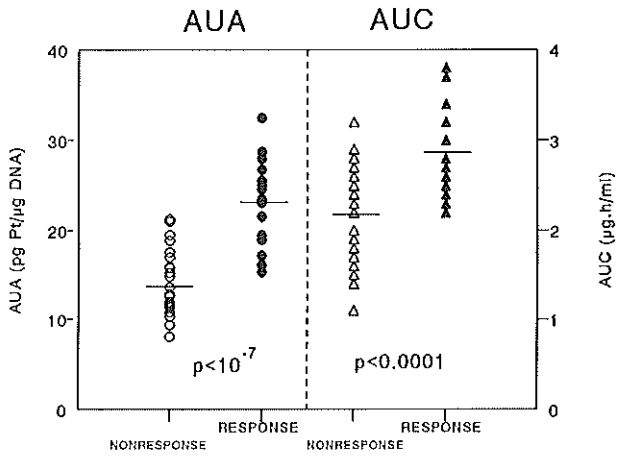

Figure 3. Area under the DNA-adduct-time curve (AUA) and area under the plasma concentration-time curves (AUC) in responders and nonresponders to cisplatin chemotherapy.

tion of 45 patients and ≤ 0.02 in the subgroup of cisplatin + VP16). The corresponding 'p' values of the goodness-of-fit tests were all > 0.50, indicating good fits. The likelihood of a response reached 100% in the three relationships with AUC, AUA and Amax.

Pharmacokinetics, DNA-adduct formation and toxicity

Myelosuppression was the most frequently encountered side-effect. CTC grade 1 anemia was observed in 9 of 45 evaluable patients (20%), grade 2 in 26 (58%) and grade 3 in 6 patients (13%). Grade 1 leucopenia was observed in 11 patients (24%), grade 2 in 9 (20%), grade 3 in 16 (35%) and grade 4 in one patient (2%). Grade 1 thrombocytopenia was found in 4 patients (9%), grade 2 in 10 (22%), grade 3 in 4 (9%) and grade 4 in 2 patients (4%). The AUC and AUA were significantly correlated with the CTC-grade of thrombocytopenia (Spearman rank R = 0.38, p = 0.01 [AUC]; R = 0.43 p = 0.005 [AUA], n = 45). These relationships were also significant in the subgroup treated with cisplatin as single agent, hence without the influence of VP16. In this subgroup the correlation coefficient between AUC and thrombocytopenia was 0.62 (p = 0.02, n = 16) and AUA and thrombocytopenia 0.70 (p = 0.007). The correlation coefficient between dose/m^2 or absolute dose given and thrombocytopenia was not significant (p = 0.11). Correlation coefficients between AUC/AUA and anaemia or leucocytopenia were not statistically significant.

Eight patients developed grade 1 nephrotoxicity (16% of 50 patients), one grade 2 (2%) and one patient grade 3 (2%). No significant Spearman rank correlation coefficients were observed between AUC, AUA or absolute dose given and CTC-grade of nephrotoxicity (n = 50).

Forty-five patients were evaluable for the sum score of neurotoxicity and in 21 patients the log VPT was determined. Fifteen patients developed grade 1 neurotoxicity (33%). No grade 2 or higher was observed. The log VPT varied between -0.05 and 0.65 (mean 0.23 and SD 0.21). No significant correlation was observed between the cumulative dose nor dose/m^2 and sum score or log VPT. The cumulative AUC (i.e. AUC of course 1 times number of administered courses) was significantly correlated with the log VPT (Spearman rank R = 0.52, p = 0.01). The AUA and cumulative AUA were not significantly correlated with the log VPT (p = 0.63).

DISCUSSION

For our pharmacologic analyses, we applied a dose-intensive schedule of weekly cisplatin, which was previously developed in our department. Presently over 200 patients with solid tumours have been treated in phase I/II trials according to this schedule (Planting et al., 1991,1993,1993a,1994). The importance of the weekly administration was recently stressed by Logothesis and Amato (Logothetis, 1992). The results clearly indicate that the AUC of unbound cisplatin and the DNA-adduct formation in WBC are closely correlated. The relationship can best be described by a linear relationship (figure 2). In addition, the likelihood of a tumour response was strongly determined by the magnitude of the AUC of cisplatin (figure 2 and 3). Not unexpected, because of the close correlation with the AUC, also the AUA and Amax were strong predictors of response (figure 2 and 3). The AUC of unbound cisplatin was highly variable, despite the small dose range of cisplatin of 70-80 mg/m^2. Also in the two subgroups treated at 70 or 80 mg/m^2 the AUC range was high (table 2). The pharmacokinetic parameters of cisplatin were of the same magnitude as reported previously (Himmelstein, et al, 1981, Reece, et al, 1987, Reece, et al, 1989). The highly significant correlation between the AUC and the level of DNA-adduct formation combined with the strong predictive power of the AUC gives evidence that the variability in the dose-response relationship of cisplatin in our patient population is mainly determined by pharmacokinetic variability. The results were obtained in a heterogeneous population with a variety of solid tumours. It implies that clinical resistance to cisplatin in these tumours is determined to a substantial extent by the magnitude of exposure to unbound cisplatin. We speculate that pharmacokinetic variability contributes significantly to clinical resistance of other tumour types which are potentially sensitive to cisplatin.

Two DNA-adduct parameters were defined and used throughout the study: Amax and AUA. The AUA may reflect processes leading to induction of DNA-adduct formation shortly post-infusion and DNA-repair in the 15 hours post-infusion of cisplatin. The correlation coefficient between AUC and AUA (0.78) was slightly higher than between AUC and Amax (0.73). Although the AUA is theoretically more of interest, the difference in AUA between responders and nonresponders was only marginally greater than in the Amax (table 2). The DNA-adduct levels did not show a significant accumulation with increasing number of courses, although the mean of the level during the first course was slightly lower than during the subsequent courses (figure 1, table 2). The DNA-adduct levels in responders were consistently higher than in nonresponders throughout the study (table 2), which supports the results of Reed et al. (Reed, et al, 1993). The most reasonable explanation for the overlap in DNA-adducts and AUC between responders and nonresponders is pharmacodynamic variability.

The relationships between the AUC of cisplatin or DNA-adduct formation (AUA and Amax) and the response were almost similar in the two subgroups treated at 70 or 80 mg/m^2.

The addition of VP16 had no measurable influence on the relationship between AUC and DNA-adduct formation or on the pharmacokinetics of cisplatin. The response rates in the present study are comparable with those reported by Planting et al. (Planting, et al, 1993a, Planting, et al, 1994, Planting, et al, 1991, Planting, et al, 1992).

The weekly schedule was overall well tolerated. Significant but manageable myelosuppression was encountered. The AUC and AUA were significantly correlated with the CTC-grade of thrombocytopenia. The nephrotoxicity was manageable in almost all patients. No significant correlations were observed between nephrotoxicity and AUC or DNA-adduct formation. One third of the patients developed grade 1 neurotoxicity. This incidence is of the same order as reported in previous studies (Cavaletti, et al, 1992, Roelofs, et al, 1984). The cumulative AUC of cisplatin was more closely correlated with the log VPT than the cumulative dose. Of note, the cumulative dose was not significantly correlated with any of the neurotoxicity parameters, which is in contrast to a previous study (Cavaletti, et al, 1992). It is important to note that the AUC of unbound cisplatin was more closely correlated with any toxicity parameter than the dose, cumulative dose or dose/m^2. These relationships, however, need affirmation in future studies.

The outlined results clearly confirm that a standardized dose/m^2 results in wide interpatient variation in the AUC of cisplatin (Himmelstein, et al, 1981, Reece, et al, 1987, Reece, et al, 1989). Considering the relationship between

the AUC and the likelihood of a tumour response in a population with a variety of solid tumours, the pharmacokinetic variability has major implications for the treatment with cisplatin. Patients should benefit from individualized dosing of cisplatin, to increase the response rate. Based on the significant correlations between AUC and toxicity parameters this will also lead to more frequent, but mostly predictable toxicity. Drug monitoring applying a limited sampling strategy is indicated to achieve target levels of AUC or DNA-adducts. The presented study provides support for tumour-type specific trials, for example in non small cell lung cancer. This procedure is currently investigated in a prospective study.

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Chapter 5

PHARMACOKINETIC-DYNAMIC RELATIONSHIPS OF DOCETAXEL AND CISPLATIN IN PATIENTS WITH ADVANCED SOLID TUMORS

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SUMMARY

The study was designed to investigate possible schedule dependent pharmacokinetic and dynamic relationships and interactions between docetaxel and cisplatin when given in combination. Docetaxel and cisplatin were administered in a phase I study to 64 patients with various types of solid tumors. Forty-nine patients received the sequence docetaxel-cisplatin, where cisplatin was given 3 h after docetaxel. Another 15 patients received the reverse sequence, where docetaxel was administered 18 h after cisplatin. Doses of docetaxel were escalated from 55 to 100 mg/m^2 and of cisplatin from 50 to 100 mg/m^2. Pharmacokinetic parameters of docetaxel and cisplatin were determined during course one. In addition, cisplatin-DNA-adducts in peripheral blood leukocytes (WBC) were determined during the first two courses at one and 21 h after the end of the cisplatin infusion. Total plasma clearance (Cl) and volume of distribution at steady state (Vss) and other pharmacokinetic parameters of docetaxel and cisplatin were not significantly different between the two schedules. The formation of cisplatin-DNA-adducts in WBC was significantly inhibited by docetaxel. The mean level at 1 h post infusion was 1.1 $\pm$ 0.37 pgPt/μgDNA and 0.50 $\pm$ 0.40 pgPt/μgDNA with prior docetaxel ($p<0.001$). The main toxicity observed was myelosuppression. Significant sigmoid relationships were established between the area under the curve (AUC) and time-period (T) > 100ng/ml of docetaxel and leukocytopenia en thrombocytopenia. A combination of 2 Emax models using T100 ng/ml for docetaxel and AUC of unbound platinum best described the observed leukocytopenia. Tumor responses (in total 1 complete and 9 partial) were observed in both schedules.

INTRODUCTION

Docetaxel (taxotere®) is a new semisynthetically derived taxoid drug which is registered for breast and NSCLC, and is currently under further clinical investigation. It enhances polymerization of the tubulin into stable microtubules and inhibits microtubule depolymerization. This results in a disruption of the equilibrium within the microtubule system and ultimately to cell death (1-3). In the phase I studies on single agent docetaxel when given as a 1-hr. iv infusion the dose-limiting toxicity was complicated neutropenia. At longer infusion durations the dose-limiting toxicities were short-lasting and non-cumulative neutropenia and mucositis (4-9). Other side effects reported were a.o. fluid retention, general alopecia, nausea, vomiting, diarrhea and nail and skin reactions. The recommended dose and schedule for phase II studies on single agent docetaxel was 100 mg/m^2 given as a 1-hour infusion every 3 weeks. Phase II studies on docetaxel revealed significant activity in breast cancer (10-14), non-small cell lung cancer (15-17) and in a

number of other tumor types (18-22). In previous studies, the pharmacokinetics could best be described by an open 3-compartment model and revealed wide interpatient variability after standard dosing per unit body surface area (4). Docetaxel is mainly eliminated through cytochrome P450 mediated biotransformation and excretion occurs essentially in the faeces via the bile, as has been established in rat and man (23,24). Less than 10% of the dose is excreted unchanged in the urine. The protein binding is high (>90%) (25). Most studies report linear pharmacokinetics of docetaxel (25). Significant sigmoid relationships have been established between the AUC and % decrease in neutrophils (4,7). This indicates that the observed variability in the pharmacokinetics has clinical implications.

Cisplatin is a highly effective drug for the treatment of a number of solid tumors (26). It exerts its activity through formation of DNA-adducts, which can be quantitated in tumors and healthy tissues and WBC (27-29). Results of a previous combination study with cisplatin and paclitaxel, revealed that the elimination of paclitaxel was significantly inhibited by cisplatin (30). This resulted in increased myelosuppression. In addition, a pharmacodynamic interaction has been described in the combination of carboplatin and paclitaxel. When given simultaneously, carboplatin and paclitaxel could be dosed almost up to their respective single agent maximum tolerated doses (31).

To date, no detailed clinical pharmacologic data are available on the interaction between docetaxel and cisplatin. These data are of relevance because of the potential clinical application of the combination of the two anticancer agents.

We performed a pharmacologic analysis in patients who were treated with the combination of either cisplatin-docetaxel or docetaxel-cisplatin in a phase I study. The objectives were: 1) to determine the pharmacokinetic profiles, 2) to characterise any pharmacokinetic and dynamic interaction between the two drugs and 3) to establish quantitative relationships between the pharmacokinetics and the toxicity profile.

MATERIALS AND METHODS

Eligibility

Only patients with histologically documented solid tumors for which no therapies with greater potential benefit than docetaxel and cisplatin existed were candidates for this study.

Eligibility criteria further included: 1) age $\geq$ 18 years and $\leq$ 75 years; 2) WHO performance status 0-2; 3) life expectancy of at least 12 weeks; 4) no more than one prior combination regimen or than two single agent

regimens; 5) off previous anticancer therapy for at least 4 weeks (6 weeks in case of Mitomycin C, nitrosoureas or extensive radiotherapy); 6) no prior treatment with platinum derivatives or taxoid drugs; 8) adequate bone marrow- (neutrophils $\geq$ 2 x 10^9/l, platelets $\geq$ 100 x 10^9/l), hepatic- (total bilirubin $\leq$ 1.25 times and ASAT (SGOT) $\leq$ 2 times the upper-normal limits) and renal functions (creatinine $\leq$ 120 μmol/l or creatinine clearance $\geq$ 60 ml/minute); 9) absence of symptomatic peripheral neuropathy > grade 0 according to NCI Common Toxicity Criteria (CTC) (32). All patients had to give written informed consent.

Drug administration

Docetaxel was supplied by Rhône-Poulenc Rorer (Antony, France) as a concentrated sterile solution containing 40 mg/ml in a 2 ml vial in polysorbate 80 (Tween 80®). The appropriate amount of the drug to be administered to the patient was diluted in 250 ml of 5% dextrose solution (or 0.9% saline serum) so that the maximum docetaxel concentration was $\leq$ 0.9 mg/ml. The drug was administered to the patients as a one hour infusion.

Cisplatin was administered to the patient as a three-hour infusion diluted in 1 liter of 0.9% saline. Prior to cisplatin administration patients were hydrated with 1 liter of 0.9% saline infused over 3 hours and subsequent to cisplatin with 3 liters of 0.9% saline infused over 18 hours. Antiemetics included 8 mg i.v. ondansetron or 5 mg tropisetron and 10 mg i.v. bolus dexamethason just prior to the cisplatin infusion. During the study data from extensive phase II studies on single agent docetaxel became available suggesting beneficial effects of additional medication to prevent side effects. The final combination of prophylactic drugs included: dexamethason 10 mg given as i.v. bolus 24 and 6 hours before and 24 hours after docetaxel administration, while clemastine fumarate was given orally at a dose of 2 mg 30 minutes before docetaxel administration.

Dosage

The docetaxel and cisplatin doses were escalated according to a pre-established schedule and according to the toxicities observed at the previous dose-level, after a minimum of 3 patients had tolerated the previous dose. Definitions of the dose limiting toxicities and maximum tolerated dose (MTD) have been described elsewhere (Pronk et al., manuscript to be submitted). The starting doses of docetaxel and cisplatin were 55 mg/m^2 and 50 mg/m^2 respectively. At first, dose escalation of docetaxel was performed with the dose of cisplatin being fixed. Dose levels of docetaxel/cisplatin studied were 55/50, 70/50, 85/50, 100/50, 55/75, 70/75, 85/75, 100/75, 75/100, 85/100 and 100/100 mg/m^2.

Drug sequence

Dose escalation was initially performed with docetaxel preceeding cisplatin (schedule A) with a 3-hour interval between the end of docetaxel infusion and the start of cisplatin infusion. When the MTDs and doses to be used for further phase II evaluation were defined with schedule A, the dose-level just below the MTD was reassessed with cisplatin preceeding docetaxel (schedule B). In this schedule the interval between the end of cisplatin infusion and the start of docetaxel infusion was 18 hours. The given intervals were derived from the optimal intervals in preclinical studies (data on file, Rhône-Poulenc Rorer). When acceptable toxicities were observed, further dose-escalation was pursued with schedule B.

Pretreatment and follow-up studies

Prior to the start of treatment history was taken and physical examination, neurologic examination, laboratory studies, ECG, chest x-ray, tumor measurements (other x-rays, scans, ultrasounds) and an audiogram were performed (Pronk et al., submitted). Formal tumor measurements were performed after every 2 courses of chemotherapy. Standard WHO response criteria were used (33). The creatinine clearance was determined using the Cockroft formula (34).

Pharmacologic studies

For docetaxel and cisplatin pharmacokinetics and protein-binding studies were performed. For the analysis of plasma concentrations of docetaxel, 3 ml blood samples were taken from the arm opposite to the infusion site immediately prior to infusion, at 30 minutes after start and at the end of the infusion and then at 5,10,20,30,60,90 minutes and 2,4,6,8,12,24 and whenever possible at 36 and 48 hours post-infusion. For the protein-binding studies of docetaxel 3 ml blood samples were collected immediately prior to infusion, at the end of the infusion and at 20 minutes, 1 hour, 2 hours and 6 hours post-infusion. The analyses were carried out applying an established HPLC method with UV detection (35).

For the analysis of cisplatin, blood samples were collected immediately before start of the infusion (8 ml) and at 1,2,3,4,5,6,8,10 and 21 hours after start of the infusion (4 ml). For cisplatin, both total and non-protein bound plasma concentrations of platinum were determined applying a method of Ma et al. (36).

Cisplatin-DNA-adducts were measured in WBC prior to infusion and at 1 and 21 hours post-infusion, according to the method of Ma et al. (29).

Pharmacokinetic parameters were estimated by weighted least-squares regression analysis (SIPHAR version 4, Simed, Créteil, France). Individual plasma

concentrations of docetaxel were fit to two- or three-exponential equations (corresponding to two- and three-compartment pharmacokinetic models) and of cisplatin using a one-exponential equation. The best model was chosen on the basis of the Akaike information criterion (37). The fitted parameters permitted the computation of the following parameters: AUC, total plasma clearance, Vss and half-lifes (t½ for cisplatin and $t_{½\alpha}$, $t_{½\beta}$ and $t_{½\gamma}$ for docetaxel).

The following pharmacokinetic parameters were used to investigate the relationship with toxicitiy parameters: AUC of docetaxel, time-period above a threshold concentration (T>threshold) of 30, 50 and 100 ng/ml (figure 1) and AUC of unbound platinum.

The pharmacodynamic parameters evaluated were: relative decrease (% decrease) of granulocytes, leukocytes and thrombocytes and the area under the leukocytopenia-time curve (AUCleuc) and the area under the thrombocytopenia-time curve (AUCthro). These toxicity parameters were determined using the decrease in leukocyte (or thrombocyte) count after administration of docetaxel and cisplatin on day 4,7,11,13,15,17,21 until the administration of the second course. The linear trapezoidal method was applied for the calculation of the AUCleuc and AUCthro.

The sigmoid Emax model was used to investigate pharmacokinetic-dynamic relationships (38). The relative decrease in leukocytes, granulocytes and thrombocytes were calculated as follows:

$$\% \text{ decrease} = \frac{\text{Pretreatment count} - \text{nadir count}}{\text{Pretreatment count}}$$

which was related to the AUC of docetaxel as follows:

$$\% \text{ decrease} = \frac{Emax * AUC^{\gamma}}{AUC_{50}^{\gamma} + AUC^{\gamma}}$$

In addition, the pharmacokinetic parameter T>threshold and the pharmacodynamic parameters AUCleuc and AUCthro were used in the Emax model. Nonlinear least-squares regression analysis using SIPHAR was applied to estimate the AUC_{50} (the AUC that produces 50 % of the maximum effect) and the sigmoidicity factor γ. When % decrease leukocytes or granulocytes or thrombocytes were fitted, the maximum effect (Emax) was assumed to be 100 % decrease.

In addition, pharmacokinetic-dynamic relationships were investigated using the sigmoid Emax model for the combined action of two drugs, because in principle both docetaxel and cisplatin may contribute to the observed toxicity parameter (38).

$$\% \text{ decrease} = \frac{Emax,1*(AUCdoc/AUC50,doc)^{\gamma 1} + Emax,2*(AUCfuPt/AUC50,fuPt)^{\gamma 2}}{1 + (AUCdoc/AUC50,doc)^{\gamma 1} + (AUCfuPt/AUC50,fuPt)^{\gamma 2}}$$

Emax,1 and Emax,2 were assumed to be 100% decrease. AUCdoc,50 and AUCfuPt,50 denote the AUC's of docetaxel and unbound platinum that produce 50% of the maximum effect, $\gamma 1$ and $\gamma 2$ are the respective sigmoidicity factors. Instead of AUCdoc, T>threshold was used as kinetic parameter and AUCleuc and AUCthro as dynamic parameters. Non-linear least squares regression analysis was employed using Stata (Stata corp. 1993, Release 3.1, College Station TX, USA). The two models (sigmoid Emax models for one drug and for the combination of two drugs) were compared on the basis of the value of the root mean squared error (RMSE) between observed and predicted values, which is a measure of precision and mean prediction error (MPE), which is a measure of bias (39). The best model had the lowest RMSE and MPE value.

Further statistical analysis

Differences in pharmacokinetic parameters between the two schedules were evaluated with the Mann Whitney test. The Pearson correlation coefficient was calculated between pharmacokinetic parameters of docetaxel and cisplatin. The Spearman rank correlation coefficient was calculated between the pharmacokinetic parameters and pharmacodynamic parameters, such as % decrease granulocytes, leukocytes and thrombocytes and the CTC-grade mucositis. P values < 0.05 were considered statistically significant.

RESULTS

Clinical data

Sixty-six patients were entered into this study. Two patients were never treated as they appeared to be infected at baseline. The median age of the 64 treated patients was 53 years (range 21-74). There were 33 males and 31 females, with a median WHO performance score of 1 (range 0-2). Thirty-two patients had reveived prior chemotherapy, 19 prior radiotherapy and 10 patients had received both. Colorectal carcinoma (22 patients) and carcinoma

of unknown primary (13 patients) were the most common tumor types. A total of 49 patients was treated in schedule A and 15 in schedule B. A total of 244 courses of docetaxel in combination with cisplatin were given and 243 courses were evaluable. Of 215 courses the duration of the granulocyte and leukocyte nadir could be determined in close detail.

Toxicity

The most relevant toxicities are summarised. They have been described in detail by Pronk et al., (to be submitted).

Four patients developed sepsis (one died). Other dose limiting toxicities were grade 2 nephrotoxicity (1 patient), grade 4 neutropenia lasting 11 days (1 patient) and grade 3 diarrhea (1 patient).

Leuko- and granulocytopenia occurred in 90% of the courses. Neutropenia grade 3 and 4 occurred at all dose levels and in 83.5% of the courses. The duration of the granulocyte nadir $< 0.5 \times 10^9$/l was longer at the high dose levels. Thrombocytopenia $\geq$ grade 2 only occurred during 5 courses at high dose levels. Leukocytopenia tended to be more pronounced during schedule B (cisplatin followed by docetaxel). The chi-square test of the CTC-graded leukocytopenia revealed a p-value of 0.09.

Mucositis was reported in 24.2% and severe (grade 3) in 1 course. Alopecia occurred in 90.6% of the patients. The severity of the mucositis was not schedule dependent. Skin toxicity occurred in 19.7% of courses and nail toxicity in 40.6% of the patients.

Nephrotoxicity grade 1 occurred in 28.7% of courses while grade 2 was only documented in 5 courses (2.3%). Skin toxicity was reported in 13.6% of courses and (mainly mild) nail toxicity in 39.6% of the patients. Edema grade 1 occurred in 23.4% and grade 2 in 4.5% of courses. These toxicities were not schedule dependent.

Responses

Nine partial responses were observed and one complete response. Responses occurred in both schedules.

Pharmacokinetics

The pharmacokinetic data of docetaxel and cisplatin are summarized in tables 1 and 2. Most of the curves of docetaxel could be fitted with a three-compartment model, others with a two-compartment model and 7 were calculated with a model independent approach. All platinum curves (cisplatin was measured as platinum by AAS) could be fitted using a one-compartment model. The correlation coefficient between dose and AUC of docetaxel was

Table 1. Pharmacokinetic data of docetaxel after a one-hour infusion.

	Dose (mg/m^2)		Dose (mg)	Cmax ($\mu g/ml$)	$t½\alpha$ (min)	$t½\beta$ (h)	$t½\gamma$ (h)	AUC ($\mu g.h/ml$)	Cl ($l/h/m^2$)	Vss (l/m^2)
Schedule A	55	mean	105	1.70	3.65	0.40	3.63	2.07	29.2	23
		SD	13	0.46	1.29	0.03	2.47	0.72	10.0	14
		N	5	4	3	3	3	4	4	4
	70	mean	132	3.18	6.81	2.75	10.66	4.60	19.0	53
		SD	15	1.03	4.32	4.61	5.98	2.14	10.0	30
		N	8	6	5	5	5	6	6	6
	75	mean	140	2.56	8.37	0.82	13.04	4.06	21.4	102
		SD	14	1.07	4.40	0.27	5.08	1.67	8.7	84
		N	7	7	6	6	6	6	6	6
	85	mean	153	2.80	6.72	1.67	11.42	4.04	22.1	77
		SD	25	0.59	1.93	2.48	5.73	1.16	6.9	40
		N	13	13	10	10	10	13	13	12
	100	mean	183	3.04	7.46	1.04	14.58	4.53	23.6	98
		SD	20	0.78	3.18	0.53	9.63	1.52	7.0	77
		N	13	13	9	9	9	13	13	9
Schedule B	70	mean	129	1.70	6.71	0.47	4.79	2.47	30.5	39
		SD	5	0.32	5.93	0.26	1.70	0.63	10.2	20
		N	4	3	3	3	3	4	4	3
	85	mean	157	1.66	5.95	0.84	16.12	3.43	26.9	116
		SD	17	0.39	0.65	0.35	10.27	1.06	9.4	94
		N	4	4	3	3	3	4	4	4
	100	mean	171	3.49	7.51	1.04	14.21	5.85	19.3	94
		SD	30	1.00	1.93	0.35	4.21	2.68	8.2	45
		N	6	6	5	5	5	6	6	6

Table 2. Pharmacokinetic data of cisplatin after a 3-hour infusion.

Schedule A	Dose (mg/m^2)		Dose (mg)	t½ (h)	AUCfu ($\mu g.h/ml$)	AUC(t) ($\mu g.h/ml$)	Cl ($l/h/m^2$)	Cl_{ren} ($l/h/m^2$)	Vss (l/m^2)
	50	mean	97	0.56	2.00	25.79	17.6	5.2	14
		SD	20	0.14	0.39	4.53	4.0	0.9	3.4
		N	12	12	12	12	12	11	12
	75	mean	140	0.56	2.55	34.04	20.2	5.1	16
		SD	13	0.13	0.61	4.79	5.0	1.4	4
		N	20	20	20	20	20	18	20
	100	mean	184	0.65	3.34	45.24	20.2	5.3	19
		SD	14	0.15	0.68	8.89	4.1	1.4	6
		N	15	15	15	13	15	9	15
Schedule B	75	mean	137	0.49	2.58	39.56	19.7	5.3	14
		SD	12	0.10	0.48	5.92	4.8	1.6	4
		N	14	14	14	14	14	8	14

AUCfu = AUC of unbound platinum to infinity
AUC(t) = AUC of total platinum up to the latest measured time point (21h)

not significant (R=0.07) and between dose and AUC of unbound platinum 0.59(p<0.001). The Cl of docetaxel showed wide interpatient variability (%CV = 35 %). Interpatient variability in the Cl of unbound platinum was also significant (%CV = 23%). There were no significant differences in pharmacokinetic parameters between the 2 groups (schedule A and B). Total plasma clearance (Cl) of unbound platinum in schedule A was 19.5 ± 4.6 (l/h/m^2) and 19.7 ± 4.8 (l/h/m^2) in schedule B (not significantly different: NS). Cl of docetaxel in schedule A was 22.1 ± 7.7 (l/h/m^2) and in schedule B 24.9 ± 9.4 (l/h/m^2) (NS). Docetaxel protein binding was high (96%) and not influenced by cisplatin. In addition, the Cl and Vss data of unbound platinum and the AUC up to the last measured point (AUC(t)) of total platinum in plasma indicate no significant influence of docetaxel on the protein binding of cisplatin.

Relationships between demographic/biochemical and pharmacokinetic parameters are enlisted in table 3. A low correlation coefficient was observed between Cl of docetaxel and Cl of unbound platinum (R=0.36). Also, relatively low correlation coefficients were observed between body weight or body surface area (BSA) and Cl of docetaxel and Cl of unbound platinum. Relationships between age and pharmacokinetic parameters were not significant.

Table 3. Pearson correlation coefficients (R) between demographic/biochemical and pharmacologic parameters.

R p-value	cisplatin				docetaxel	
	ClfuPt	$Cl_{ren}Pt$	AUCfuPt	VssPt	Cldoc	Vssdoc
age	NS	NS	NS	NS	NS	NS
weight	0.47 <0.001	0.43 0.003	NS	0.28 0.03	0.38 0.004	NS
BSA	0.43 <0.001	0.36 0.02	NS	0.34 0.008	0.38 0.004	NS
Cl creat	0.33 0.01	0.50 <0.001	NS			
Cl doc	0.36 0.006					
T_{100} doc	NS					
Vss doc				NS		

ClfuPt = plasma clearance of unbound platinum
$Cl_{ren}Pt$ = renal clearance of unbound platinum
Vss = volume of distribution at steady state
BSA = body surface area (m^2)
Cl creat = creatinine clearance, calculated using the Cockroft method (34)
AUCfuPt = AUC of unbound platinum
NS = not significant (p>0.05)

WBC DNA-adduct levels

The DNA-adduct levels during course one in schedule A (first docetaxel then cisplatin) measured at 1 and 21 h after the end of the cisplatin infusion turned out to be extremely low compared to the levels in schedule B (table 4). The difference in DNA-adduct levels during course two was not significant anymore. Of note, the number of observations was very much lower than during course one.

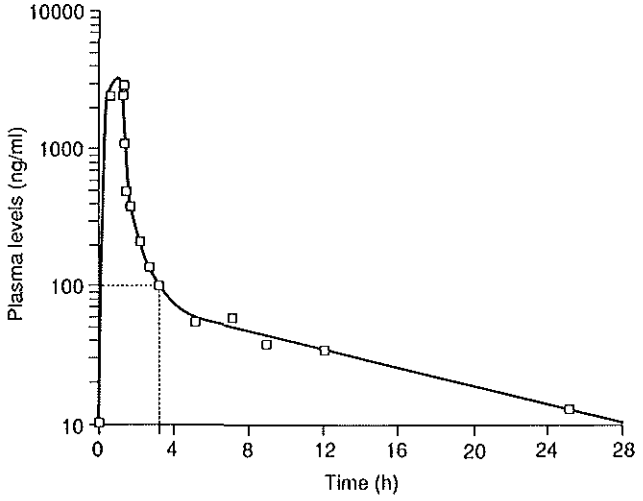

Figure 1. Plasma concentration-time curve of docetaxel. The dotted line illustrates the time-period > 100 ng/ml (T100), which was used in pharmacokinetic-dynamic analyses.

Relationships between pharmacokinetics and dynamics

Significant Spearman rank correlation coefficients were observed between AUC of unbound platinum and the % decrease of leukocytes (R=0.51), granulocytes (R=0.48) and thrombocytes (0.32), but not with the CTC-grade mucositis. Also, significant Spearman rank correlation coefficients were established between the AUC or T100 of docetaxel and % decrease leukocytes (R=0.40 respectively 0.53) and thrombocytes (R=0.46 respectively 0.54), but not with the CTC-grade mucositis. T100 performed slightly better in these relationships than T30 and T50 (time-period > 30 and 50 ng/ml).

Relationships between % decrease granulocytes, leukocytes or thrombocytes and AUC of docetaxel could best be fitted using a sigmoid Emax model (figures 2, 3 and table 5). Use of T100 of docetaxel resulted in lower RMSE values, indicating better precision than obtained with the AUC of docetaxel. A low but significant correlation was observed between T100 and AUC of docetaxel ($R=0.54$, $p<0.05$). Also, the AUC of unbound platinum could

Table 4. WBC DNA-adduct levels of cisplatin 1 and 18 hours after a 3-hour infusion, during course 1 and 2.

Schedule A	Dose (mg/m^2)		Course 1		Course 2	
			A1 (pg Pt/μg DNA)	A21 (pg Pt/μg DNA)	A1 (pg Pt/μg DNA)	A21 (pg Pt/μg DNA)
	50	mean	0.38	0.59	0.87	0.84
		SD	0.60	0.60	1.08	0.85
		N	11	11	9	9
	75	mean	0.50	0.30	0.79	0.54
		SD	0.41	0.38	0.78	0.60
		N	20	19	15	16
	100	mean	0.71	0.57	1.00	0.97
		SD	0.33	0.36	0.51	0.25
		N	14	12	9	7
Schedule B	75	mean	1.10*	0.77*	1.11	0.82
		SD	0.37	0.25	0.52	0.52
		N	14	14	6	6

* : $p<0.001$ compared to schedule A at the same dose-level of 75 mg/m^2 of cisplatin.
A1 and A21 = DNA-adduct level at 1 and 18 hours after the end of infusion.

adequately be fitted to the % decrease leukocytes and thrombocytes. Because of the low correlation coefficient between the AUC or T100 of docetaxel and the AUC of unbound platinum the % decrease granulocytes, leukocytes and thrombocytes were also fitted using a combination of two Emax models, assuming that the myelotoxicity was induced by both drugs. The fit between % decrease granulocytes and AUC_{doc} + AUCfuPt or $T100_{doc}$ + AUCfuPt resulted in lower RMSE values than obtained using AUC_{doc}, AUCfuPt or $T100_{doc}$ as single independent parameters (table 5). However, most relationships showed significant bias except when AUCfuPt was used. The same result was obtained for the relationship between % decrease leukocytes and $T100_{doc}$ + AUCfuPt. Bias in these relationships was much lower (table 5).

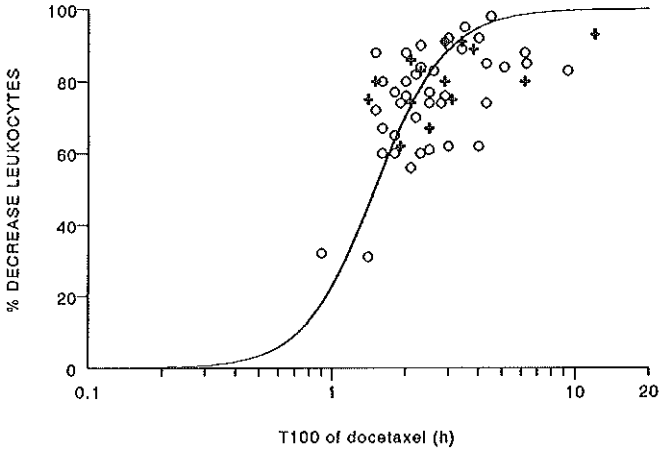

Figure 2. Relationship between % decrease leukocytes and the time-period of docetaxel > 100 ng/ml (T100). (o) data of schedule A and (+) data of schedule B. The sigmoid Emax model was used to fit the data. $T100_{r50}$ (value of T100 inducing 50% decrease) = 1.5 (h) and the slope factor γ = 2.0 (combined dataset).

No relevant fits were obtained using the dose of docetaxel or of cisplatin as independent parameter vs. the toxicity parameters.

Area under the curves of the leukocytopenia and thrombocytopenia during the first course varied widely among patients. Mean ± SD of AUCleuc was 45 ± 34 (units of leucocyte count*time) and of AUCthro 1119 ± 1334 (units of thrombocyte count*time). Poor fits were obtained when this parameter was related to the outlined pharmacokinetic parameters (figure 4).

The Spearman rank correlation coefficients between $T100_{doc}$, AUC of docetaxel or AUC of unbound platinum and mucositis were very low and not significant.

The number of responses was too small to establish meaningful relationships between pharmacokinetic parameters and likelihood of response.

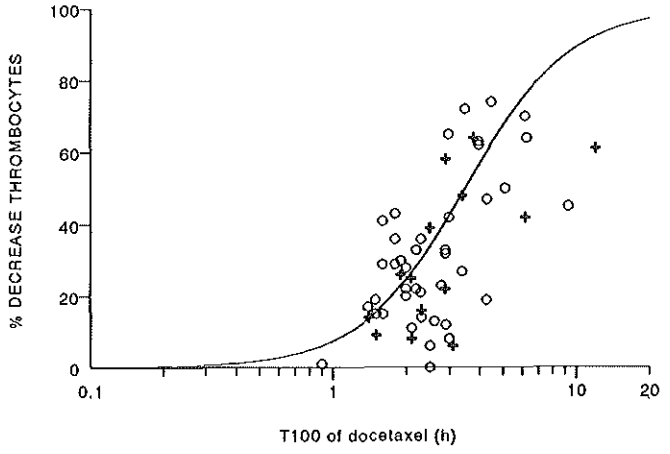

Figure 3. Relationship between % decrease thrombocytes and the time-period of docetaxel > 100 ng/ml (T100). [o] data of schedule A and [+] data of schedule B. The sigmoid Emax model was used to fit the data. $T100_{50}$ (value of T100 inducing 50% decrease) = 3.5 (h) and the slope factor γ = 2.0 (combined dataset).

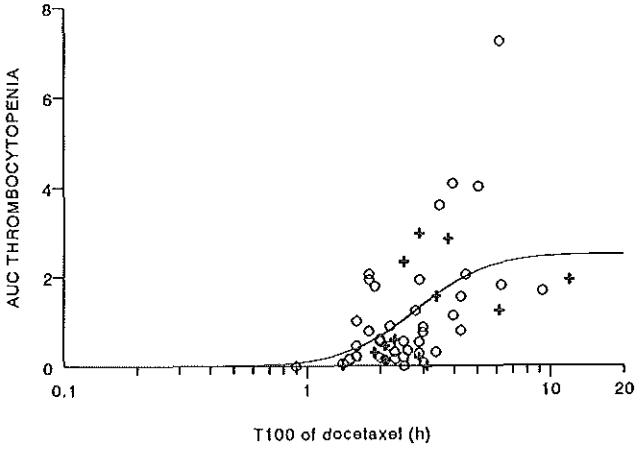

Figure 4. Relationship between area under the curve of the thrombocytes (AUCthro) during course one and the time-period of docetaxel > 100 ng/ml (T100). [o] data of schedule A and [+] data of schedule B. The sigmoid Emax model was used to fit the data. $T100_{50}$ (value of T100 inducing 50% effect) = 2.5 (units of thrombocyte count*time) and the slope factor γ = 3.0 (combined dataset). (for reasons of clear presentation the thrombocyte data were divided by 1000)

Table 5. Pharmacokinetic-dynamic relationships between AUC or T100 of docetaxel and AUC of unbound platinum and myelotoxicity parameters, determined during course 1.

		one Emax model			combination of two Emax models (1)	
		AUC_{doc} (μg.h/ml)	$T100_{doc}$ (h)	AUCfuPt (μg.h/ml)	AUC_{doc} + AUCfuPt (μg.h/ml) (μg.h/ml)	T100 + AUCfuPt (h) (μg.h/ml)
% decrease granulocytes (N=52)	RMSE	0.16	0.15	0.16	0.15	0.14
	MPE	0.87	1.06	-0.07	-2.94	0.73
% decrease leucocytes (N=56)	RMSE	0.19	0.18	0.20	*	0.17
	MPE	0.30	0.20	-0.23	*	0.18
% decrease thrombocytes (N=56)	RMSE	0.27	0.26	0.30	*	*
	MPE	-0.08	0.19	-0.02	*	*

(1) : see methods section
* : model not appropriate, because of negative or extremely out of range values of parameters
T100 : time-period of a docetaxel plasma concentration > 100 ng/ml
RMSE : root mean square error
MPE : mean prediction error

DISCUSSION

Cisplatin is one of the most widely applied anticancer agents in patients with solid tumors. Recent clinical studies revealed significant or high activity of docetaxel against a variety of solid tumors (10-22). Because of high activity of the two drugs and the only partly overlapping toxicity profiles the feasibility of combination therapy was investigated in a phase I and pharmacologic study employing both sequences of administration. Preclinical studies have revealed that specific intervals in the sequencing of both drugs did lead to optimal efficacy (Rhône-Poulenc Rorer, data on file). In the present study two sequences were studied, which both had optimal efficacy in the preclinical studies: cisplatin given shortly after docetaxel (schedule A) and docetaxel given 18 hours after cisplatin (schedule B). An additional argument for the time interval between cisplatin and docetaxel in schedule B was that a previous phase I study with the combination of cisplatin and paclitaxel administration of paclitaxel shortly after cisplatin resulted in a significantly increased toxicity. This was probably due to the reduced clearance of paclitaxel by cisplatin (30).

Results of the present clinical study revealed that repeated cycles of 100 mg/m^2 of docetaxel with 75 mg/m^2 of cisplatin, or 85 mg/m^2 of docetaxel with 100 mg/m^2 of cisplatin appear feasible especially in patients with a good physical condition. The sequence docetaxel followed by cisplatin appeared the most attractive for phase II studies. The dose-limiting toxicity was granulocytopenia, which was $<500/mm^3$ in the majority of cases (Pronk et al., to be submitted).

The pharmacokinetics of docetaxel and cisplatin revealed significant interpatient variability. This is in line with previous observations (4,7,40). The pharmacokinetic data obtained in schedule A were not significantly different from those in schedule B, indicating that the applied sequence of administration did not influence the pharmacokinetics. In addition, the magnitude of the observed pharmacokinetic parameters is comparable to those previously published (41).

Interpatient variability in body weight, BSA and creatinine clearance contributed to a low extent to the observed variability in clearance of docetaxel and cisplatin (table 3). In contrast to previous reports, but in line with our own observations, age was not significantly correlated with the clearance of unbound platinum nor with the AUC of unbound platinum (41,42).

Significant correlation coefficients were observed between pharmacokinetic parameters such as AUC of unbound platinum, AUC or T100 of docetaxel and % decrease granulocytes, leucocytes and thrombocytes. Sigmoid relationships could be described between the % decrease in

leukocytes and the AUC of docetaxel. Previous studies have also illustrated significant sigmoid relationships between the magnitude of exposure to docetaxel and myelosupprssion (4). The relationships between % decrease granulocytes, leukocytes or thrombocytes and pharmacokinetic parameters using data of schedule A and B were completely overlapping (figures 2, 3, 4). In the present study the time period above 100 ng/ml was the best single pharmacokinetic parameter which could be related to the myelotoxicity parameters % decrease granulocytes, leukocytes and thrombocytes. This has not been reported in other studies. In particular, in the relationship with % decrease leukocytes T100 resulted in the lowest RMSE as well as the lowest MPE value. For paclitaxel several investigators have observed that the time period above a threshold concentration was better correlated with the myelosuppression than the AUC (43,44). The fits between pharmacokinetic parameters and % decrease granulocytes were relatively poor and substantially biased, most likely because very low nadir values were achieved in almost all patients. The AUC of unbound cisplatin could also be fitted to the % decrease leukocytes and thrombocytes, which suggests that cisplatin contributed to the observed leukocytopenia and thrombocytopenia. Myelotoxicity induced by cisplatin has been associated with chronic administration, although dose-intensive cisplatin induces myelotoxicity on a short term. In a previous study with weekly cisplatin the AUC of unbound platinum was significantly correlated to the leukocytopenia and thrombocytopenia (41). The correlation coefficients between the AUC of unbound platinum and the AUC or T100 of docetaxel were low and for this reason the AUC of docetaxel (as well as T100 of docetaxel) and the AUC of unbound platinum were considered as independent pharmacokinetic parameters and related to the myelosuppression. It was assumed that the combination of two sigmoid Emax models was the appropriate model, suggesting the combined action of two drugs (38). The maximum effects induced by the two drugs (Emax1 and Emax2) were assumed equal and 100% decrease. The nonlinear least squares fits using two Emax models compared to one Emax model resulted in lower values of the RMSE for the relationship between % decrease granulocytes and leukocytes and T100 of docetaxel (or AUC docetaxel) and AUC of unbound platinum (table 5). Although the differences in RMSE values were not great, the consistency of the results indicates that the applied Emax model for the combined action of docetaxel and cisplatin is the best model to describe the observed granulocytopenia and leukocytopenia. The combined Emax model, when applied in the relationship with % decrease leukocytes also resulted in the lowest bias. This model should be applied in future studies, also when other combinations of drugs are applied which show at least partly overlapping toxicity profiles, to

investigate its usefulnes. Application of the combined Emax model to describe the thrombocytopenia did not result in realistic fits, because of negative or extremely out of range values for the estimated parameters.

In an attempt to optimize the relationship between pharmacokinetics and myelosuppression, the AUCleuc and AUCthro were calculated. At least 6 time-points were available to describe the decline and recovery of leucocytes and thrombocytes after the first course of chemotherapy was given. Such dynamic parameter has previously been used in the pharmacokinetic-dynamic analysis of etoposide (45). The AUCleuc and AUCthro (calculated using both absolute values and values relative to the level prior to chemotherapy) showed wide interpatient variation. The relationships between AUCleuc or AUCthro and pharmacokinetic parameters showed poor fits (figure 4). Therefore AUCleuc and AUCthro are not better dynamic endpoints than the nadir value in the present study.

Spearman rank correlation coefficients between pharmacokinetic parameters and mucositis, the other main toxicity observed, were low and not significant.

A significant reduction in the formation of DNA-adducts by cisplatin in WBC was observed. The DNA-adduct levels were significantly lower when docetaxel preceded cisplatin (table 4). This was particularly striking during course one. During course two the data were not significant anymore, however the number of observations was very much reduced compared to course one. Preclinical studies in our laboratory revealed that docetaxel and paclitaxel inhibit the intracellular accumulation of cisplatin and carboplatin in WBC (46). Additional results obtained in our laboratory illustrate that the interaction was not observed in two human tumor cell lines (data not shown). Data on intracellular kinetics of cisplatin in combination with taxanes in tumors *in vivo* in man are presently not available. The leukocytopenia tended to be less pronounced during schedule A (docetaxel followed by cisplatin), however it did not reach statistical significance ($p=0.09$). Also, in the sigmoid relationships between pharmacokinetic parameters and % decrease granulocytes and leukocytes, data of the two schedules were overlapping. Hence, although there was a trend towards more pronounced leukocytopenia in schedule A, the pharmacodynamic interaction had no significant clinical implications in the present study.

Results of the present study reveal that docetaxel and cisplatin do not show a pharmacokinetic interaction in the studied population. The T100 of docetaxel was the best pharmacokinetic parameter in the relationship with myelotoxicy parameters. The best pharmacokinetic-dynamic model was obtained using the combined Emax model with T100 of docetaxel and AUC of unbound platinum as independent pharmacokinetic parameters and %

decrease leukocytes as dynamic parameter. Docetaxel inhibited the formation of DNA-adducts in WBC by cisplatin. Further studies are needed to unravel clinical implications of the pharmacodynamic interaction.

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CHAPTER 6

DOCETAXEL AND PACLITAXEL INHIBIT DNA-ADDUCT FORMATION AND INTRACELLULAR ACCUMULATION OF CISPLATIN IN HUMAN LEUKOCYTES

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ABSTRACT

The purpose of this study was to determine the mechanism of the pharmacodynamic interaction between docetaxel/paclitaxel and cisplatin. Cisplatin induced DNA-adducts and cisplatin accumulation were quantitated in peripheral blood leukocytes (WBC). The WBC were obtained from patients treated with docetaxel or paclitaxel in phase I/II studies and were incubated *in vitro* with cisplatin. In addition, blank whole-blood samples were obtained from patients and healthy subjects and incubated *in vitro* with cisplatin or docetaxel/paclitaxel and cisplatin. The cisplatin induced DNA-adduct levels in WBC after treatment with docetaxel or paclitaxel were significantly lower than in non-pretreated WBC. Docetaxel and paclitaxel reduced the intracellular accumulation of cisplatin in WBC by 46-47%. If the pharmacodynamic interaction between docetaxel/paclitaxel and cisplatin also occurs in other normal tissues such as bone marrow, it may well explain to the sequence dependent toxicity.

INTRODUCTION

Docetaxel and paclitaxel are novel antimicrotubule agents inducing tubulin polymerization and inhibiting microtubule depolymerization. Clinical studies revealed high response rates particularly in breast and ovarian cancer [8,11]. *In vitro*, paclitaxel has shown synergism when given before cisplatin [3,4], whereas *in vivo* in humans administration of cisplatin before paclitaxel induced more neutropenia than has the alternate sequence. This was attributed to a reduced clearance of paclitaxel [10].

We have previously reported a clinical and pharmacologic study in patients with solid tumors, applying the sequence docetaxel/cisplatin in one group of patients and that of cisplatin/docetaxel in another group. It was shown that the cisplatin-DNA-adduct levels in WBC were markedly lower when docetaxel preceded cisplatin [13]. Indeed, the DNA-adduct level at 1 hour post cisplatin (dose of 75 mg/m^2) was 0.54 $\pm$ 0.4 pg Pt/μg DNA for the sequence docetaxel/cisplatin and 1.21 $\pm$ 0.34 pg Pt/μg DNA for the reverse sequence ($p<0.0001$) [13]. In addition, significantly less granulopenia ($p<0.001$) and mucositis ($p<0.01$) were observed for the sequence docetaxel/cisplatin than for the reverse sequence. There was no significant mutual pharmacokinetic interaction between docetaxel and cisplatin. Here we report on additional *in vitro* experiments that contribute to a better understanding of the mechanism of interaction.

MATERIALS AND METHODS

Patients were treated either with docetaxel (55-100 mg/m^2) and cisplatin (50-100 mg/m^2) in a phase I/II study ($n=22$) [13] or with single agent docetaxel

(100 mg/m^2) (n=6) or paclitaxel (135 mg/m^2) (n=3) in phase I/II studies. All patients gave written informed consent, had a WHO performance status of ≤1, and adequate renal and liver function, i.e. serum creatinine < 120 μmol/l (1.4 mg/dl) and total bilirubin value of < 25 μmol/l (1.5 mg/dl), as well as a WBC > 4.0x10^9/l, and a platelet count of >100x10^9/l.

For *in vitro* incubation with cisplatin, heparinized whole blood samples (20 ml) were drawn prior to docetaxel or paclitaxel infusion and 3 hours post-infusion (i.e. prior to cisplatin in the docetaxel/cisplatin group). In the six patients treated with single agent docetaxel and in one of the three patients treated with single agent paclitaxel, a third sample was drawn at 24 h postinfusion. From 8 patients, additional blank blood samples were collected, i.e., prior to any drug treatment. The samples were incubated *in vitro* with docetaxel (1 μg/ml), immediately followed by cisplatin (10 μg/ml), for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The level of cisplatin-DNA-adduct in WBC and the concentrations of total and unbound cisplatin in the incubation tubes were measured with atomic spectroscopy (AAS) [6,7,9].

The intracellular cisplatin concentration in WBC was measured in blank whole-blood samples obtained from five patients and two healthy volunteers. Three 10 ml portions were incubated for 2 h with cisplatin (10 μg/ml). Prior to cisplatin, docetaxel (1 μg/ml) was added to one portion, paclitaxel (1 μg/ml) to the second, and the third served as control. WBC were collected after the incubation. The remaining red blood cells were lysed by addition of 30 ml of a buffer [0.83% NH_4Cl, 0.1% $KHCO_3$, 1mM ethylenediaminetetra-acetic acid (EDTA)]. The WBC were washed twice with ice-cold phosphate-buffered saline and lysed in 1 ml deionized water. The protein was quantitated by the Lowry method [5]. After evaporation at 70°C, the sample was digested by nitric acid (65%) at 75°C for 2 h. The platinum (Pt) concentration was measured AAS. Student's paired and unpaired two-sided t-tests were used for data analysis.

RESULTS AND DISCUSSION

The DNA-adduct levels determined *in vitro* after treatment of blank blood with cisplatin in the 22 patients receiving the combination were 12.1 ± 3.7 and 3.8 ± 1.6 pg Pt/μg DNA before and after docetaxel infusion, respectively ($p<0.0001$, Fig. 1, Table 1). In the six patients treated with single-agent docetaxel, DNA-adduct levels measured pretreatment were 10.0 ± 2.0 pg Pt/μg DNA, those determined at 3 h 3.7 ± 1.0 pg Pt/μg DNA ($p<0.0001$ as compared with preinfusion levels) and those measured at 24 h post infusion were 7.7 ± 2.6 pg Pt/μg DNA ($p=0.01$ as compared with 3 h post infusion). In the three patients treated with paclitaxel, the DNA-adduct level was 6.8 ± 1.2 pg Pt/μg DNA before treatment and 2.8 ± 1.1 pg Pt/μg DNA at 3 h after

infusion ($p<0.0001$). In the one of these three patients from whom a blood sample was also collected at 24 h after the infusion the DNA-adduct level was 6.6 pg Pt/μg DNA before treatment and 3.4 and 6.1 pg Pt/μg DNA at 3 and 24 h postinfusion, respectively. These results indicate a reversible interaction. The 4- to 14-h plasma elimination half-lives of docetaxel and paclitaxel of 4-14 hours [1,2,12] indicate that the concentrations determined at 24 h after infusion are already substantially reduced, which suggests that the interaction is reversed at that time.

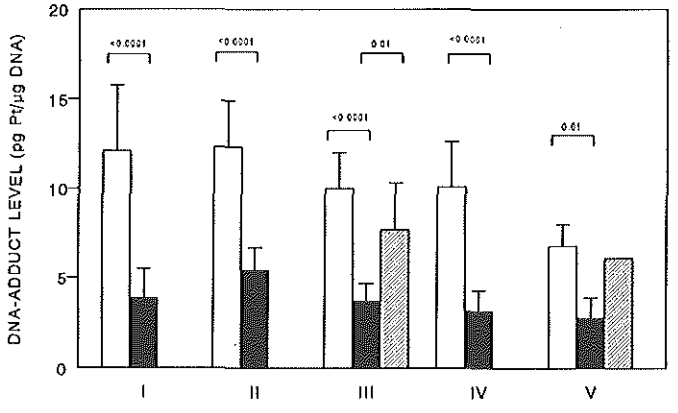

Figure 1. Cisplatin DNA-adduct levels determined in vitro in WBC in blank blood and blood samples obtained after docetaxel or paclitaxel administration. II Blood collected prior to (white bar) and at 3 h after docetaxel infusion (black bar) and incubated with cisplatin *in vitro* (n=22), II Patients' blank blood incubated *in vitro* with cisplatin alone (white bar) or docetaxel + cisplatin (black bar; n=8), III Blood collected prior to (white bar), and at 3 (black bar) and 24 h (shaded bar) after docetaxel infusion and then incubated with cisplatin *in vitro* (n=6), IV Patients' blank blood incubated *in vitro* with cisplatin alone (white bar) or paclitaxel + cisplatin (black bar n=6), V Blood collected prior to (white bar) and at 3 (black bar) and 24 h (shaded bar, n=1) after paclitaxel infusion and then incubated with cisplatin *in vitro* (n=3)I

Incubation of WBC *in vitro* with docetaxel (n=8) or paclitaxel (n=6) and cisplatin resulted in the same magnitude of inhibition of DNA-adduct formation (Fig. 1). The intracellular concentrations of platinum in WBC were reduced by 46% (docetaxel) and 47% (paclitaxel), respectively, as compared with the control values ($p<0.0001$).

If the pharmacodynamic interaction between docetaxel/paclitaxel and cisplatin also occurs in other normal tissues such as bone marrow, it may well contribute to the sequence dependent toxicity. This suggests that the drug

schedule is an important determinant not only of activity, but also of toxicity.

Table 1. Cisplatin-induced DNA adducts determined in WBC of patients treated with docetaxel in the sequence docetaxel/cisplatin or with single-agent docetaxel or paclitaxel. All blood samples were incubated in vitro with cisplatin (10 μg/ml). Blood samples were collected prior to (time 0) and at 3 h after docetaxel administration from the 22 patients receiving docetaxel/cisplatin and prior to (time 0) and at 3 and 24 h after the infusion of docetaxel and paclitaxel given as single agents.

		Cisplatin-DNA adducts in WBC (pg Pt/μg DNA)		
	n	0	3 h	24 h
Docetaxel + cisplatin	22	12.1 ± 3.7	3.8 ± 1.6**	-
Docetaxel, single-agent	6	10.0 ± 2.0	3.7 ± 1.0**	7.7 ± 2.6*
Paclitaxel, single-agent	3	6.8 ± 1.2	2.8 ± 1.1**	6.1[a]

*P = 0.01 as compared with 3 h after infusion; **P < 0.0001 as compared with time 0
[a]Single observation (in this patient the level at 0 hours was 6.6 and at 3 hours 3.4 pg Pt/μg DNA)

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Preclinical Studies

CHAPTER 7

PHARMACOKINETIC-DYNAMIC RELATIONSHIP OF CISPLATIN *IN VITRO*
simulation of an iv bolus, 3h and 20h infusion

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SUMMARY

The profiles of an iv bolus and 3 h and 20 h infusion of cisplatin (CDDP) were simulated *in vitro* by using a culture of the IGROV1 human ovarian cancer cell line. Disappearance of pharmacologically active unbound CDDP was accomplished by adding human albumin to the medium. Total and unbound CDDP and CDDP-DNA adduct levels were quantitated by atomic absorption spectroscopy (AAS), and tumour cell survival was measured by the clonogenic assay. The design of the experiment resulted in non-significant differences in the magnitude of the area under the concentration time curve (AUC) of unbound CDDP between the three dose-input functions (AUC i.v. bolus, 6.34 ± 0.36, 3 h infusion 6.35 ± 0.59, and 20 h infusion, 6.76 ± 0.40 μg.hr/ml). Also, the differences between the area under the CDDP-DNA adduct time curves (AUA) of the three dose-input functions were not significant. The initial rate of decline of the CDDP-DNA adduct time curve was significantly higher for the i.v. bolus and 3 h infusion than for the 20 h infusion. There was a log-linear relationship between the AUC of unbound CDDP and cell survival. These relationships were not significantly different between the three dose-input functions. Variation in the rate of input of CDDP leads to differences in the shape of the AUC and AUA without significant effects on cell survival.

INTRODUCTION

CDDP is one of the most potent cytotoxic compounds *in vivo* and is frequently used for the treatment of ovarian and testicular cancer, head and neck cancer and other malignancies (Loehrer & Einhorn, 1984; Forastiere *et al.*, 1987; Reed *et al.*, 1988a; 1990). Its application is accompanied by dose limiting side-effects, such as nephro-, neuro- and ototoxicity (Kovacs *et al.*, 1982; Meijer *et al.*. 1983; Vermorken *et al.*, 1983). Despite its long-standing and wide clinical application, the optimal schedule of CDDP, with a maximal attainable anti-tumour effect and tolerable side-effects, has never been clearly established. From early studies it became evident that a rapid i.v. infusion was associated with the development of profound, and sometimes irreversible, nephrotoxicity. Because of this infusion times were prolonged, with intensive pre- and post-hydration and administration of CDDP in saline solutions (Jacobs *et al.*, 1978; Salem *et al.*, 1984; Vogelzang *et al.*, 1984), rendering side-effects better manageable. The influence of the variation of the rate of input (i.e. dose-input function per treatment cycle) on the cytotoxic activity of CDDP is less clearly defined. Clinical observations suggest that the anti-tumour effect is not influenced by the dose-input function (Vermorken *et al.*, 1982).

Data on pharmacokinetic-dynamic relationships of CDDP in clinical and preclinical studies, taking the CDDP-DNA adduct formation and repair and exposure to unbound CDDP into consideration, are lacking.

CDDP binds almost irreversibly to plasma and cellular components (Yotsuyanagi *et al.*, 1991). This results in an extremely long retention of CDDP in tissues (Loehrer & Einhorn, 1984). The elimination half-life of the pharmacologically active unbound CDDP in the plasma is short and is approximately 1 h in man (Vermorken *et al.*, 1982; 1986). The cytotoxic activity of CDDP is very likely correlated to the covalent binding to DNA, so-called inter- and intrastrand adduct formation (Plooy *et al.*, 1984). This is not an irreversible proces, because of the cellular capacity to remove the formed adducts (DNA-repair) (Plooy *et al.*, 1984; Fichtinger-Schepman *et al.*, 1987).

Variation in the dose-input function results in a different shape of the plasma concentration-time curve of unbound CDDP. Because of the diffusion of CDDP to tumour tissues, this will also result in different concentration time curves at the site of action. If one assumes linear pharmacokinetics, i.e. the magnitude of the AUC is not dependent on the dose-input function, then variation in the dose-input function will only lead to differences in the shape of the AUC.

In the present study the kinetics of CDDP-DNA adduct formation and repair was studied *in vitro* as a function of the variation in the dose-input of CDDP, using a well characterised IGROV1 ovarian cancer cell line. The profiles of an i.v. bolus and 3 h and 20 h infusion were simulated *in vitro* and the pharmacokinetics of unbound CDDP was correlated to the CDDP-DNA adduct formation and repair and tumour cell survival (i.e. pharmacodynamics).

MATERIALS AND METHODS

Chemicals

Roswell Park Memorial Institue (RPMI) 1640 medium was obtained from Brunschwig (Amsterdam, The Netherlands), bovine calf serum (BCS) from Hyclone (Logan, Utah, USA), dimethyl sulphoxide (DMSO) and platin (Pt) standard solution (500 p.p.m.) from Baker (Deventer, The Netherlands), phosphate-buffered saline (PBS) from Boom (Meppel, The Netherlands) and insulin Neerlandicum from Organon (Oss, The Netherlands). Streptomycin, penicillin, gentamycin, glutamine and trypsin were obtained from Gibco (Breda, The Netherlands), DNAase I from Sigma (St. Louis, MO, USA), proteinase K and caesium chloride (CsCl) from Merck (Darmstadt, Germany), and sodium dodecyl sulphate (SDS) and hematoxylin from Brunschwig (Amsterdam, The Netherlands). Ethylene-diaminetetraacetic acid (EDTA) and all other chemicals were obtained from Baker and were of analytical grade or higher. T25 (25

cm^2), T75 (75 cm^2) and T175 (162 cm^2) culture flasks were obtained from Costar (Badhoevedorp, The Netherlands).

IGROV1 cell culture

The IGROV1 ovarian adenocarcinoma cell line was originated by Dr. J. Bénard (Institut Gustave Roussy, Villejuif, France) (Bénard *et al.* 1985; Teyssier *et al., 1989)* and kindly supplied by Dr. R.L.H. Bolhuis, (Rotterdam Cancer Institute, Rotterdam, The Netherlands). The cell line was maintained in a continuous logarithmic culture in RPMI- 1640 medium with HEPES and phenol red supplemented with 10% BCS, 10 mM sodium bicarbonate, 2 mM glutamine, penicillin 111 IU/ml, streptomycin 103 μg/ml, gentamycin 43 μg/ml and insulin 10 μg/ml. The cells were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The cells were mildly trypsinised for passage and for use in experiments. The cloning efficacy and cell doubling time were determined. For the latter the sulforhodamine B (SRB) test was used.

Apparatus

A flameless Perkin-Elmer 3030B atomic absorption spectrophotometer (AAS) was used equipped with an AS60 autosampler and HGA600 controller system (Überlingen, Germany). The UV spectrophotometer was a Backman DU62 (Fullerton, CA, USA) set at 260 nm.

Assay of CDDP in the culture medium

Total and unbound CDDP were analysed by AAS. One millilitre of medium was taken for the analysis of the total and unbound CDDP concentration. For the total CDDP concentration 100 μl were taken and diluted 10-40 times with 0.2% triton X-100 containing 0.06% calsium chloride in order to obtain CDDP concentrations in the range of 10 - 100 ng/ml. Unbound CDDP was analysed after deproteination of 0.5 ml of the medium sample with 1.0 ml of ice-cold absolute ethanol. A volume of 250 μl was taken and diluted 10-40 times with deionised water. A volume of 50 μl was injected into the AAS. All measurements were carried out in duplicate.

Assay of DNA levels in tumour cells

DNA levels in tumour cells were determined according to a method described by Fichtinger-Schepman *et al.* (1987).

Analysis of CDDP-DNA-adduct levels

CDDP-DNA-adduct levels were quantitated according to a method described by (Reed *et al.*, 1988a) with modifications. Briefly: a DNA sample was

digested with DNAase I and $ZnCl_2$ (10 μM, 10 μl) was added to the mixture to optimise the enzymatic reaction (Fichtinger-Schepman *et al.*, 1987). A volume of 160 μl (approximately 60-250 μg DNA) was injected into the furnace using the 4 times multiple sampling feature of the instrument. The samples were calibrated on a standard curve of 4 samples with a Pt concentration of 0, 1.5, 3, 6 ng/ml (equivalent to 0, 240, 480, 960 pg Pt in the injected volume of 160 μl). CDDP-DNA-adduct levels were expressed as pg of Pt per μg DNA (pgPt/μgDNA). The analysis was carried out in duplicate.

CDDP protein binding experiment

CDDP (10 μg/ml) was incubated for 24 hrs at 37°C with human plasma (15 ml) and a 7% (v/v) human albumin solution in RPMI-1640, containing 10% BCS (total volume of the mixture, 15 ml). During the incubation period total and unbound CDDP were analysed at 0, 0.5, 1, 2, 3, 5, 8 and 24 h.

Influence of protein binding on the CDDP-DNA adduct formation

In order to study the relationship between the unbound CDDP and adduct formation, IGROV1 cells were incubated with CDDP in three different experiments.

1.A volume of 5 ml of PBS was added to each of three T75 flasks containing the cell culture in 10 ml RPMI-1640. Subsequently CDDP was added.

2. Instead of PBS, 5 ml of a 20% albumin solution was added, immediately followed by CDDP.

3. CDDP was preincubated with 5 ml of the 20% albumin solution at 37°C for 24 h and added to the cell culture. The final concentration of CDDP in each flask was 5 μg/ml. All experiments were carried out in triplicate. At 1, 2 and 4 h after the start of the incubation a flask was taken and analysed for total and unbound CDDP and the CDDP-DNA adduct level.

Design of the simulation of the profiles of an i.v. bolus and 3 h and 20 h infusions

Intravenous bolus profile: Six T75 culture flasks containing approximately $6x10^6$ cells in 16 ml medium were used for the concentration-time and adduct-time curves. An 8 ml aliquot of albumin was added immediately followed by 90 μl of a CDDP solution of 1 mg/ml. The final albumin concentration in the mixture was then 7%. After 3 h the medium was carefully removed, the cells were washed twice with PBS and cultured again in CDDP-free medium. At 1, 3, 8, 20, 44 and 68 h from the start a flask was used to harvest cells for measurement of the CDDP-DNA adduct level. During the incubation 1 ml of medium was taken at 0, 0.5, 1, 2 and 3 h for measurement of the concentration of total and unbound CDDP.

Three hour infusion profile: Every 15 min for 1½ h small, but constant, amount of CDDP (18.4 μl containing 0.2 mg/ml CDDP) was added to the 16 ml of culture medium. After another 1½ h of incubation 8 ml 20% albumin was added to the flask immediately followed by CDDP (51.2 μl of a solution of 0.2 mg/ml CDDP), to prevent dilution of CDDP in the flask owing to the addition of the relatively large volume of the albumin solution. Five hours later (8 h from start) the medium was removed and the cells were washed and cultured again as outlined. The same CDDP-DNA adduct time points were determined as in the i.v. bolus experiment. During the incubation 1 ml of medium was taken at 0, 0.5, 1, 2, 3 and 8 h for measurement of the total and unbound CDDP concentration.

Twenty-hour infusion profile: Seven flasks were used. CDDP was added at time point 0 h and after 20 h the medium was removed. Subsequently, the cells were washed and cultured as outlined. The CDDP-DNA-adduct-time points were 1, 3, 8, 20, 24, 44 and 68 h. Total and unbound CDDP were determined at 1, 3, 8 and 20 hrs, as outlined.

All experiments are carried out in quadruplicate.

The three experiments were further denoted as i.v. bolus and 3 h and 20 h infusions.

Cell survival measurement

The clonogenic assay was used. Exponentially growing IGROV1 cells in RPMI- 1640 were plated in T25 culture flasks (in 4 ml medium), with a density range of $5x10^2$ to 10^5 cells, 24 h before CDDP administration. The same procedure of CDDP incubation and albumin addition was followed as outlined above. In the i.v. bolus experiment individual flasks were taken after 1, 2 and 3 h of incubation, in the 3 h infusion experiment after 1, 3 and 8 h of incubation, and in the 20 h infusion after 1, 3, 8 and 20 h of incubation. The medium was removed and the cells were washed twice with PBS and cultured again in CDDP free medium for approximately 2 weeks. Subsequently, the colonies were fixed with absolute methanol/acetic acid (17 M) (v/v = 2/1) and stained with hematoxylin. Colonies of >50 cells were counted. All experiments were carried out in quadruplicate.

Pharmacokinetic and statistical analysis

The AUC of unbound CDDP in the medium and AUA (up to 68 h) in the tumour cells were calculated using the trapezoidal method. The initial slope, after withdrawal of CDDP, was calculated between the adduct time points 3-20 h (i.v. bolus, three adduct time points), 8-20 h (3 h infusion, 2 points) and 20-44 h (20 h infusion, 3 points). Student's t-test, Kolmogorov-Smirnov (KS) test and log-linear regression analysis were used. Student's t-test was used if at

least four observations were evaluable and the standard deviations per treatment group were <400% different, otherwise the non-parametric KS-test was applied.

RESULTS

Igrov1 cell culture (IGROV1)

The cloning efficacy was 20 ± 4%. The cell doubling time was 24 ± 3 h.

CDDP protein binding experiment

The binding of CDDP to proteins in human plasma was similar to the binding to proteins in the mixture of RPMI-1640 containing human albumin (Figure 1). After 1 h approximately 50% of CDDP was bound in both experiments. After 24 h only 3% was unbound.

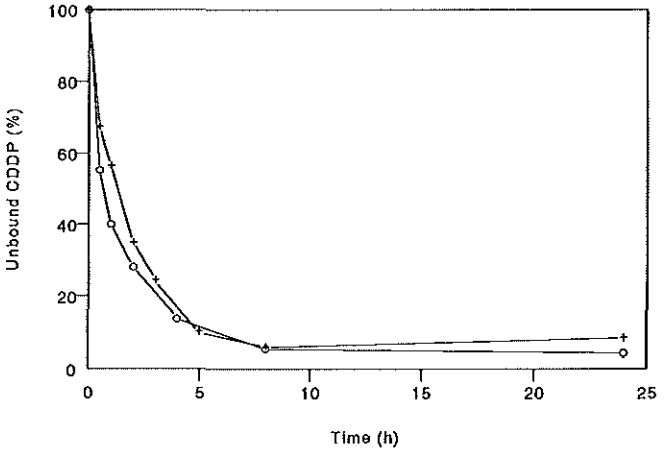

Figure 1. Protein binding of CDDP (10 μg/ml) in human plasma (o) and in a 7% solution of human albumin in RPMI 1640 (+). Incubation at 37°C.

In the protein-free medium there was a linear increase of adduct formation with time (Figure 2). Addition of albumin, immediately followed by CDDP, resulted in a significantly lower increase of adducts with time. When CDDP was preincubated with albumin, thereby eliminating unbound CDDP, no significant adduct formation was observed.

Assay of CDDP in the culture medium

Total and unbound CDDP could be analysed reproducibly in the observed concentration range of 0.1 to 5 μg/ml. Coefficients of variation were <5%. The measured concentration-time curves of total and unbound

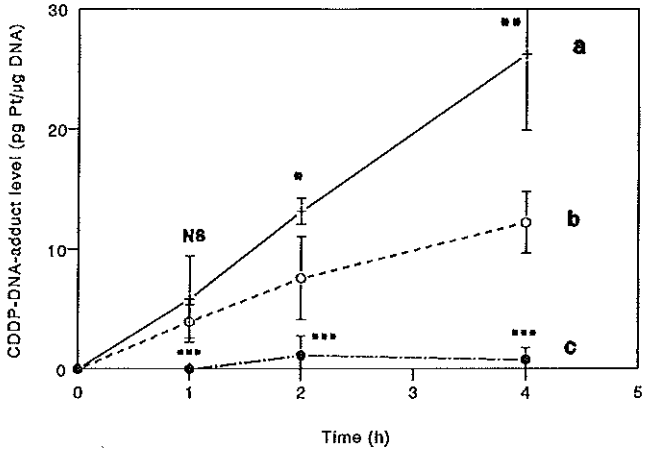

Figure 2. Relationship between the exposure of CDDP I5 µg/ml] and CDDP-DNA adduct levels in the IGROV1 cell line. [a] CDDP in RPMI-1640 medium with $6x10^6$ cells, [b] CDDP in a 7% solution of human albumin in RPMI-1640, [c] CDDP was preincubated with human albumin for 24 h and added to RPMI-1640.
* $p < 0.05$, ** $p < 0.01$ (a compared to b), *** $p < 0.01$ (c compared to b). NS not significant.

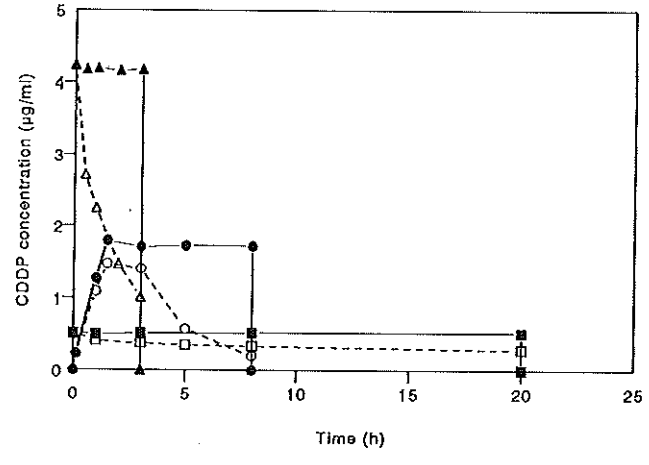

Figure 3. Total and unbound CDDP of the three dose-input functions.
▲,△ total and unbound CDDP of the i.v. bolus;
●,○ total and unbound CDDP of the 3 h infusion;
■,□ total and unbound CDDP of the 20 h infusion.

Table 1. Pharmacokinetic and -dynamic data of CDDP *in vitro* (Mean ± s.d. of four observations)

Dose-input	Cmax (μg/ml)	AUC (μg.hr/ml)	Amax (pgPt/μg DNA)	AUA (pgPt.hr/μg DNA)	Initial Slope (%)
Intravenous bolus	4.23 ± 0.12[a]	6.33 ± 0.36	8.8 ± 0.9	293 ± 32	21 ± 1[b,c]
Three hour infusion	1.47 ± 0.03	6.34 ± 0.59	8.5 ± 0.7	271 ± 10	31 ± 9[b]
Twenty hour infusion	0.52 ± 0.05	6.76 ± 0.40	7.4 ± 1.2	319 ± 49	13 ± 1
		NS	NS	NS	

[a] $p < 0.05$ 0.52 < 1.47 < 4.23
[b] $p < 0.05$ compared to 20 hr-infusion
[c] $p = 0.07$ compared to 3 hr infusion
NS = not significantly different

CDDP in the three experiments are given in Figure 3. The profiles of unbound CDDP in the simulated i.v. bolus and 3 h and 20 h infusions were not significantly different from those expected in plasma. The elimination half-life of unbound CDDP in the i.v. bolus experiment was 1.5 ± 0.20 h, and in the 3 h infusion experiment 1.7 ± 0.20 h (not significantly different). In the i.v. bolus experiment only 22% CDDP was unbound after 3 h of incubation. In the 20 h infusion experiment 55% was unbound after 20 h of incubation (Figure 3). The Cmax of CDDP was highest in the i.v. bolus and lowest in the 20 h infusion experiment (Table 1). The AUC values of the three dose-input functions were not significantly different.

Analysis of DNA and CDDP-DNA adduct levels

CDDP-DNA adduct levels could be determined reproducibly in the observed range of 0.5 to 15 pgPt/μgDNA. The coefficient of variation was 20% at 1.5 ng Pt/ml (240 pg Pt per injected sample) and 8% at higher concentrations. The detection limit was 100 pg Pt per sample.

The maximal adduct levels (Amax) were reached at the end of the incubation with CDDP (Figure 4). The differences between the three experiments were not significant (Table 1), although there appeared to be a rank order with the i.v. bolus having the highest and the 20 h infusion experiment the lowest Amax level. Also, the AUA values, calculated up to 68 h, were not significantly different between the experiments. The initial decline

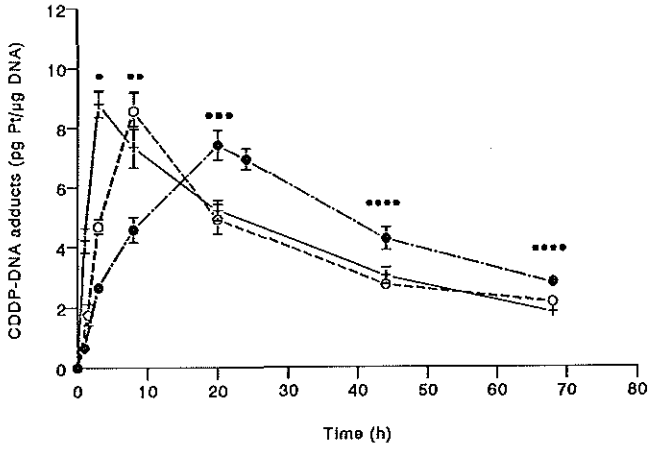

Figure 4. CDDP-DNA adduct-time curves of the three dose-input functions.
(+) = intravenous bolus (o) = 3 h infusion (•) = 20 h infusion
* $p < 0.01$: i.v. bolus significantly higher than 3 h infusion and 3 h infusion significantly higher than 20 h infusion.
** $p < 0.01$: i.v. bolus and 3 h infusion significantly higher than 20 h infusion.
*** $p < 0.01$: 20 h infusion significantly higher than i.v. bolus and 3 h infusion.
**** $p < 0.05$: 20 h infusion significantly higher than i.v. bolus and 3 h infusion.

in the adduct-time curve (initial slope in Table 1) was significantly more rapid in the i.v. bolus and 3 h infusion than in the 20 h infusion experiment.

Cell survival measurement

The decline of the cell survival with time was highest in the i.v. bolus and lowest in the 20 h infusion experiment (Figure 5). A log-linear relationship was observed between the decline in the cell survival and the AUC of unbound CDDP (Figure 6). The slopes of the three dose-input functions were not significantly different (Figure 6). The log-linear slope of the i.v. bolus experiment was 12.9 $\pm$ 0.13, of the 3 h experiment 13.2 $\pm$ 0.66 and of the 20 h experiment 11.7 $\pm$ 0.53 (all n=3; not significantly different).

DISCUSSION

In the present study relationships were established between the exposure to unbound CDDP, CDDP-DNA adduct kinetics and tumour cell survival. To assess these relationships former and presently used clinical dose-input functions were simulated *in vitro*, using a cell culture of the IGROV1 human ovarian cancer cell line.

After administration to patients, unbound CDDP is eliminated by irreversible protein binding and renal elimination. The clearance by protein binding exceeds renal clearance of unbound CDDP approximately by a factor of 4 to 5 (Bajorin *et al.*, 1986). Irreversible protein binding can easily be simulated *in vitro*, as is illustrated in Figure 1. Human albumin was added to the RPMI-1640 culture medium of the IGROV1 cell line as outlined. The results show that the protein binding kinetics in this mixture and in human plasma are similar. The concentration range of CDDP *in vitro* in the present study was of the same order as is observed in plasma of patients after administration of a dose of approximately 80 mg/m^2 (Vermorken *et al.*, 1986). The elimination kinetics of CDDP *in vitro*, using the outlined approach, reflects the kinetics *in vivo* (Vermorken *et al.*, 1982; Vermorken *et al.*, 1986). The elimination half-life is slightly longer because of the absence of renal clearance. In the present model the remaining low concentration of unbound CDDP was removed from the medium after 3, 8 and 20 h in the i.v. bolus, 3 h and 20 h experiment respectively, to prevent low remaining concentrations of unbound CDDP and to facilitate the calculation of the AUC.

The simulation of the clinical dose-input functions of CDDP *in vitro* has some limitations. The cell line is exposed to concentration-time profiles of CDDP which are observed *in vivo* in the blood compartment. This profile may be different from the exposure of tumour cells in solid tumours in peripheral tissues in patients. The concentration of unbound CDDP to which the tumour cells are exposed in the *in vitro* model will be slightly different from the

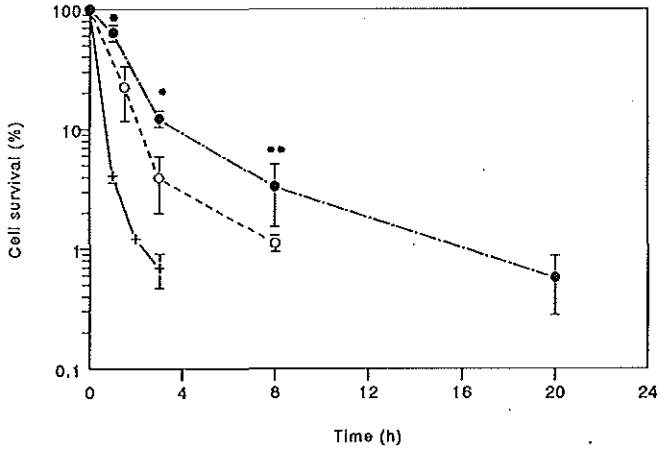

Figure 5. Influence of the three dose-input functions of CDDP on cell survival of IGROV1 tumour cells in the clonogenic assay.
(+) = intravenous bolus (o) = 3 h infusion (•) = 20 h infusion
* $p < 0.01$: 20 h infusion significantly higher than 3 h infusion and 3 h infusion significantly higher than i.v. bolus.
** $p < 0.05$: 20 h infusion significantly higher than 3 h infusion.

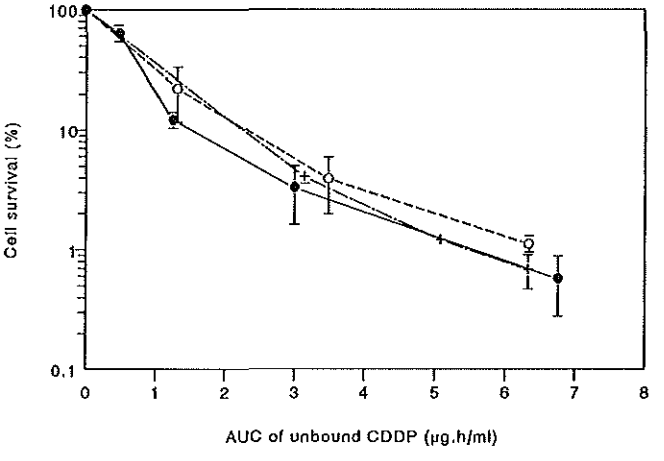

Figure 6. Log-linear relationship between the AUC of unbound CDDP of the three dose-input functions of CDDP and cell survival.
(+) = intravenous bolus (o) = 3 h infusion (•) = 20 h infusion.

concentration in the tumour in patients, because no steady state has been reached after a single CDDP administration. Another limitation of the model is associated with the use of a logarithmic growing cell culture instead of the use of a solid tumour. However, because of the similarities between the pharmacokinetics of unbound CDDP *in vivo* in patients and *in vitro* in the present model, the model is considered appropriate for the assessment of pharmacokinetic-dynamic relationships of unbound CDDP, despite the outlined restrictions.

Protein-bound CDDP is not able to form CDDP-DNA adducts, which is illustrated in Figure 2. Hence the unbound CDDP should be used for the assessment of the pharmacokinetic-dynamic relationship. In the light of this observation it should be realised that clinical trials which use CDDP preincubated with albumin (Holding *et al.*, 1992) may not lead to significant anti-tumour responses.

The design of the simulation of the three dose-input functions resulted in non-significant differences in the AUC of unbound CDDP. This is an ideal starting point to assess the influence of the rate of input of CDDP on CDDP-DNA adduct formation and repair and cell survival.

The Amax in the three experiments was not significantly different, although the level in the i.v. bolus and 3 h infusion was slightly higher than in the 20 h infusion. The AUA was clearly not significantly different in the three dose-input functions. This illustrates that the rate of input of CDDP does not influence the magnitude of the adduct formation.

The initial rate of decline of the adduct-time curve was significantly lower in the 20hr infusion. The rate of decline was studied in all experiments after withdrawal of CDDP. It is not clear what mechanism has caused the difference. Furthermore, the increase of the adduct formation with time in the 20 h infusion experiment is clearly non-linear (Figure 4). However, the experimental design does not permit assessment of differences in adduct removal during the period of CDDP exposure. The observed differences in the adduct kinetics had no implications for cell survival (Figure 6). This seems to be in contrast to the results of Troger *et al.* (1992). They have found that the longest exposure time of total CDDP in a head and neck cancer cell line resulted in the lowest cytotoxicity. However, protein binding of CDDP in the culture medium was not taken into account and no adduct levels were determined. Correction for the unavoidable protein binding of CDDP might have changed the results of that study.

The log-linear relationship between the AUC of unbound CDDP and cell survival was strikingly similar in the three dose-input functions. This implies that cell kill is determined not by the rate of input of CDDP, but by the magnitude of the exposure to unbound CDDP. If these results are confirmed

in other tumour models, then they may have significant implications in designing optimal modes of clinical CDDP administration.

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CHAPTER 8

ABROGATED ENERGY-DEPENDENT UPTAKE OF CISPLATIN IN A CISPLATIN-RESISTANT SUBLINE OF THE HUMAN OVARIAN CANCER CELL LINE IGROV-1

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ABSTRACT

The parental IGROV-1 human ovarian adenocarcinoma cell line was intermittently exposed to increasing concentrations of cisplatin to obtain resistant sublines. A stable resistant subline with a resistance factor of 24 had been developed after 9 months and 28 passages, which was denoted $IGROV_{CDDP}$. A high correlation coefficient of 0.97 was found between the log cell survival and the DNA-adduct peak level during the process of resistance development. $IGROV_{CDDP}$ was significantly cross-resistant to carboplatin and doxorubicin and moderately cross-resistant to etoposide, docetaxel and topotecan but not to 5-fluorouracil (5-FU) and methotrexate (MTX). Intracellular accumulation of cisplatin was 65% lower in the $IGROV_{CDDP}$ compared to the parental IGROV-1 at 37°C and standard incubation medium. Co-incubation of cisplatin with the Na^+/K^+-ATPase inhibitor ouabain resulted in a more pronounced decrease of platinum accumulation in IGROV-1 (44% decrease) than in the $IGROV_{CDDP}$ (26% decrease). Under energy-deprived conditions, the accumulation of cisplatin in the parental cell line was approximately 60% lower than under standard ATP-rich culture conditions. In contrast, the accumulation in $IGROV_{CDDP}$ was not affected by ATP-depletion. There was no significant difference between the intracellular accumulation of platinum in the resistant and sensitive cells, neither under energy-deprivation, nor when the uptake was studied at 0°C. In conclusion, abrogation of energy-dependent accumulation in $IGROV_{CDDP}$ seems to be a major mechanism of resistance to cisplatin in this cell line.

INTRODUCTION

Cisplatin is one of the most widely applied anticancer agents and is particularly effective in the treatment of testicular and ovarian cancer [1]. A major limitation associated with the application of cisplatin is the existence or development of resistance. The cytotoxic activity of cisplatin is most closely correlated with the covalent binding to DNA, the so-called DNA-adduct formation [2]. Several mechanisms of resistance to cisplatin have been documented, for example increased repair of the induced adducts, increased intracellular inactivation by glutathione and metallothioneins, increased efflux of cisplatin-glutathione complexes, decreased influx into the cell by presently not fully characterized alterations in active cell membrane transport mechanisms and overexpression of oncogenes or tumour suppressor genes [3-5]. Resistance to cisplatin overlaps in many cases with resistance to other anticancer agents, hence mechanisms of resistance are not unique for cisplatin [6]. Resistant cell lines of sensitive parental cell lines have in many cases been established by continuous exposure to the drug [7-9]. To simulate the clinical condition more closely an intermittent exposure

seems to be more appropriate. This strategy was followed to develop a resistant cell line, which can be used as a tool to study mechanisms of cisplatin resistance. In a preliminary report we have described that GSH-levels in the resistant cell line were not increased, and that the apparent level of MDR-related P-gp in the resistant and parental cell line were not significantly different [10].

The aim of the present study was to further characterize the mechanism of resistance to cisplatin in $IGROV_{CDDP}$, which was developed through intermittent exposure of the parental IGROV-1 cell line to cisplatin [10].

MATERIALS AND METHODS

Chemicals

Platinum standard solution (500 ppm) was obtained from Baker (Deventer, The Netherlands). DNAase I (EC 3.1.21.1), sulforhodamine B (SRB), glucose-free RPMI 1640 medium, 2-deoxy-D-glucose and sodium azide were from Sigma (St. Louis MO, USA). Proteinase K was purchased from Merck (Darmstadt, Germany). RPMI 1640 was obtained from GibcoBRL (Life Technologies B.V., Breda, The Netherlands). Bovine calf serum (BCS) was from Hyclone (Logan, Utah, USA). Cisdiamminedichloroplatinum II (cisplatin) and MTX were purchased from Lederle (Wolfratshausen, Germany). Carboplatin, paclitaxel (Taxol®), etoposide (VP16) were purchased from Bristol Myers (Troisdorf, Germany). Doxorubicin was from Pharmacia (Brussels, Belgium). 5-FU was from Roche (Mijdrecht, The Netherlands). Ouabain was purchased from Pharmachemie (Haarlem, The Netherlands). Docetaxel (Taxotere®) was kindly supplied by Rhône-Poulenc Rorer (Alfortville, France).

Instruments

A Perkin-Elmer 3030B atomic absorption spectrophotometer (AAS), equipped with a HGA600 flameless system and AS 60 autosampler was applied (Uberlingen, Germany) for determination of intracellular cisplatin (measured as platinum) and cisplatin DNA-adducts. A Beckman DU62 UV spectrophotometer (Fullerton CA, USA) was used for spectrophotometrical quantitation of DNA. A Perkin-Elmer LS-3B fluorescence spectrophotometer (Uberlingen, Germany) was used for the determination of DNA interstrand cross-links. Cell cycle analyses were performed on a FACSAN flow cytometer (Becton Dickinson (Etten-Leur, The Netherlands).

Cell lines and development of resistance

The sensitive IGROV-1 human ovarian cancer cell line was originated by Dr. J. Benard et al. [11]. Cells were cultured in RPMI 1640 medium with Hepes 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 mycoplasma contamination.

The cells were exposed to cisplatin for one hour once a week or 2 weeks, dependent on the recovery. The treated cells were cultured in fresh medium until the surviving cells had recovered and showed a normal exponential growth rate. The incubation concentration was gradually increased from 1 μg/ml (IC_{90} of the sensitive cell line) to 10 μg/ml. After 20 passages the cells were split and a part was repeatedly exposed to the concentration of 10 μg/ml, whereas the other part was exposed to further increasing concentrations of cisplatin. The resistant cell line which was developed after 28 passages was denoted $IGROV_{CDDP}$.

Cloning efficacy

The colony formation was determined after plating 1000 exponentially growing cells of the sensitive and resistant cell lines. Colonies were counted after 10-14 days after start of the experiment. The experiment was carried out four times in duplicate.

Assessment of cytotoxicity

Two different methods were applied for the assessment of the cytotoxicity in the cell culture experiments. The clonogenic assay was used as previously described [12,13]. Briefly, single logarithmic growing cells were seeded into T25 tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) in 4 ml of growth medium, in 2 different cell densities each in duplicate. After an overnight incubation, cisplatin was added and incubated for 2 hours. Subsequently, the cells were washed twice with PBS and cisplatin-free medium was added. The cells were then incubated for another 2 weeks during which period medium was refreshed three times. After fixation and staining with haematoxylin, colonies were counted. The assay was used to determine the development of resistance.

The sulforhodamine B test (SRB) assay was carried out as previously described [14] and was used to determine the level of cross-resistance in the resistant cell line for different antitumour agents. Carboplatin, doxorubicin, 5-FU, methotrexate, topotecan, paclitaxel and docetaxel were used as substrates.

The survival was tested every 4 passages during the development of resistance and the cells were used in experiments after culturing in cisplatin-free medium for a period of 2 weeks.

Intracellular platinum accumulation

The intracellular platinum accumulation was studied using 6 T175 flasks (Greiner, Alphen a/d Rijn, The Netherlands) each containing approximately 1-1.5x10^7 50-70% confluent cells. The parental and resistant cells were used 3-4 days after plating. The cells were incubated with cisplatin for 2 hours at a concentration of 0, 2.5, 5.0, 10, 25 and 50 μg/ml. Immediately afterwards, the cells were washed twice by ice-cold PBS and harvested by scraping. The cells were collected in two volumes of 1.5 ml ice-cold PBS, centrifuged and resuspended in 1.0 ml of deionized (Milli-Q) water. A volume of 50 μl was taken for protein determination by the Lowry method [15] and another 800 μl were dried by centrifugation under vacuum for 2 hours at 70°C. The cell pallet was digested in 200 μl of 65% nitric acid at 75° C for 2 hours. After dilution with water, platinum was analyzed by AAS [16]. The cellular platinum level was expressed as ng platinum (Pt) per mg protein.

The effects of co-incubation with ouabain, temperature reduction and energy-deprivation on the intracellular accumulation of platinum were investigated as previously described [17,18]. In the ouabain experiment, $IGROV_{CDDP}$ and IGROV-1 cells were exposed to 10 μg/ml of cisplatin for 2 hours with or without 0.5 μg/ml of ouabain.

The energy-depletion experiment was performed by replacing the standard medium by glucose-free RPMI 1640 medium which contained 1 mg/ml of 2-deoxy-D-glucose and 10 mM of sodium azide 15 minutes prior to exposure of 10 μg/ml of cisplatin to the cells for 2 hours. The intracellular concentration of cisplatin was measured by AAS as described above.

The influence of temperature reduction on the accumulation of platinum was studied by incubating cells with cisplatin at 0°C. Experiments at 0°C were performed simultaneously with incubations at 37°C and with the same batch of cells. After exposure of the cells to cisplatin, cells were washed and collected. Subsequently, platinum accumulation was determined by AAS as described above.

Efflux of platinum

Parental and resistant cells were incubated in culture flasks for 30 minutes with 30 and 90 μg/ml of cisplatin, respectively, to achieve equal levels of intracellular platinum. Subsequently, the cells were washed twice with PBS and cultured in drug free medium. After 0, 10, 30, 60 and 120 minutes a flask

was withdrawn, the cells were washed with ice-cold PBS and collected by scraping. Cellular platinum was analyzed by AAS.

Total cisplatin-DNA adduct and interstrand cross-link kinetics

Total cisplatin-DNA adducts were determined in the parental cell line and during development of drug resistance. Exponentially growing cells (1-1.5x10^7) in 6 T175 flasks were exposed to 4 μg/ml of cisplatin for 2 hours. Subsequently, the cells were washed twice with PBS and fresh cisplatin-free medium was added. At time -1, 0, 6, 24, 48 and 72 hours after withdrawal of cisplatin, 1 flask was taken and cells were harvested by trypsinization. Cells were washed with nuclear buffer (400 mM NaCl; 2 mM EDTA; 10 Mm Tris PH 7.3) once, resuspended in 3 ml of nuclear buffer and subsequently stored at -20°C until DNA isolation within 2 weeks. DNA isolation was carried out according to a previously published method [12,19]. Total cisplatin DNA-adducts were determined according to the method of Reed et al. [20], with modifications according to Ma et al. [12]. DNA-adduct levels were expressed as pg Pt/μg DNA.

The relationship between the incubation concentration and the total DNA adducts and ISC was studied, using two series of 6 T175 flasks containing the sensitive and resistant cell lines, using the same incubation conditions as described for the intracellular platinum accumulation experiment. Cells were exposed to cisplatin at a concentration of 1, 2, 4, 10, 20, or 30 μg/ml for 2 hours. Subsequently, cells were washed twice with PBS and collected by trypsinization and washed once more with nuclear buffer. The cells were resuspended in 3 ml of the nuclear buffer and stored at -20°C until isolation of DNA for determination of total DNA adducts and interstrand cross-links. Interstrand cross-links were analyzed using the ethidium bromide (EB) method as described by de Jong et al. [21] and Ali-Osman et al. [22] with some modifications. Briefly, 5-10 μg DNA (isolated as outlined) were dissolved in 200 μl of nuclear buffer. Each sample was divided into two portions. To each sample 3 ml of an EB solution (10 μg/ml, pH 12) were added. The first sample was immediately analyzed for the native fluorescence of DNA using an excitation wavelength of 525 nm and emission wavelength of 580 nm. The second sample was heated to 100°C and kept at that temperature for 5 minutes. Subsequently, the sample was immediately cooled down to room temperature, the fluorescence analyzed and the ISC's calculated as previously reported [21]. Experiments were carried out in duplicate and at least in 3 independent experiments.

Flow cytometric analysis

Determination of the cell cycle distribution was carried out after incubation of IGROV-1 and $IGROV_{CDDP}$ with equitoxic levels of cisplatin (the IC_{50}). Samples were stained with propidium iodine according to the method of Krishan [23]. Cisplatin was continuously incubated for 2 days and samples were analyzed after 24 and 48 hours.

Statistical analysis

Student t-test and Pearson correlation analysis were used and $p<0.05$ was applied as the significance level.

RESULTS

During 9 months of incubation and 5 stepwise increases of the incubation concentration of cisplatin from 1 to 10 μg/ml a stable resistant cell line denoted $IGROV_{CDDP}$ was established with a resistance factor of 24 in the colony forming assay and a factor of 8.4 in the SRB assay. The IC_{50} of cisplatin in IGROV-1 in the clonogenic assay was 0.26 $\pm$ 0.10 and in $IGROV_{CDDP}$ 6.4 $\pm$ 1.2 μg/ml. No significant changes of the doubling time and cloning efficacy were observed (with doubling times of 28 $\pm$ 3.4 and 26 $\pm$ 4.5 hours, and cloning efficacies of 19 $\pm$ 8% and 23 $\pm$ 4% for the IGROV-1 and $IGROV_{CDDP}$, respectively). Without cisplatin exposure the $IGROV_{CDDP}$ remained resistant during the follow up period of 4 months.

$IGROV_{CDDP}$ was highly cross-resistant to carboplatin and doxorubicin (Table 1). The cell line was also moderately cross-resistant to paclitaxel, docetaxel, etoposide and topotecan. Minor or nonsignificant cross-resistance to 5-FU and methotrexate was observed.

The resistance of IGROV-1 to cisplatin increased and the DNA-adduct peak height after cisplatin exposure decreased with increasing passage number (Figure 1). High correlations were found between the log cell survival and DNA-adduct peak height ($R=0.97$) and area under the DNA-adduct-time curve (AUA) up to 72 hours ($R=0.96$) after cisplatin exposure.

TABLE 1. CROSS RESISTANCE OF IGROV$_{CDDP}$ TO OTHER ANTICANCER DRUGS

DRUGS	IGROV-I (IC_{50} μM)	IGROV$_{CDDP}$	Rf	p
CISPLATIN	0.53	4.46	8.4	<0.001
CARBOPLATIN	5.04	32.24	6.4	<0.001
DOXORUBICIN	0.13	0.77	6.0	<0.001
PACLITAXEL	0.07	0.22	3.1	<0.001
DOCETAXEL	0.02	0.06	3.2	0.01
ETOPOSIDE	1.33	3.55	2.7	0.002
TOPOTECAN	0.02	0.06	2.7	<0.05
5-FU	2.48	4.14	1.7	<0.05
MTX	0.04	0.04	1.0	NS

Rf = resistance factor, determined with the SRB method in which resistant and sensitive cell lines were exposed to the drug for 5 days
NS = not significant

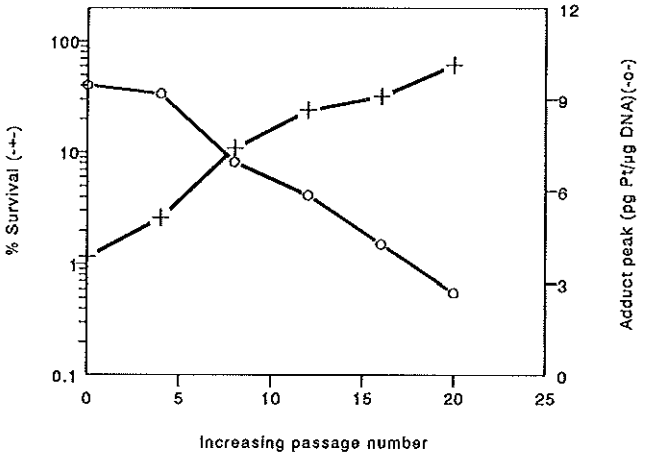

Figure 1. Log-linear relationship between increasing passage number and cell survival (clonogenic assay) and DNA-adduct peak height, as determined by AAS. Determinations were performed after incubation with 4 μg/ml of cisplatin for 2 hours.

The intracellular accumulation of cisplatin in IGROV$_{CDDP}$ was 65% lower than in IGROV-1 (Figure 2). There was a linear and apparently non-saturable relationship between the incubation concentration and the intracellular platinum concentration in the 2 cell lines over the studied range of 1 to 50 μg/ml. The slope of the regression line in IGROV$_{CDDP}$ was 1/3 of the slope in IGROV-1 ($p<0.01$). In accordance with this lower intracellular accumulation of cisplatin, the DNA-adduct peak height and AUA decreased 3-fold with increasing resistance (data not shown). Also the relationship between the

total DNA-adducts or ISC and the incubation concentration was linear over the studied range with a difference in slope between the 2 cell lines of approximately 1/3 (Figure 3a and 3b).

Co-incubation of cisplatin with the Na^+/K^+-ATPase inhibitor ouabain resulted in a decrease of platinum accumulation by 44% in the IGROV-1 cell line. However, in the resistant $IGROV_{CDDP}$ a significantly lower decrease of 26% was noted (data not shown). Lowering the temperature to 0°C resulted in a significantly reduced intracellular accumulation of cisplatin in the sensitive (by 95%, as compared to 37°C) and resistant cells (by 91%, as compared to 37°C) (Figure 4). At 0°C there was no significant difference between the intracellular concentration of platinum in the IGROV-1 and $IGROV_{CDDP}$ cell lines.

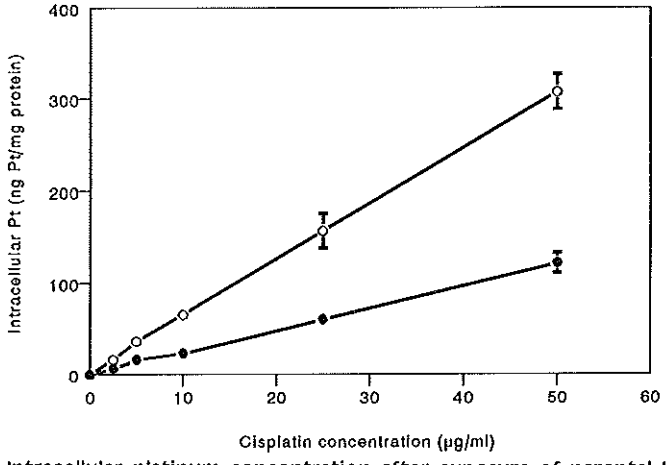

Figure 2. Intracellular platinum concentration after exposure of parental IGROV-1 (o) and $IGROV_{CDDP}$ (•) cell lines to increasing concentrations of cisplatin (exposure time 2 hours). Intracellular concentrations of platinum were measured by AAS as described in Materials and Methods.

Under energy-deprived conditions the intracellular platinum concentration in IGROV-1 was significantly reduced to 35% of the level obtained under standard energy-rich culture conditions (Figure 4). However, in the $IGROV_{CDDP}$ cell line, depletion of energy did not result in a significantly decreased accumulation of platinum. Notably, under energy-depriving conditions the difference between the sensitive and resistant cell lines was no longer significant (Figure 4).

The efflux of platinum within the first 10 minutes after withdrawal of cisplatin in the $IGROV_{CDDP}$ was significantly higher than in the IGROV-1 (17.3 vs 0%; $p<0.05$). After 2 hours no efflux of platinum could be observed in any of the two cell lines.

Monitoring the cell cycle distribution revealed that the cell cycle distribution in IGROV-1 and IGROV$_{CDDP}$ without cisplatin exposure was not different. Following a 2 days exposure to equitoxic (IC_{50}) concentrations of cisplatin, both cell lines accumulated in G_2/M phase. The % of increase in G_2/M 48 hours after stopping the incubation in IGROV$_{CDDP}$ was higher than in IGROV-1 (41% vs 23%) at 48 hours.

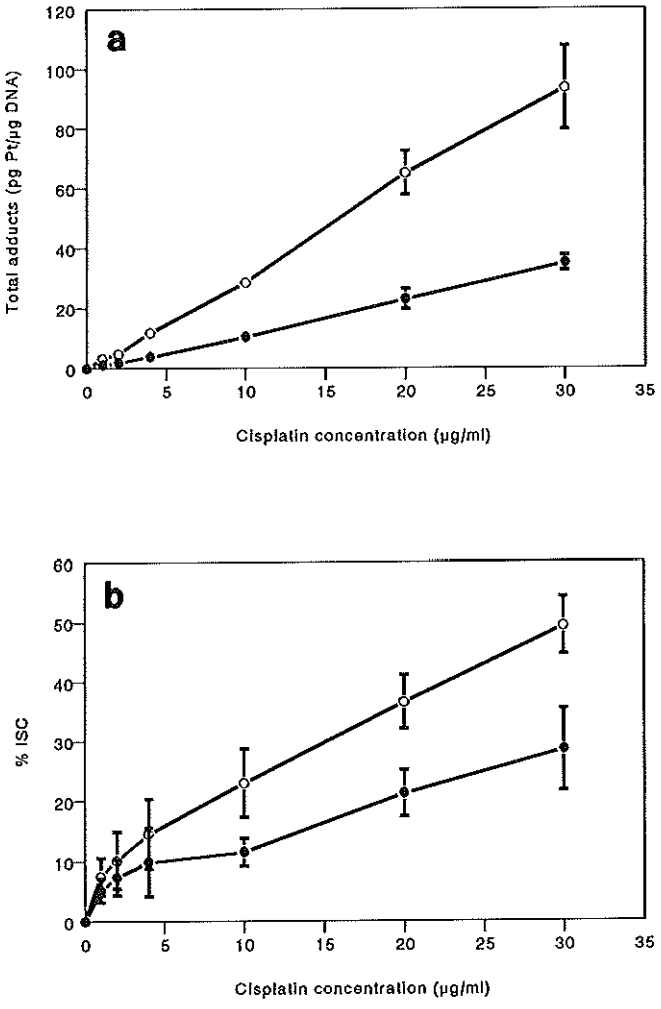

Figure 3. Total DNA adducts (a) and ISC (b) after exposure of IGROV-1 (o) and IGROV$_{CDDP}$ (•) cells to increasing concentrations of cisplatin for 2 hours. Total adduct and ISC levels were determined as described in Materials and Methods.

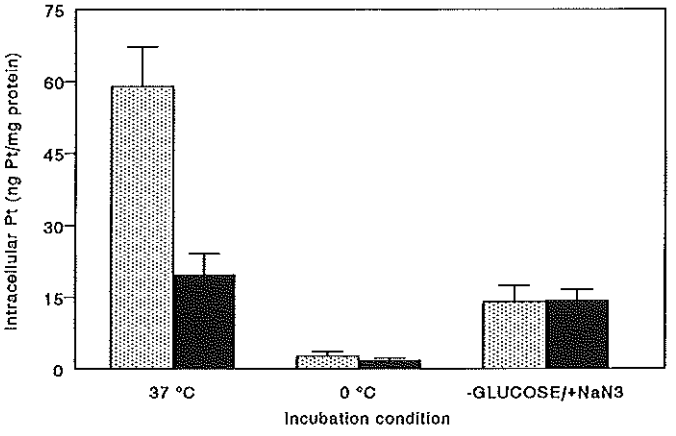

Figure 4. Intracellular concentrations of cisplatin in the IGROV-1 [:::] and $IGROV_{CDDP}$ [▓] cell lines, following 2 hours incubation with 10 μg/ml of cisplatin. Furthermore the accumulation at 0°C and under energy-deprived conditions (as described in Materials and Methods) is displayed.

DISCUSSION

Both intrinsic and acquired resistance to cisplatin limit the clinical utility of this anticancer agent. Previous studies have shown that decreased accumulation of cisplatin is an important factor in *in vitro* and *in vivo* acquisition of resistance [24,25]. In the present study we developed and characterized a resistant subline of the IGROV-1 human ovarian adenocarcinoma cell line which was established previously by Benard et al. [11]. The cell line was intermittently exposed to mimic the clinical application of cisplatin. Intermittent exposure of the cisplatin sensitive IGROV-1 ovarian cancer cell line resulted in a stable resistant cell line, denoted $IGROV_{CDDP}$ with a resistance factor of 24 in the colony forming assay and 8.4 in the SRB assay. The difference in the resistance factors between the two assays in the same cell line are possibly due to the different exposure times to cisplatin. In the clonogenic method, the exposure time was 2 hours, whereas in the SRB assay cells are incubated for 5 days. For practical reasons the SRB assay was used after the resistance factor had initially been measured with the clonogenic assay.

Accumulation of cisplatin in the $IGROV_{CDDP}$ is decreased significantly, as compared to the parental cell line. Ouabain, a Na^+/K^+-ATPase inhibitor [26-28], in combination with cisplatin resulted in a significant decrease in the intracellular concentration of platinum. The effect was more pronounced in

the IGROV-1, which suggests that the functional activity of Na^+/K^+-ATPase has changed in $IGROV_{CDDP}$. The reduced influence of ouabain in the resistant cell line is also suggestive for an important role of K^+ homeostasis and Na^+/K^+-ATPase in the mechanism of active uptake of cisplatin. Further studies are needed to unravel whether this mechanism is associated with the presented decreased uptake of cisplatin.

Under normal culture conditions (i.e. at 37°C), the intracellular concentration of platinum in IGROV-1 is 3-fold higher than in the $IGROV_{CDDP}$ cell line (Figures 2 and 4). However, the difference was not significant at 0° and under energy-deprived conditions (Figure 4). It has been suggested that cisplatin enters the cells partly by passive diffusion and partly by active transport [29]. Our energy-depletion experiments illustrate that in the IGROV-1 cells, approximately 65% of cisplatin is taken up actively, whereas approximately 35% enters the cell via passive diffusion. Furthermore, our results indicate that the energy-dependent uptake is abrogated in the $IGROV_{CDDP}$ cell line.

At 0°C both the sensitive and the resistant cell line show a decreased cisplatin accumulation. Notably, this accumulation of cisplatin is even lower than the accumulation observed under energy-deprived conditions (Figure 4). An explanation for this effect may be that at 0°C not only active, energy-dependent transport, but also passive transport of cisplatin is decreased, due to a changed membrane fluidity at this lower temperature. Therefore also this observation is suggestive of both active and passive accumulation of cisplatin in the IGROV-1, and only passive transport in the $IGROV_{CDDP}$ cell line.

No saturation of the uptake of cisplatin up to the highest tested concentration of 50 μg/ml has been observed, which is in agreement with other studies [30]. The DNA-adduct level decreased gradually during the development process of the resistant cell line, indicating a gradual reduction of the intracellular uptake of cisplatin. A log-linear relationship between cell survival and DNA-adduct kinetics of cisplatin was established during the development process of $IGROV_{CDDP}$.

Also in an other cisplatin-resistant human ovarian cancer cell line, reduced influx of cisplatin (without changes in efflux) has been reported [31-32]. The explanation for this reduced influx remains to be elucidated. Sharp et al. identified a plasma membrane protein (M_r 36,000) which was overexpressed in the cisplatin-resistant cell line [32]. However, the role of this protein in the reduced accumulation of cisplatin is not clear yet. If involved, this protein may be capable of inhibiting energy-dependent transport channels for cisplatin. Additional studies should be aimed at investigating whether this enzyme is also overexpressed in our $IGROV_{CDDP}$ cell line. As reduced accumulation is a well known phenomenon in *in vivo* acquired resistance to

cisplatin, extended research into the mechanism of this reduced accumulation is warranted. The $IGROV_{CDDP}$ cell line may be a good model for such studies.

In addition to the decreased uptake of cisplatin, the results revealed a small but significant increase in the efflux of platinum in $IGROV_{CDDP}$. Notably, the multidrug resistance-associated protein (MRP) has been suggested to be involved in cisplatin resistance (33,34,35). MRP may be capable of transporting glutathione-cisplatin complexes out of the cells. In the $IGROV_{CDDP}$ cell line, however, no overexpression of MRP was noted (unpublished results). The magnitude of this increase appears to be minor compared to the reduced uptake, because the intracellular concentration after exposure to cisplatin in $IGROV_{CDDP}$ is 70% lower whereas the efflux is only 17% higher over a short time period compared to IGROV-1. Therefore, changes in drug uptake in $IGROV_{CDDP}$ appear to be more important than changes in efflux.

We previously reported that the levels of P-gp in the IGROV-1 and $IGROV_{CDDP}$ were not significantly different, and that glutathione levels in the resistant cell line were not increased as compared to the parental cell line (10). However, the observed difference in the cell cycle distribution after the exposure to equitoxic concentrations of cisplatin suggests that also other than cellular membrane alterations have developed in the resistant cell line. The indication of multifactorial adaptations is supported by the observed pattern of cross-resistance with the tested anticancer agents. Further studies are needed to identify these additional alterations.

In conclusion, the $IGROV_{CDDP}$ is 24-fold resistant to cisplatin in the clonogenic assay, as compared to its parental cell line IGROV-1. The main mechanism of resistance appears to be altered uptake kinetics of the drug, possibly caused by abrogated active uptake of cisplatin. However, other cellular adaptations may have occurred. The cell lines which have been and currently are being developed can be used as a models in future studies directed at elucidating cell uptake related mechanisms of cisplatin resistance.

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Chapter 9

SYNERGISTIC CYTOTOXICITY OF CISPLATIN AND TOPOTECAN OR SN38 IN A PANEL OF 8 SOLID TUMOUR CELL LINES IN VITRO

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Abbreviations: IC_{50}(5 day), IC_{50} after 5 days of continuous exposure; IC_{50}(3 day), IC_{50} after 3 days of exposure; TPT, topotecan; ISC, interstrand cross-links; topo I, topoisomerase I; SRB, sulforhodamine B; ER, estrogen receptor; F_u, fluorescence yield untreated cells; F_t, fluorescence yield treated cells

(submitted)

SUMMARY

The cytotoxicity of cisplatin alone and in combination with topotecan (TPT) or SN38, two novel topoisomerase I (topo I) inhibitors, was determined in a panel of 8 well characterized human solid tumour cell lines. Interactions between cisplatin and these topo I inhibitors were investigated using three different administration schedules *1)* simultaneous incubation (C+T and C+S); *2)* cisplatin followed by TPT or SN38 (C→T and C→S); *3)* TPT or SN38 followed by cisplatin (T→C and S→C). Applying median-effect analysis, synergistic cytotoxicity was observed in 7 out of 8 cell lines used. In addition, a significant schedule-dependent synergistic cytotoxicity was found in 3 of the cell lines used, with C→T (or C→S) being the most active schedule. The formation and repair of total cisplatin-DNA adducts in the IGROV-1 ovarian cancer cell line and its cisplatin-resistant subline $IGROV_{CDDP}$ was not significantly affected by TPT, using simultaneous incubation. In contrast, repair of cisplatin-DNA interstrand cross-links in the IGROV-1 and $IGROV_{CDDP}$ was significantly lower after co-incubation of the cells with TPT. Assessment of the cell cycle distribution revealed an accumulation of cells in G_2/M phase after exposure to cisplatin. After exposure to TPT, a different pattern was observed that was cell type specific. Although up to four-fold differences in topo I activity were observed in this panel of cell lines, these differences do not appear to be related to the observed synergism between cisplatin and TPT or SN38. The observed synergy may at least partly be explained by the increased retention of cisplatin-DNA interstrand cross-links in the presence of topo I inhibitors.

INTRODUCTION

Cisplatin is one of the most widely used anticancer drugs in the clinic nowadays, and shows good responses in treatment of testicular cancer, NSCL or SCL cancer, ovarian cancer and head and neck malignancies [1,2]. The antitumour activity of cisplatin is supposed to be correlated with its interaction with DNA. Cisplatin is capable of forming potentially lethal intra- and interstrand DNA cross-links [3,4]. The clinical use of cisplatin is frequently limited by intrinsic or acquired resistance. Amongst other reasons, this resistance to cisplatin has been ascribed to increased repair of cisplatin-DNA adducts [5-7]. The importance of DNA repair in cisplatin toxicity is also evident in a testicular cancer cell line, that has decreased repair rate of cisplatin-DNA adducts, as compared to a bladder carcinoma cell line [8]. This deficiency in DNA repair was shown to contribute to the remarkable sensitivity of this testicular cell line to cisplatin [9]. Because of this apparent relation between sensitivity of tumour cells to cisplatin and DNA repair, inhibition of this repair may be clinically important in cisplatin chemotherapeutic treatment. A

promising class of agents in this respect are the topoisomerase I (topo I) inhibitors. Topoisomerases are involved in many important processes in the cell, e.g. in topologically changing of the cellular DNA structure, in DNA replication and transcription [10-12]. Topo I inhibitors such as topotecan (TPT) and SN38 (the active metabolite of irinotecan), show a broad antitumour activity in preclinical studies with a unique mechanism of action, targeting topo I. Both TPT and irinotecan are now in clinical phase I and phase II trials, and yield good responses in many tumour types as single agent or in combination chemotherapy [13-15]. Due to their non-overlapping toxicity and different mechanisms of action, the combination of topo I inhibitors with cisplatin is an attractive subject for further clinical study. There are many reports about the function of topo II inhibitors in cisplatin-DNA adduct repair [16-18]. However, the role of topo I in the formation and repair of these cisplatin-DNA adducts has not fully been identified yet, and consequently, there is a need for data on this subject. Recently, studies about the interaction between cisplatin and topo I inhibitors as TPT, irinotecan or SN38 revealed a synergistic effect between topo I inhibitors and cisplatin [19-21].

In the present study, the synergism between cisplatin and TPT or SN38 was investigated, using a panel of 8 human solid tumour cell lines covering a broad tumour spectrum and a broad range of sensitivities to cisplatin and topo I inhibitors. In addition, the schedule dependency of this combination was investigated, by administering cisplatin and TPT or SN38 using three different administration schedules. Total and interstrand adduct kinetics, in the presence and absence of TPT or SN38 were monitored, in an attempt to explain synergism between these drugs. Furthermore the possible relationship between the cell cycle distribution and synergism was investigated.

MATERIALS AND METHODS

Chemicals and drugs

TPT was a generous gift from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA, USA). SN38 was generously supplied by Rhône-Poulenc Rorer (Alfortville, France). Cisplatin was obtained from Lederle (Wolfratshausen, Germany). Plasmid pBR322 DNA, dithiothreitol (DTT), bovine serum albumin (BSA), bromophenol blue, sulforhodamine B (SRB), ethidium bromide, and DNase I (E.C. 3.1.21.1) were from Sigma (St. Louis, MO, USA). Proteinase K was purchased from Merck (Darmstadt, Germany). RPMI 1640 and DMEM medium was from GibcoBRL (Life Technologies B.V, Breda, The Netherlands). Bovine calf serum (BCS) was obtained from Hyclone (Logan, Utah, USA). EGTA was

purchased from Serva (Heidelberg, Germany). Propidium iodide was from Calbiochem (la Jolla, CA, USA).

Instruments

A Perkin-Elmer 3030B atomic absorption spectrophotometer was equipped with a HGA 600 flameless system and AS 60 autosampler (Uberlingen, Germany). Microplates were read on a Bio-Rad 450 microplate reader. Fluorescence measurements were performed on a Perkin-Elmer LS-3B fluorescence spectrophotometer. Flow cytometry was conducted on a FACScan flow cytometer (Becton Dickinson, Etten-Leur, The Netherlands), with excitation at 488 nm. The following parameters were measured: forward light scatter (FLS), perpendicular light scatter (PLS), and fluorescence of the DNA-PI complex (563-607 nm). Cell debris was excluded from analysis by an appropriate FLS threshold setting.

Cell lines and culture conditions

The human WiDr colon cancer, the H226 un-differentiated lung cancer, the A498 renal cell cancer, the M-19 melanoma, the IGROV-1 ovarian adenocarcinoma and its 8-fold cisplatin-resistant subline $IGROV_{CDDP}$ [22], as well as the estrogen receptor (ER) positive MCF7 breast cancer cell lines were cultured in RPMI 1640 supplemented with 10% BCS, 10 mM $NaHCO_3$, 2 mM glutamine, 110 IU/ml penicillin, 100 μg/ml streptomycin, 45 μg/ml gentamycin and 10 μg/ml insulin. Cells were kept in continuous logarithmic growth at 37°C in a humidified atmosphere of 5% CO_2 in air. The ER-negative EVSAT breast cancer cell line was cultured in DMEM supplemented with 5% BCS. Other supplements are as outlined above.

Cytotoxicity assay

Exponentially growing cells were trypsinized and plated (2000 cells/200 μl per well) in 96 well microplates (Costar Corporation, Cambridge, MA, USA). Subsequently, the cells were allowed to attach for 48 hours at 37°C under 5% CO_2. Following this attachment period, 100 μl of drug solution (diluted with RPMI 1640 or DMEM) was added to the wells. Next, the cells were incubated for 5 days at 37°C under 5% CO_2. Dependent on the administration schedule, the second drug was added after 2 days without washing away the first drug, and cells were incubated for another 3 days. Cytotoxicity was evaluated using the SRB method [23]. Briefly, at day 7, cells were fixed with 10% TCA, and stained with SRB (0.4% in 1% acetic acid) for 1 hour. After washing unbound SRB, bound SRB was dissolved in 10 mM Tris-buffer, and the absorption at 540 nm was determined using a microplate reader. Each agent was tested in quadruplicate in at least 3 independent experiments.

Drug combination schedules and data analysis

In the combination study, the following combination schedules were applied: *1)* cisplatin and TPT or SN38 were administered simultaneously for 5 days starting from day 2 (C+T or C+S); *2)* cells were incubated with cisplatin for 5 days starting from day 2, whereas TPT or SN38 was added on day 4, and cells were incubated for another 3 days (C→T or C→S); *3)* cells were incubated with TPT or SN38 for 5 days starting from day 2, followed by incubation with cisplatin from day 4 through day 7 (T→C or S→C).

Individual survival curves of cisplatin and TPT or SN38 following a 3 or 5 days exposure were generated using the SRB assay. In drug combination experiments, TPT or SN38 was combined with cisplatin at a constant molar ratio, dependent on the IC_{50}-values of cisplatin and TPT or SN38 following the respective incubation period (Table 1, Figures 1a and 1b). Drug interactions were evaluated using the median-effect analysis as described by Chou et al. [21,24,25]. The combination index for each fractional effect was calculated using the following equation:

$$\textit{Combination index} = d_1/D_1 + d_2/D_2$$

D_1 and D_2 are the doses of drug 1 and drug 2 which by themselves produce a given fractional effect (such as the IC_{50}); d_1 and d_2 are the doses which produce the same fractional effect in combination. Combination indices are generally interpreted as zero interaction or additive cytotoxicity when CI=1, a synergistic effect when CI<1, and an antagonistic effect when CI>1.

Quantitation and kinetics of total cisplatin-DNA adducts and cisplatin-DNA interstrand cross-links

DNA was isolated as previously described [26] with minor modifications [27], and was quantitated spectrophotometrically. The total amount of platinum bound to DNA was determined using AAS [27,28]. Prior to this measurement, the isolated DNA was DNAse I digested. Subsequently the sample was injected using the 4-times multiple injection feature of the AAS apparatus. Adduct levels are expressed as pg platinum per μg DNA (pg Pt/μg DNA).

The relative amount of cisplatin-DNA interstrand cross-links was determined using an ethidium bromide assay [16,29]. Ethidium bromide fluorescence was measured before and after denaturation of the DNA, using an excitation wavelength of 525 nm, and an emission wavelength of 580 nm. The relative amount of interstrand cross-links was calculated using the following formula [29]:

$$\% ISC = [(F_t-F_u)/(1-F_u)] \times 100\%$$

In which % ISC = the percentages interstrand cross-links DNA in cisplatin treated cells, F_t = the fluorescence in cisplatin treated cells, and F_u = the fluorescence in untreated cells (blanks). F_t and F_u are calculated as the ratio of ethidium bromide fluorescence after and prior to denaturation of the DNA.

In order to monitor the kinetics of total cisplatin-DNA adducts and ISC, IGROV-1 or $IGROV_{CDDP}$ cells (60-75% confluent in T75 flasks (Greiner, Frickenhausen, Germany)) were exposed for 2 hours to 10 μg/ml of cisplatin alone or to 10 μg/ml of cisplatin combined with 10 ng/ml of TPT. After 2 hours of incubation, cells were washed twice with PBS, drug-free medium was added and culturing was continued. In the case of combined cisplatin/TPT treatment, 10 ng/ml of TPT was added again to the medium immediately after this washing procedure. Directly, and 4, 8, 24 and 48 hours after stopping exposure to cisplatin, flasks were withdrawn, cells were harvested by trypsinization and washed with PBS. Subsequently, cells were resuspended in 3 ml TE buffer (10 mM Tris-HCl, 2 mM EDTA, and 0.4 M NaCl, pH 7.3) and stored at -20°C until DNA isolation. DNA was isolated, and the adducts were quantitated as described above.

Flow cytometric analysis

The cell cycle distribution was monitored following exposure of the cells to IC_{50}(5 day) doses of cisplatin or TPT. Furthermore, the effect of simultaneous exposure to a combination of cisplatin and TPT (at 50% of their IC_{50}(5 day) dose each) was monitored. Cells were exposed to cisplatin and/or TPT continuously, and flasks were withdrawn at different times following the start of the incubation. To obtain single cell suspensions, cells were trypsinized and washed twice with RPMI culture medium without phenol red. Cells were stained with propidium iodide (100 mg/l) and the DNA content was analyzed according to the method of Krishan [30].

Topoisomerase I catalytic activity

Crude nuclear extracts were prepared as described by others [31]. After isolation, the nuclear extracts were diluted 1:1 with 99% glycerol and stored in small aliquots at -80°C for a maximum period of 1 month. The protein concentrations were determined according to the Bradford method, using the Bio-Rad assay.

Topo I catalytic activity was assayed by monitoring the relaxation of 250 ng of supercoiled pBR322 DNA [31], using serial dilutions of nuclear extracts. The reaction mixture was incubated at 37°C for 30 min. After stopping the reaction, samples were analyzed by electrophoresis on a 1% agarose gel.

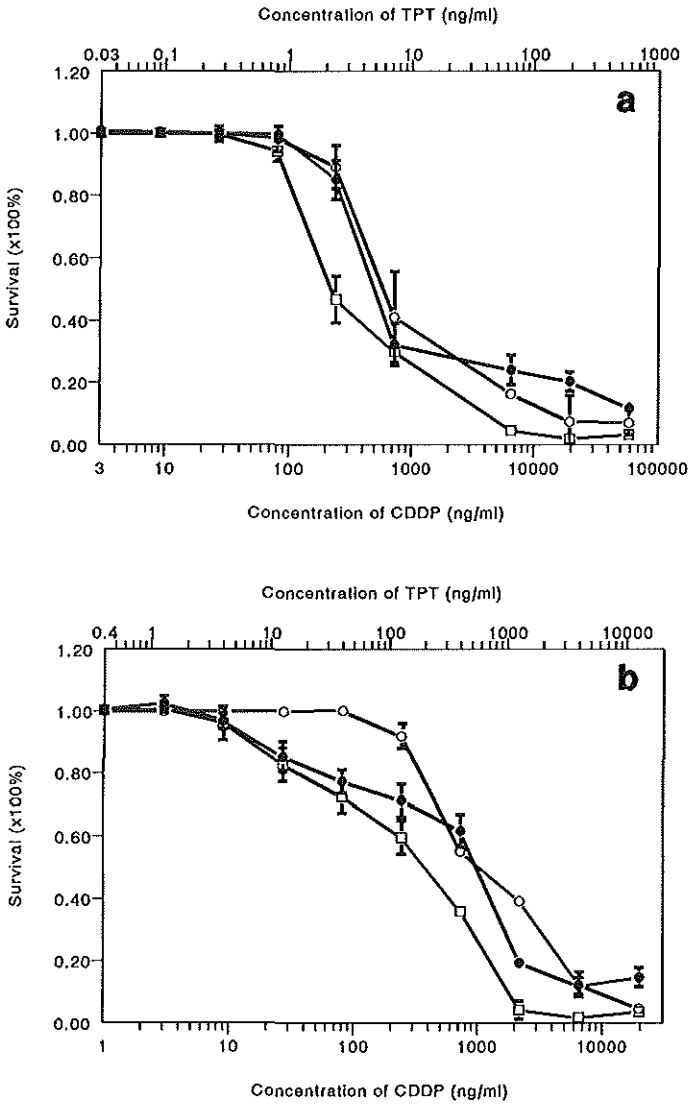

Figure 1. Cell proliferation curves of the MCF7 cell line. **a.** Cells were exposed continuously to cisplatin (○), TPT (●) or to a combination of cisplatin and TPT (□) for 5 days. A fixed ratio cisplatin:TPT of 100:1 (approximately equals IC_{50}(5 day) ratio of the individual drugs in the MCF7 cell line, see Table 1) was used in the combination experiment. **b.** Cells were exposed continuously to cisplatin for 5 days (○), to TPT for 3 days (starting at day 3) (●), or to a combination of cisplatin (5 days exposure) and TPT (3 days exposure, from day 3) (□). The fixed ratio cisplatin/TPT in this scheduled combination experiment was 2.7:1 (approximately equals IC_{50}(5 day)/IC_{50}(3 day) ratio of the individual drugs in the MCF7 cell line, see Table 1). Values are means ± s.d from at least 3 independent experiments.

After staining with ethidium bromide, bands were visualized on an UV light table and photographed with Polaroid (667) positive/negative films.

RESULTS

Characterization of the cell lines

The doubling times and sensitivities of the cells to cisplatin, TPT and SN38 are summarized in Table 1. The WiDr cell line was the fastest, whereas the H226 was the slowest growing cell line, with doubling times of 18 and 43 hours, respectively. Doubling times of the other cell lines were between 24 and 33 hours. The IGROV-1 cell line was most sensitive to cisplatin, whereas MCF7 was the most sensitive to TPT and SN38 using a 5-day incubation period.

Cytotoxicity of combined exposure to cisplatin and TPT or SN38

The results of combination experiments of cisplatin and TPT are shown in Figure 2. In all cell lines tested, except the A498 cell line, a markedly synergistic interaction between cisplatin and TPT was observed. In case SN38 was used, analogous results were obtained (summarized in Table 2). Comparing different combination schedules, the (C→T) and (C→S) treatment resulted in the most pronounced synergy. In the IGROV-1, the $IGROV_{CDDP}$, and the MCF7 cell lines, this schedule yielded significantly higher synergism ($p<0.05$) than the other combination schedules. Also in the other cell lines, except the H226, the C→T schedule tended to the most active. However, in these cases the difference between the (C→T) and other schedules did not reach statistical significance. Strikingly, in the H226 cell line the most pronounced synergism was observed in the (T→C) schedule (CI at $F_a=0.5$: 0.46 ± 0.07).

Effect of TPT on the kinetics of total cisplatin-DNA adducts and cisplatin-DNA interstrand cross-links

The kinetics of total cisplatin-DNA adducts and of ISC were evaluated in the IGROV-1 and the cisplatin-resistant $IGROV_{CDDP}$ cell lines. TPT did not affect the formation and removal of total cisplatin-DNA-adducts, as measured by AAS (data not shown). Furthermore, TPT did not affect the amount of adducts formed by cisplatin. However, monitoring of the ISC's revealed that TPT inhibits the removal of cisplatin-DNA ISC's both in the IGROV-1 and the $IGROV_{CDDP}$ cell lines (Figure 3). At all time-points, the number of ISC's in the presence of TPT tended to be higher than in the absence of TPT. In the IGROV-1 cell line, the number of ISC's, 8 to 48 hours following exposure to cisplatin was 10-29% higher in the presence of TPT (Figure 3a), whereas this difference was 30-39% in the resistant $IGROV_{CDDP}$ cell line (Figure 3b). Differences in DNA

Table 1. Doubling time (DT) and IC50 of cisplatin, TPT and SN38 in a panel of 8 human tumour cell lines, determined using the SRB assay.

Cell line	DT (hour)	IC50 (5 day)			IC50 (3 day)		
		cisplatin	TPT (ng/ml)	SN38	cisplatin	TPT (ng/ml)	SN38
IGROV-1	28	145 ± 17	6.5 ± 1.9	1.5 ± 0.2	1984 ± 326	541 ± 337	143.5 ± 86
IGROV/CDDP	26	1285 ± 285	23.0 ± 3.4	4.6 ± 0.5	7449 ± 1269	831 ± 163	400 ± 157.5
MCF7	24	676 ± 205	5.4 ± 0.6	0.9 ± 0.1	7525 ± 1648	422 ± 51	184.8 ± 45.2
EVSAT	25	687 ± 104	10.3 ± 2.1	1.0 ± 0.1	6505 ± 219	281 ± 141	49.3 ± 17.7
M-19	24	1210 ± 492	19.6 ± 10	3.2 ± 1.7	4598 ± 1068	552 ± 353	270.9 ± 28.9
H226	43	1275 ± 518	35.1 ± 31	7.3 ± 3.8	6319 ± 822	466 ± 66	195.8 ± 65.9
A498	33	925 ± 357	21.9 ± 4.7	6.2 ± 0.8	2355 ± 345	172 ± 32	110.8 ± 51.5
WIDR	18	609 ± 95	12.6 ± 2.9	4.1 ± 2.3	12770 ± 125	299 ± 63	106.9 ± 9.3

Table 2. Combination index at a fraction affected (Fa) of 0.5 of cisplatin, TPT, and SN38 in a panel of 8 human solid tumour cell lines.

Cell lines	C + T	CL (Fa = 0.5)		
		C + S	C → T	C → S
IGROV-1	0.83 ± 0.08	0.77 ± 0.15	0.41 ± 0.16	0.51 ± 0.12
IGROV/CDDP	0.68 ± 0.05	1.03 ± 0.12	0.52 ± 0.02	0.60 ± 0.07
MCF7	0.78 ± 0.08	0.81 ± 0.07	0.51 ± 0.07	0.51 ± 0.11
EVSAT	0.64 ± 0.12	0.77 ± 0.10	0.57 ± 0.10	0.54 ± 0.12
M-19	0.84 ± 0.06	0.85 ± 0.18	0.68 ± 0.09	0.67 ± 0.11
H226	0.78 ± 0.12	0.73 ± 0.09	0.66 ± 0.17	0.67 ± 0.09
A498	0.88 ± 0.09	0.89 ± 0.08	0.87 ± 0.12	0.91 ± 0.19
WIDR	0.96 ± 0.27	0.71 ± 0.11	0.62 ± 0.10	0.46 ± 0.04

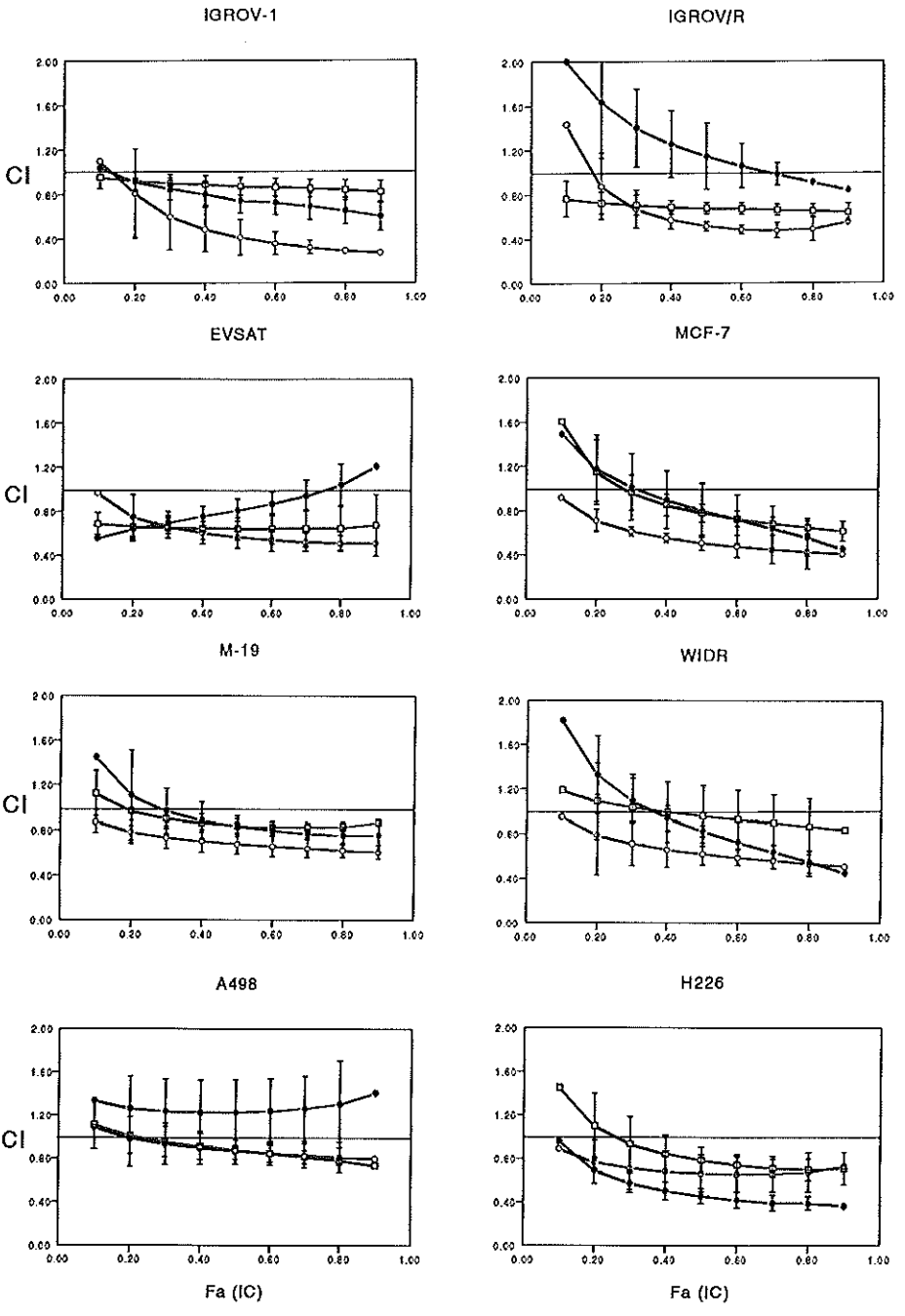

Figure 2. Combination index (CI) as a function of the fraction affected (F_a) in the indicated cell lines, using three different schedules, as described in Materials and methods (○) C→T; (□) C+T; (●) T→C. Values are means ± s.d. from at least 3 independent experiments.

-adduct levels in the absence and presence of TPT were significant after 48 hours (with p = 0.008 and 0.02 for the IGROV and the $IGROV_{CDDP}$, respectively (paired t-test)).

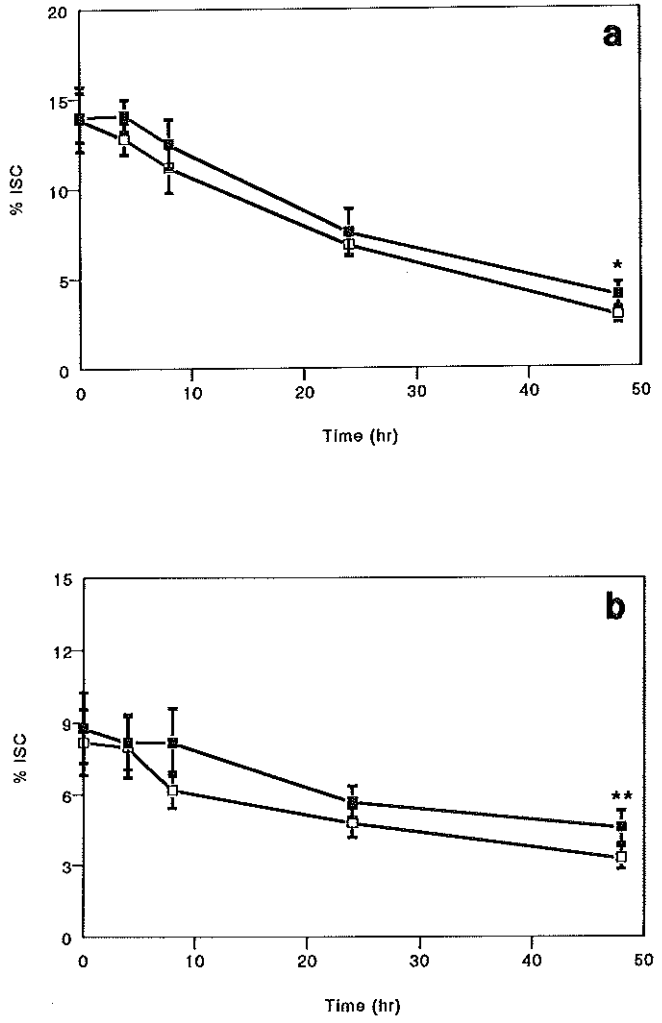

Figure 3. Kinetics of interstrand-cross links (ISC) after a 2 hour incubation of IGROV-1 (a) and $IGROV_{CDDP}$ (b) to 10 μg/ml of cisplatin with (■) or without (□) 10 ng/ml of TPT for 48 hours. Data are means ± s.d. from at least 4 independent experiments. * p = 0.008, ** p = 0.02.

Cell cycle distribution

The cell cycle distribution, following exposure to cisplatin, TPT or to a combination of the two are shown in Figure 4. Continuous exposure of cells to IC_{50}(5 day) doses of cisplatin for 24 or 48 hours resulted in an increase of the fraction of cells in the G_2/M phase, with a corresponding decrease of cells in G_1. Furthermore, in the H226 cell line, a significant increase of the number of cells in S phase was observed. Notably, also continuous exposure of cells to TPT resulted in accumulation in G_2/M for most of the cell lines tested. Only the H226 cells arrested in S. Following treatment of H226 and A498 cell lines with TPT, almost no cells were observed in G_1. Combination of cisplatin and TPT (both at 50% of their IC_{50}(5 day) dose) resulted in a combined effect of the drugs alone, i.e. most of the cell lines accumulated in G_2/M.

To mimic the most active schedule in the IGROV-1, i.e. C→T (see Figure 2), IGROV-1 cells were exposed to an IC_{50}(5 day) dose of cisplatin for 5 days and to an IC_{50}(3 day) dose of TPT for 3 days (starting at day 2) alone and in combination (each drug at 50% of their respective IC_{50} dose). Cells were monitored up to 5 days after starting the incubation (Figure 5). Untreated IGROV-1 cells displayed no change in cell cycle distribution in this time period. Following administration of cisplatin, cells accumulated in G_2/M. However, exposure of the cells to high concentrations of TPT (IC_{50}(3 day)) appeared to result in an arrest of the cells in G_1 and S instead of the accumulation in G_2/M as observed with lower concentrations of TPT.

Topoisomerase I catalytic activity

Topo I catalytic activity was evaluated as described in Materials and methods. For most cell lines, an equal catalytic activity was observed. However, the MCF7 displayed a two-fold higher activity, whereas the A498 cell line showed a two-fold lower activity than the other cell lines (data not shown).

DISCUSSION

In this study, the interaction between cisplatin and topo I inhibitors was investigated using a panel of eight human solid tumour cell lines. Two different topo I inhibitors, TPT and SN38, were used, and the drugs were administered using three different schedules. In analogy to previously published reports [20,21,32], synergistic effects were observed in most of the tested cell lines. In addition to this, the observed cytotoxicity appears to be schedule-dependent, with the C→T and C→S schedule yielding the highest level of synergy in most of the cell lines. This type of schedule-dependency has not been reported by others. In the report by Masumoto [32], all combined schedules of cisplatin and SN38 showed synergy, but the maximum

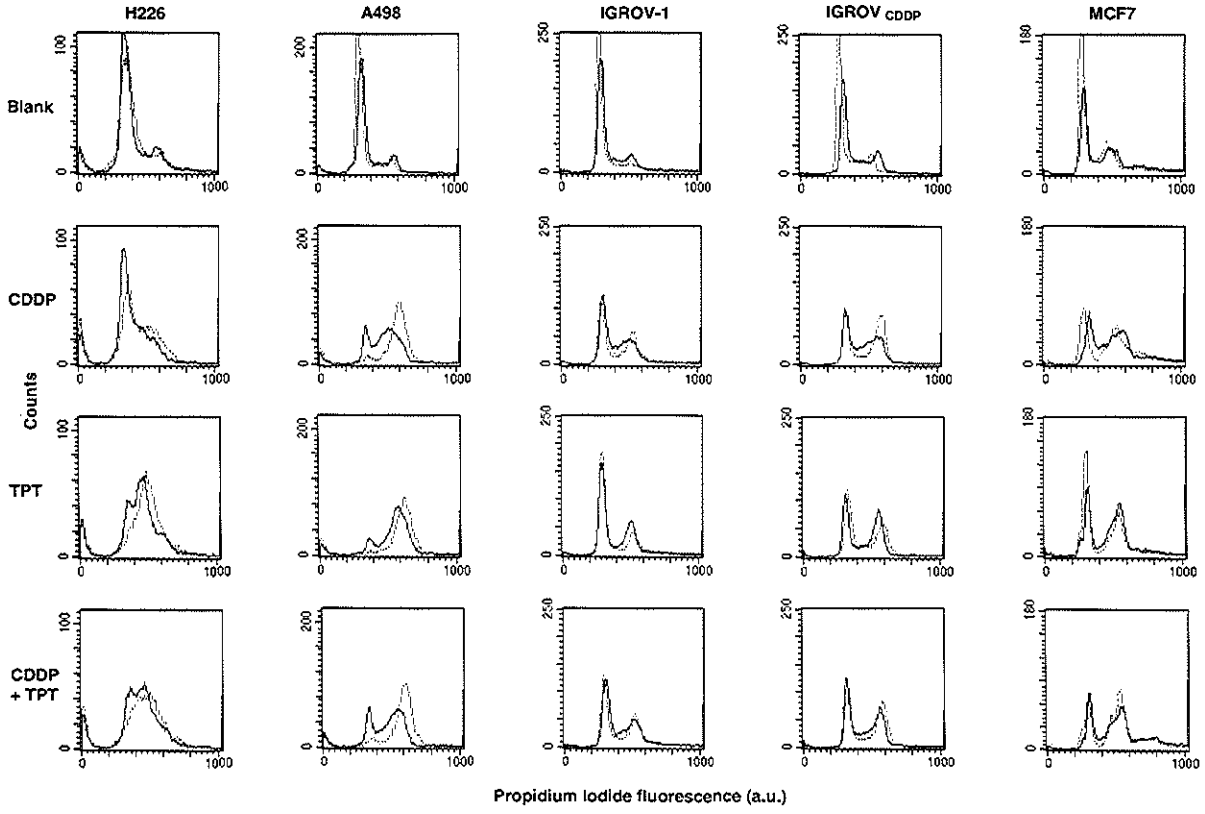

Figure 4. DNA cell cycle analysis following continuous exposure IC_{50}(5 day) concentrations of TPT or cisplatin for 48 hours. Cells were fixed after 24 (—) and 48 (---) hours of exposure and stained with propidium iodide.

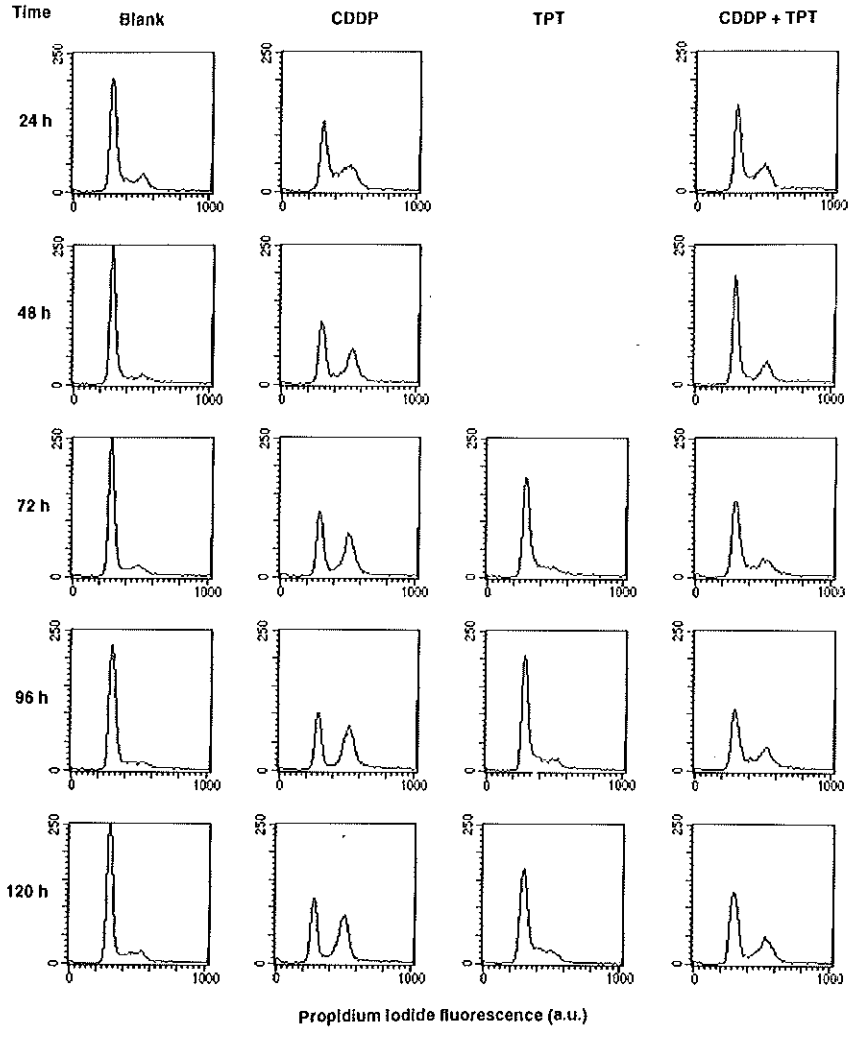

Figure 5. DNA cell cycle analysis following continuous exposure of IGROV-1 to an IC_{50}(5 day) concentration of cisplatin (150 ng/ml) for 5 days, to an IC_{50}(3 day) concentration of TPT (450 ng/ml) for 3 days, and to 0.5 of these concentrations in the combination schedule of C→T (in this cell line: cisplatin: 75 ng/ml for 5 days and TPT: 225 ng/ml for 3 days, starting from day 3). Cells were fixed after 24, 48, 72, 96 and 120 hours of exposure and stained with propidium iodide.

effect was obtained upon simultaneous administration. The difference between these results and ours may be caused by the different cell type that was used. A second possible explanation may be the different incubation periods of the drugs. Most scheduling experiments have been performed using a relative short exposure period (one to two hours) to TPT or SN38, followed by removal of the drug. As TPT and SN38 have been shown to be preclinically and clinically more active upon prolonged administration [33], we reasoned it to be more relevant using longer incubation periods for TPT and SN38. This different approach may have resulted in different synergistic effects.

The synergistic cytotoxicity of cisplatin and TPT upon simultaneous administration may, at least partly, be caused by the inhibition of repair of DNA interstrand cross-links. This inhibition of DNA repair by TPT has also been noted for SN38 [20]. No inhibition of cisplatin-DNA adduct repair was observed on basis of the total amount of cisplatin-DNA lesions, as determined by AAS. Importantly, although ISC only represent a small portion of the total amount of DNA-adducts they may be critical for cell survival [34,35].

As reported by others [36-38], upon treatment with cisplatin, all cell lines accumulated in G_2/M phase. The effect of TPT on cell cycle distribution appeared to be cell-type dependent. Although arrest in S-phase has been reported for camptothecin [39], in our panel, at IC_{50} doses of TPT only the H226 cells accumulated in that cell cycle phase (Figure 4), whereas the other cell lines tested accumulated in G_1 and/or G_2/M. The A498 cell line displays a very marked accumulation in G_2/M both with cisplatin and TPT.

Testing the cell cycle distribution effects in the C→T scheduled administration showed that at higher concentrations of TPT (i.e. the IC_{50} (3 day) dose, see Table 1), relatively more cells appear to be arrested in G_1, instead of G_2/M. Further studies are needed to elucidate whether the type of arrest contributes to the observed schedule-dependency of the synergistic effects between cisplatin and TPT or SN38.

Although up to four-fold differences in topo I catalytic activity were observed in this cell line panel, this topo I level does not appear to be related to the sensitivity of cells to topo I inhibitors nor to the observed synergy.

In conclusion, the combination of cisplatin with TPT or SN38 yields a synergistic cytotoxicity in most human solid tumour cell lines in our panel. Furthermore, this synergy appears to be schedule-dependent in 3 out of 8 evaluated cell lines. In this case, the C→T schedule is the most active. Analyses of the cell cycle distribution did not reveal an explanation for the synergy and the schedule-dependency of this phenomenon. Also topo I catalytic activity appears not to be related to the observed synergistic effects. However, inhibition of repair of cisplatin-DNA ISC's may contribute to the

synergy between cisplatin and topo I inhibitors. Additional studies, such as assessment of the effect of topo I inhibitors on repair of the major cisplatin-DNA adducts, i.e. the GG- and AG-intrastrand cross-links, are currently being performed.

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CHAPTER 10

REDUCED CELLULAR ACCUMULATION OF TOPOTECAN: A NOVEL MECHANISM OF RESISTANCE IN A HUMAN OVARIAN CANCER CELL LINE

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ABSTRACT

A 30-fold topotecan-resistant human IGROV-1 ovarian cancer cell line, denoted $IGROV_{T100r}$ was developed by stepwise increased exposure to topotecan (TPT). The $IGROV_{T100r}$ cell line is strongly cross-resistant to SN38 (51-fold), but is only 3-fold resistant to camptothecin (CPT). Remarkably, this cell line is 30-fold resistant to mitoxanthrone, whereas no significant cross-resistance against other cytostatic drugs is observed.

Resistance to TPT and SN38 in the resistant $IGROV_{T100r}$ cell line is accompanied by decreased accumulation. No reduced accumulation was observed for CPT. The efflux of TPT is very rapid in both cell lines. However, the efflux rate of TPT in the parental and resistant cell lines was not significantly different. Decreased accumulation was not related to P-glycoprotein or multidrug resistance-associated protein (MRP). Topoisomerase I and II catalytic activity, as well as topoisomerase I cleavable complex stabilization by CPT in the parental and resistant cell lines were equal.

These results strongly suggest that the resistance to TPT and SN38 is mainly caused by reduced accumulation. The reduced accumulation appears to be mediated by a novel mechanism, probably related to the uptake of these drugs.

INTRODUCTION

DNA topoisomerase I (topo I[1]) catalyzes changes in DNA topology through cycles of transient DNA strand breaks and relegations (1). Camptothecin (CPT) is a potent anti-cancer agent which stabilizes the covalent enzyme-DNA intermediate formed by topo I (2,3). Drug treatment results in inhibition of DNA replication and chromosomal fragmentation. A number of CPT derivatives have been developed in recent years, e.g. CPT-11 and its active metabolite SN38, topotecan (TPT), 9-aminocamptothecin (9-AC), and GI-147211. These drugs show a broad spectrum of anti-cancer activity in preclinical and clinical studies (4,5).

Several topo I inhibitor resistant cell lines have been established. The mechanism of resistance has often been ascribed to various topo I related changes, e.g. 1) reduced topo I catalytic activity (6-8), 2) decreased formation of cleavable complexes (6,8), 3) decreased expression of topo I (9,10), and 4) a point mutation or rearrangement of topo I genes (8,11). Reduced accumulation of TPT, due to P-glycoprotein (P-gp) overexpression, has been reported to yield resistance to TPT as well (12).

[1]The abbreviations used are: BSO, DL-buthionine-(S,R)-sulfoximine; CDDP, cisplatin; CPT, camptothecin; DOX, doxorubicin; 5-FU, 5-fluorouracil; MAb, monoclonal antibody; MRP, multidrug resistance-associated protein; MTX, methotrexate; MX, mitoxantrone; P-gp, P-glycoprotein; SRB, sulforhodamine B; TPT, topotecan; topo I/II, topoisomerase I/II; VPL, verapamil.

We have developed and characterized a TPT-resistant human IGROV-1 ovarian cancer cell line which is highly cross-resistant to SN38, but displays only minor resistance to CPT. The acquisition of resistance was associated with reduced cellular accumulation rather than changes of topo I catalytic activity or cleavable complex formation. This reduced accumulation was shown to be independent of P-gp or multidrug resistance-associated protein (MRP).

MATERIALS AND METHODS

Chemicals and drugs. CPT was obtained from Aldrich (Bornem, Belgium). TPT was a generous gift from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA, USA). SN38 was generously supplied by Rhône-Poulenc Rorer (Alfortville, France). Plasmid pBR322 DNA, sulforhodamine B (SRB), DL-buthionine-(S,R)-sulfoximine (BSO) and agarose were obtained from Sigma (St. Louis, MO, USA). Kinetoplast DNA (kDNA) was from TopoGEN (Columbus, Ohio, USA). Doxorubicin (DOX) was obtained from Pharmacia (Brussels, Belgium). Cis-diamminedichloroplatinum(II) (cisplatin, CDDP), mitoxantrone (MX) and methotrexate (MTX) were from Lederle (Wolfratshausen, Germany). Paclitaxel (Taxol™) and etoposide (VP-16) were purchased from Bristol Myers (Troisdorf, Germany), and 5-fluorouracil (5-FU) from Roche (Mijdrecht, The Netherlands). Verapamil (VPL) was obtained from Bufa (Uitgeest, The Netherlands). RPMI 1640 medium was from GibcoBRL (Life Technologies B.V., Breda, The Netherlands). Bovine calf serum (BCS) was purchased from Hyclone (Logan, Utah, USA). The P-glycoprotein specific monoclonal antibody (MAb) C219 was obtained from Cis Biointernational (Gif-sur-Ivette, France) and the MRP-specific MAb MRPr1 was kindly provided by Dr. R. Scheper (Dept. Pathology, Free University, Amsterdam).

Cell lines and development of resistance. The human ovarian cancer IGROV-1 and the TPT-resistant $IGROV_{T100r}$ cells were cultured in Hepes-buffered RPMI 1640 medium, supplemented with 10% BCS, 10 mM $NaHCO_3$, 2 mM glutamine, gentamycin, penicillin, streptomycin and phenol red in a humidified atmosphere of 5% CO_2 at 37°C. The doxorubicin-resistant 2780AD ovarian cancer cell line, as well as the small-cell lung cancer GLC_4 and the doxorubicin-resistant GLC_4/ADR cell lines were cultured in the same medium, supplemented with 10% FCS instead of BCS. The cells were frequently monitored for mycoplasma contamination.

The TPT resistant $IGROV_{T100r}$ was developed by continuous exposure to increasing concentrations of TPT. The starting concentration of TPT used for incubation was 5 ng/ml. Within 5 months the cells were exposed to 100 ng/ml. The cells were passaged at that concentration of TPT for another 4 months,

after which TPT was withdrawn. No change in TPT resistance was observed after withdrawal of TPT for at least 3 months.

Assessment of cytotoxicity and cross-resistance. The cytotoxicity of TPT, SN38, CPT, DOX, VP-16, CDDP, paclitaxel, 5-FU, MX, and MTX against the $IGROV_{T100r}$ cells was estimated using the SRB assay (13).

Intracellular accumulation of TPT, SN38, and CPT. Accumulation of TPT, SN38 and CPT was measured in IGROV-1 and $IGROV_{T100r}$ cells that were grown to 50 to 70% confluency. The cells were incubated for 30 min at 37°C with 400 or 800 ng/ml TPT, SN38 or CPT. All drugs were first converted into their lactone form. Following incubation, cells were washed twice with ice-cold PBS and scraped immediately. The cells were collected in a glass tube and centrifuged (5 min, 500 g, 4°C). Subsequently the cells were resuspended in 150 μl of 0.1% acetic acid to lyse the cells. Protein concentrations were determined using the Bio-Rad assay based on the Bradford method. The concentration of TPT in the sample was measured using a previously described sensitive HPLC method (14). Only small fluctuations in the pH of the medium were noted in the incubation experiments, and these pH differences did not affect the exposure of the cells to TPT in its lactone form.

The accumulation process was also studied at 0°C. The parental and resistant cell lines were incubated for 30 min at 0°C with 400 or 800 ng/ml TPT. Following incubation, samples were treated as described above.

For quantitation of the accumulation of SN38 and CPT, 200 μl of suspension was mixed with 100 μl of MeOH/acetonitrile 1/1 (v/v) on ice. After mixing, the sample was kept on ice for 10 min. Subsequently, 10 μl of a saturated $ZnSO_4$ solution in H_2O was added. After mixing, the samples were centrifuged at 4000 g for 5 min. From the supernatant, 200 μl was injected on a hypersil ODS column (Shandon, Astmoor, UK). SN38 and CPT were eluted with an acetate buffer (75 mM ammonium acetate/5 mM tetrabutyl-ammoniumhydrogensulphate (pH 6.4))/acetonitrile (78/22). CPT and SN38 were detected using fluorescence detection (excitation wavelength 355 nm, emission wavelength 515 nm).

The influence of the P-gp blocking agent VPL on the accumulation of TPT in the IGROV-1 and resistant $IGROV_{T100r}$ cell lines was evaluated by incubating the cells for 30 min simultaneously with 800 ng/ml of TPT and 10 μM VPL. The effect of glutathione depletion on the MRP-mediated efflux (15,16) of TPT was tested by incubating cells with 50 μM BSO for 24 hours followed by a 30 min incubation with 800 ng/ml of TPT. The MRP overexpressing GLC_4/ADR cell line (17), together with the parental GLC_4, were used as controls for the influence of BSO on the MRP-mediated accumulation of TPT. Intracellular accumulation of TPT was assayed as outlined above.

Efflux of TPT. In order to measure the efflux of TPT from the IGROV-1 and the $IGROV_{T100r}$ cell lines, cells were loaded with 200 ng/ml and 1500 ng/ml respectively, resulting in similar intracellular concentrations of the drug. After exposure to TPT for 30 min, cells were washed twice with PBS at 37°C and resuspended in 4 ml of prewarmed RPMI medium. After resuspension, cells were incubated at 37°C. At t=0, 5, 10, and 20 min after the start of the incubation, flasks were withdrawn, cells were washed and subsequently harvested. The intracellular concentration of TPT was determined as outlined above. The experiment was also performed at 0°C. In that case, cells were loaded at 37°C, subsequently washed with ice-cold PBS and incubated further at 0°C. The efflux of TPT was determined after 10 min.

Energy-dependence of accumulation. Energy-dependence of TPT accumulation was investigated by transferring IGROV-1 and $IGROV_{T100r}$ cells to RPMI 1640 medium, containing 10 mM sodiumazide and 2-deoxy-d-glucose instead of glucose, for 15 min. Subsequently TPT (800 ng/ml) was added, and cells were incubated at 37°C for 30 min. Samples were analyzed as described above.

P-gp and MRP expression. Immunocytochemical detection of P-gp and MRP was performed according to previously used procedures (18,19). Cytospin preparations were fixed in cold acetone (10 min, 0°C), air-dried and incubated with the MRP-specific MAb MRPr1 or with the P-gp specific MAb C219. Antibody binding was detected using alkaline phosphatase-conjugated immunoglobulin (Dako, Copenhagen, Denmark) and alkaline phosphatase substrate using new fuchsine (Dako). The slides were counterstained with haematoxylin and mounted. Prior to use, C219 and MRPr1 were diluted (1:10, and 1:1500, respectively) in Tris buffered saline (50 mM Tris pH 7.4) containing normal rabbit serum (10%, w/v), normal goat serum (1%, w/v), and normal human AB serum (1%, w/v). Each assay included the use of an isotype-matched irrelevant MAb (mouse IgG2a and rat IgG2a for respectively C219 and MRP).

DNA topoisomerase I and topoisomerase II catalytic activity. Nuclear extracts were prepared as described (20). Topo I catalytic activity in these nuclear extracts was assayed by monitoring the relaxation of supercoiled pBR322 DNA (250 ng), whereas topo II catalytic activity was determined by monitoring decatenation of kinetoplast DNA (250 ng) in the presence of 1 mM ATP. Samples were analyzed by electrophoresis on a 1% agarose gel. After staining the gels with ethidium bromide, the bands were visualized over an UV light table and photographed with Polaroid (667) positive/negative films.

The stabilization of topo I cleavable complexes by CPT was determined by incubating reaction mixtures at 37°C for 30 min with concentrations of CPT ranging from 3 to 200 μM. The reaction mixtures contained the lowest

amount of nuclear proteins that was able to yield complete relaxation of 250 ng of pBR322 DNA. Reaction products were separated on 1% agarose gel and visualized as described above.

Statistical analysis. Statistical evaluation was performed using the student t-test and Pearson correlation analysis. P-values ≤ 0.05 were considered to be significant.

RESULTS

IGROV$_{T100r}$ cell line: Resistance to TPT and cross-resistance pattern. The resistant IGROV$_{T100r}$ cell line was developed by stepwise increased exposure of TPT for 5 months. This cell line not only developed resistance to TPT (30-fold), but also for SN38 (51-fold) and MX (30-fold). IC_{50}-values for the resistant and sensitive cell lines are summarized in Table 1. Only minor cross-resistance to CPT, another topo I inhibitor, was found. Minor or no cross-resistance to the topo II inhibitors DOX and VP-16, nor to CDDP, paclitaxel, and 5-FU was observed. The cells show an increased sensitivity to MTX.

Table 1 *Cross resistance pattern of IGROV$_{T100r}$*

DRUGS	IGROV-1 (IC_{50} ng/ml)	IGROV$_{T100r}$ (IC_{50} ng/ml)	Rf[a]	p-value
TPT	6.8 ± 1.7	199 ± 49.5	30.0 ± 6.9	<0.001
SN38	1.3 ± 0.5	65.9 ± 27.1	51.7 ± 17.3	0.02
CPT	2.3 ± 0.8	5.9 ± 1.0	2.7 ± 0.4	0.008
DOX	52.3 ± 24.4	51.2 ± 21.0	1.0 ± 0.2	NS[b]
VP-16	457 ± 134	603 ± 101	1.4 ± 0.2	NS
CDDP	76.0 ± 24.3	155 ± 54.5	2.1 ± 0.6	NS
PACLITAXEL	29.5 ± 12.5	29.8 ± 11.0	1.0 ± 0.1	NS
5-FU	412 ± 88	316 ± 117	0.8 ± 0.2	NS
MX	4.1 ± 1.2	131 ± 59.7	30.7 ± 9.6	0.02
MTX	17.2 ± 2.9	6.6 ± 0.6	0.4 ± 0.0	0.003

Data are means ± s.d of at least 3 experiments.
[a]Rf, resistance factor, determined with the SRB method.
[b]NS, not significant.

Intracellular accumulation of TPT, SN38 and CPT. Results of TPT accumulation experiments are shown in Fig. 1. At extracellular TPT concentrations of 400 and 800 ng/ml, accumulation in the IGROV$_{T100r}$ cell line is approximately 85% lower than in the parental cell line ($p<0.001$). Notably, in both cell lines no saturation kinetics was observed up to TPT concentrations of 10,000 ng/ml.

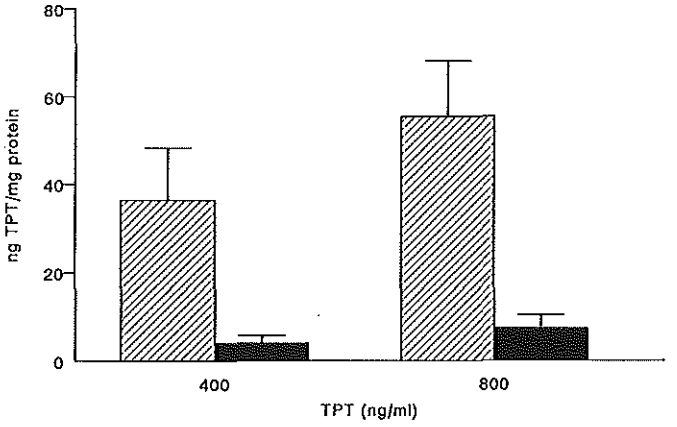

Fig 1. Accumulation of TPT in the IGROV-1 and IGROV$_{T100r}$ cell lines after exposure to 400 or 800 ng/ml of TPT. IGROV-1, dashed bars; IGROV$_{T100r}$, black bars.

Also the accumulation of SN38 in the resistant cell line was decreased, i.e. from 12.1 ± 6.67 ng/mg of protein in the IGROV-1 to 4.4 ± 2.45 ng/mg of protein in the IGROV$_{T100r}$ cell line ($p < 0.05$). No decreased accumulation was observed for CPT in the resistant cell line. Concentrations of CPT in the IGROV-1 and IGROV$_{T100r}$ were 3.23 ± 0.66 and 3.7 ± 1.28 ng/mg of protein, respectively.

Effects of VPL and BSO on the cellular accumulation of TPT. In order to correlate reduced TPT accumulation with known resistance mechanisms, cells were treated with the P-gp blocking agent VPL (10 μM) or glutathione depleting BSO (50 μM). Simultaneous incubation of TPT with 10 μM VPL did not significantly influence the intracellular accumulation of TPT in the IGROV-1 or IGROV$_{T100r}$ cell lines (data not shown). Moreover, depletion of glutathione by a 24 h preincubation with BSO did not significantly affect the accumulation of TPT, in the IGROV-1 as well as the IGROV$_{T100r}$ cell line (Fig. 2). To evaluate the impact of MRP on the accumulation of TPT, the GLC$_4$ and the MRP overexpressing GLC$_4$/ADR cell lines were used as controls. In the parental GLC$_4$ cell line, BSO did not influence the accumulation of TPT in the cell

significantly. The accumulation of TPT in the resistant GLC_4/ADR cell line was 35% lower than in the parental GLC_4 cell line (Fig. 2). Pretreatment of the MRP-overexpressing GLC_4/ADR cell line with BSO resulted in an increased accumulation of TPT ($p=0.04$). Following treatment of GLC_4 cells with BSO, no significant difference was observed between the accumulation of TPT in the sensitive GLC_4 and the resistant GLC_4/ADR cell line (Fig. 2).

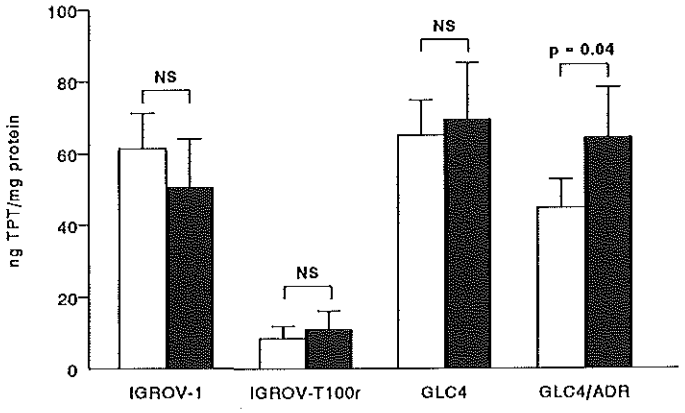

Fig 2. Influence of BSO on the accumulation of TPT in the IGROV-1, $IGROV_{T100r}$, GLC_4 and GLC_4/ADR cell lines. Control, white bars; incubated with 50μM BSO, black bars.

Efflux of TPT from the parental IGROV-1 and $IGROV_{T100r}$ cell lines. A very rapid efflux of TPT was observed both in the IGROV-1 and the $IGROV_{T100r}$ cell lines. Relative intracellular concentrations of TPT, compared to $t=0$, at $t=5$, 10 and 20 were 34.6 ± 6.68%, 13.2 ± 9.04%, and 2.6 ± 1.89%, respectively. For the $IGROV_{T100r}$ the relative intracellular TPT concentrations at 5, 10 and 20 min were 27.4 ± 7.74%, 6.9 ± 3.12%, and 1.2 ± 0.8%, respectively. Efflux rates of TPT in the IGROV-1 and $IGROV_{T100r}$ were not significantly different.

Accumulation and efflux at lower temperature. In order to assess the role of active transport in the kinetics of TPT, accumulation and efflux of TPT were assayed at 0°C. Data on the accumulation of TPT (800 ng/ml) at 37 and 0°C are shown in Fig. 3a. At 0°C, accumulation in the parental IGROV-1 cell line was only 7.3% of the accumulation at 37°C ($p<0.001$). The efflux of TPT from the parental IGROV-1 cells was completely inhibited in preloaded cells by lowering the temperature to 0°C (data not shown). In the $IGROV_{T100r}$ cell line, accumulation at 0°C was lowered to approximately 50% of the value at 37°C.

As in the parental IGROV-1, at 0°C the efflux of TPT from the $IGROV_{T100r}$ cell line was completely inhibited.

Energy-dependence of TPT accumulation. Results of accumulation experiments in energy-depleted cells are shown in Fig. 3b. Cellular accumulation of TPT in the sensitive IGROV-1 cell line tended to decrease upon depletion of energy (NS). In the resistant $IGROV_{T100r}$ cell line, accumulation increased significantly under these conditions as compared to the situation in glucose-containing medium. Notably, the accumulation of TPT under low energy conditions was equal in both cell lines.

Expression of P-gp and MRP. Using immunocytochemistry, no expression of P-gp was noted in the resistant $IGROV_{T100r}$ and parental cell line (data not shown). Clear membrane staining was observed in the P-gp positive cell line 2780AD, while the parental cell line A2780 was negative for P-gp.

With the MRP-specific MRPr1 MAb, marked staining was observed in the MRP-positive cell line GLC_4/ADR. Membrane staining as well as cytoplasmic staining was found. Only about 20% of the $IGROV_{T100r}$, and 10% of the IGROV-1 cells stained for MRP, and staining was primarily found in the cytoplasm. However, staining in these cell lines was very weak, as compared to the staining that was observed in the MRP-overexpressing GLC_4/ADR cell line (data not shown).

Topo I and topo II catalytic activities and stabilization of topo I cleavable complex by CPT. No apparent differences in topo I or topo II catalytic activities were observed between the IGROV-1 and the $IGROV_{T100r}$ (data not shown). In both cell lines, Topo I mediated relaxation of supercoiled pBR322 DNA was completely inhibited by concentrations of 25 μM of CPT and higher for both cell lines.

DISCUSSION

We developed the TPT-resistant human $IGROV_{T100r}$ ovarian cancer cell line, which is cross-resistant to SN38, but displays only minor resistance to CPT. Resistance to CPT and analogues has been attributed to reduced expression or reduced activity of topo I (6-10). However, for the $IGROV_{T100r}$ cell line this does not appear to be the case. Catalytic activity of topo I in the IGROV-1 and $IGROV_{T100r}$ cell lines was equal. Furthermore, cleavable complex formation in both cell lines was equally sensitive to CPT.

Resistance to TPT and SN38 in the $IGROV_{T100r}$ cell line appears to be caused by reduced accumulation of the respective compounds. The importance of reduced accumulation is also suggested by the unchanged accumulation of CPT, accompanied by an almost lack of resistance of the cells for this compound.

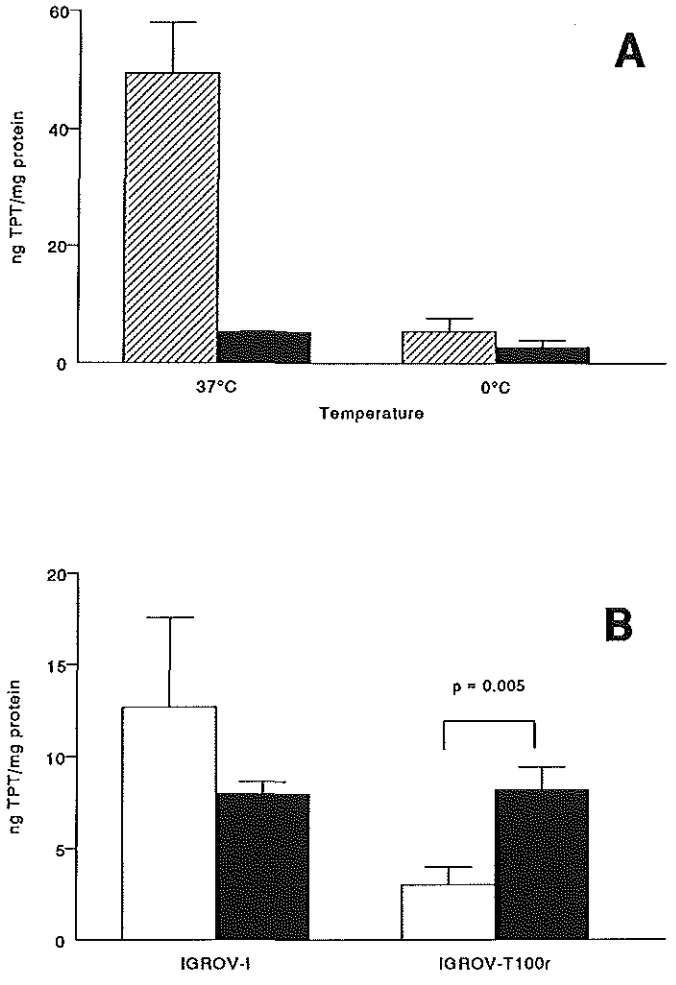

Fig 3. Accumulation of TPT in the IGROV-1 and the $IGROV_{T100r}$ cell lines. *A.* Accumulation at 37°C and 0°C. IGROV-1, dashed bars; $IGROV_{T100r}$, black bars. *B.* Accumulation in energy depleted IGROV-1 and $IGROV_{T100r}$ cells. Accumulation of TPT in the presence of glucose, white bars; Accumulation of TPT under energy depleting conditions, black bars. Energy was depleted by incubating cells in RPMI, containing 2-deoxy-d-glucose instead of glucose, and with 10 mM sodiumazide added, as described in Materials and methods.

Multidrug resistance has been associated with an increased expression of P-gp and MRP. P-gp has also been shown to be involved in resistance to TPT, as acquired resistance to TPT was accompanied by increased expression of P-gp and reduced accumulation (12). However, in the $IGROV_{T100r}$ cell line, no overexpression of P-gp was observed as compared to the parental cell line. Furthermore, no P-gp related MDR characteristics are present in the $IGROV_{T100r}$ cell line, as no cross-resistance to other drugs except MX is observed. Also the absence of a stimulating effect of VPL on accumulation of TPT in this cell line argues against the involvement of P-gp in the mechanism of resistance to TPT and SN38.

Although in the resistant cell line about 20% of the cells has increased expression of MRP as estimated by immunocytochemistry, MRP-content does not appear to be critical in relation to the acquired reduced accumulation and resistance in the $IGROV_{T100r}$ cell line, for several reasons. *1)* Although slightly increased levels of MRP are present in the resistant $IGROV_{T100r}$ cell line, the level of this protein is much lower than in the MRP-overexpressing GLC_4/ADR cell line. The relative reduction of TPT accumulation in this MRP-overexpressing GLC_4/ADR cell line is only moderate and even less marked than in the $IGROV_{T100r}$ cell line (Fig. 2). In case MRP is closely involved in the efflux of TPT, a more markedly decreased accumulation would be anticipated in the GLC_4/ADR cell line. *2)* Inhibition of MRP by depletion of glutathione did not result in significant reversal of the reduced accumulation of TPT in the $IGROV_{T100r}$ cells. *3)* Like P-gp, MRP has been related to multidrug resistance. However, the $IGROV_{T100r}$ is not a multidrug resistant cell line.

Efflux of TPT from both IGROV cell lines could completely be inhibited by lowering the temperature to 0°C. Lowering of the temperature resulted in almost complete (90%) inhibition of the accumulation of TPT in the IGROV-1 cell line as well. However, accumulation of TPT in the resistant $IGROV_{T100r}$ cell line was decreased to a lesser extent. This indicates that a significant portion of TPT in the resistant cell line apparently accumulates by temperature, and therefore presumably energy independent processes. Strikingly, the accumulation of TPT at 0°C was comparable in both cell lines. Under low-energy conditions, accumulation of TPT in the resistant $IGROV_{T100r}$ increases, whereas accumulation in the IGROV-1 shows a tendency to decrease. As no different efflux characteristics of TPT were observed in these cells, the results may be explained by the presence of an energy-dependent inward transport system in the IGROV-1 cell line that is capable of transporting TPT and SN38, but not CPT. The transport system appears to be absent in the $IGROV_{T100r}$. To our knowledge, such a mechanism of resistance has not been described before. Recently, Yang et al. reported an MX resistant breast carcinoma MCF7 cell line, that surprisingly displayed a selective resistance against TPT, SN38

and CPT-11, but lacked resistance to CPT (21). The similarities between this and our IGROV$_{T100r}$ cell line suggest that the resistance in these cell lines is mediated by the same mechanism.

Notably, the rapid efflux of both TPT and SN38 may be clinically important in relation to the known improved activity upon a prolonged incubation of TPT *in vitro* and *in vivo* in patients (22). Topo I inhibitors like CPT or TPT can not be trapped in the cell, as their binding to cleavable complexes is a reversible process. Due to the efficient efflux of TPT from the cell, a short incubation period would lead to a much lower magnitude of exposure, and therefore decreased activity of TPT, as compared to prolonged incubations.

In conclusion, we developed a TPT-resistant IGROV-1 ovarian tumor cell line, that displays significant cross-resistance to SN38, but is only marginally resistant to CPT. The resistance is accompanied by decreased accumulation of TPT and SN38, and is not mediated by P-gp or MRP. The nett energy-dependent efflux of TPT in the IGROV-1 and IGROV$_{T100r}$ cell lines was not significantly different. In addition, no significant differences have been observed between energy-independent efflux of TPT in the parental and resistant cell line. We hypothesize that the resistance to TPT in the IGROV$_{T100r}$ is caused by uptake related factors, probably an impaired uptake system for TPT and SN38 in the IGROV$_{T100r}$ cells.

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CHAPTER 11

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS

Cisplatin is one of the most widely applied anticancer agents in the treatment of different types of solid tumors (1). The pharmacokinetics and pharmacodynamics of cisplatin reveal wide interpatient variation. When patients are given a standard dose based on the body surface area, the area under the curve (AUC) of unbound platinum (cisplatin is measured with atomic spectroscopy as platinum) varies more than threefold. In addition, it is clinically well known that there is significant interpatient variation in the response to cisplatin chemotherapy. Patients with advanced germ cell tumors can be cured on average in more than 90% of cases. However, other tumor types are less sensitive or insensitive for cisplatin. Clearly there is a need to characterize the dose-response relationship in greater detail with the aim to optimize clinical application of cisplatin.

Topoisomerase I (Topo I) inhibitors form a novel and promising class of anticancer agents. They reversibly interact with the so-called cleavable complex. This is the binding complex of the nuclear enzyme topo I and DNA in which this enzyme induces a single strand break. The topo I inhibitors topotecan and irinotecan (CPT-11) are currently evaluated in clinical studies and irinotecan has already been approved in several countries for the treatment of advanced colorectal cancer. The combination of cisplatin and topo I inhibitors has a number of potential benefits for the treatment of patients with advanced cancer. Both drugs act on the DNA through a different mechanism. Therefore, combination of cisplatin and a topo I inhibitor may well be synergistic or at least additive. In addition, results of clinical studies reveal that the toxicity profile of topo I inhibitors only partly overlaps with that of cisplatin. These characteristics form the basis for extensive clinical evaluation of the combination of the two different types of anticancer agents and for preclinical studies aiming at characterization of the mechanism of interaction of cisplatin and topo I inhibitors.

Another unique and novel class of anticancer agents are the taxanes. The two representatives approved for clinical application are paclitaxel (Taxol®) and docetaxel (Taxotere®). Taxanes enhance polymerization of the tubulin into stable microtubules and inhibit microtubule depolymerization. This results in a disruption of the equilibrium within the microtubule system and ultimately leads to cell death. Taxanes are cell cycle specific drugs and employ their cytotoxicity mainly during the mitosis-phase of the cell cycle. Phase II studies revealed high activity in a number of solid tumor types, such as breast and ovarian cancer, non-small cell lung cancer, head and neck cancer, soft tissue sarcoma and gastric cancer. The unique mechanism of action combined with the high activity in clinical studies make taxanes good

candidates for combination chemotherapy with cisplatin. Taxanes may induce neurosensory toxicity which overlaps with the toxicity profile of cisplatin. However, the additional toxicity profile shows only marginal overlap with cisplatin. In addition to clinical phase I and II studies to establish the feasibility of the combination therapy, pharmacologic studies are needed to characterize concentration-response relationships and any schedule dependent influences on the pharmacokinetics and dynamics of both compounds.

In clinical studies analysis of cisplatin induced DNA-adducts most often is limited to measurement of DNA-adducts in peripheral white blood cells (WBC). A number of different assays have been published for the quantitation of DNA-adducts in WBC (2,3). One of the two most widely applied techniques is atomic spectroscopic quantitation of platinum (and quantitation of DNA employing UV-spectrophotometry) (2). The other frequently applied method is quantitation of DNA-adducts with a competitive ELISA (3). Prior to the execution of the clinical pharmacologic studies we became aware of a significant bias in a previously published method for whole blood sample preparation. Studies presented in Chapter 2 have been directed to characterize the bias and to validate a new method for measurement of DNA-adducts in WBC. Results illustrate that immediate separation of leucocytes from plasma is important to prevent continued formation of DNA-adducts *ex vivo*. During and shortly after the end of the cisplatin infusion whole blood samples collected from a patient contain unbound cisplatin which can form adducts with DNA in WBC. Previously published studies eraneously interpreted the combination of high levels of DNA-adducts shorly post-infusion and the low levels 1 day later as DNA-repair. Our studies reveal that the DNA-adduct level shortly post-infusion are approximately 50% lower than reported by others (3). In addition, the interpatient variability in DNA-adduct levels after standard dosing of patients is of the order of 10-fold lower than reported by others (2,4). This can be explained by a major reduction of variation in the assay.

In Chapter 3 the validation of the analysis of unbound platium in plasma of patients is reported. A simplified and very economical plasma protein precipitation with cold ethanol is presented. The method results in equal concentrations of unbound platinum as when protein free plasma is obtained through plasma ultrafiltration. The ethanol precipitation method is less laborious and much less expensive than the use of Millipore tubes or Amicon filters. The method can also be used for the measument of unbound platinum after administration of carboplatin.

The outlined methods have been applied to characterize the concentration-DNA-adduct-response relationship of cisplatin in patients who

were treated with weekly cisplatin (Chapter 4). The aim of weekly administration of cisplatin was to increase the dose-intensity and thereby the likelihood of response. In total 45 patients with various types of solid tumors, mainly head and neck cancer, non-small cell lung cancer, adenocarcinoma of unknown primary site and mesothelioma could be evaluated for the pharmacokinetics of cisplatin, the DNA-adduct formation in WBC and tumor response and toxicity. The results reveal that after administration of a standardized dose of 70-80 mg/m^2 the exposure to unbound cisplatin (AUC) varied more than threefold. Hence, significant interpatient pharmacokinetic variability was observed. A second important observation was that the AUC of unbound platinum and the level of the DNA-adducts in WBC were highly correlated (R=0.78). This is a new observation, which however could be anticipated. In preclinical studies, linear relationships have been observed between the incubation concentration of cisplatin over a wide concentration range and the level of DNA-adducts in tumor cell lines (see also chapter 7). In addition, WBC circulate in the blood, i.e. central compartment to which unbound cisplatin is infused. Importantly, both the AUC of unbound cisplatin and the DNA-adduct levels were predictive for the likelihood of tumor response. This means that the likelihood of tumor response is at least partly determined by variability between patients in the pharmacokinetics of cisplatin. Furthermore, significant but relatively low correlation coefficients could be described between the AUC of unbound platinum and the severity of the thrombocytopenia and the log VPT (vibration perception threshold), the latter being a measure of the severity of neurosensory toxicity induced by cisplatin. A new surrogate end point has been defined in this study, the area under the DNA-adduct-time curve (AUA). This parameter was calculated using the base-line level, the level at 1 hour and 18 hours post-infusion and the trapezoidal method. The AUA was only a slightly better parameter than the DNA-adduct level determined at one single time-point at 1 hour after the end of the cisplatin infusion. The significant relationship between AUC and likelihood of response, combined with the significant interpatient variability in the pharmacokinetics form a strong basis for individualized treatment with cisplatin. This approach is presently ongoing and preliminary results obtained in 34 patients mainly with advanced non-small cell lung cancer reveal a favourable response rate of more than 60% (5). Extended studie are needed to establish whether the individualized approach is superior to standard dosing in terms of response rate, disease-free and total survival and whether it is coupled with managable toxicity.

The pharmacokinetics and dynamics of docetaxel and cisplatin in patients with solid tumors are described in Chapter 5. As outlined, cisplatin

and docetaxel are two anticancer agents with documented high activity against a number of solid tumor types. The toxicity pattern is only partly overlapping, which combined with the high activity of the single agents, forms a strong basis for the evaluation of the feasibility of combination therapy with cisplatin and docetaxel. In the clinical study the maximum tolerated doses of docetaxel and cisplatin given in the two sequences of administration were investigated. The aim of the pharmacologic study was to investigate whether the pharmacokinetics and dynamics of docetaxel and cisplatin were sequence dependent and whether significant relationships existed between pharmacokinetics and toxicity. The pharmacokinetics and dynamics could be evaluated during the first course in 64 of 66 patients entered. The total plasma clearance of docetaxel was 19.6 $\pm$ 4.7 (l/h/m^2) and of unbound platinum 23.0 $\pm$ 8.2 (l/h/m^2). These data are of the same order as when the two drugs are administered as single agents (6,7). The plasma protein binding of docetaxel and cisplatin were not significantly influenced by their simultaneous administration. In addition, all other pharmacokinetic parameters, such as volume of distribution at steady state, half-lives and renal clearance of unbound platinum were not significantly different between the two sequences of administration. Granulocytopenia and leucocytopenia were the dose-limiting toxicity. In addition, significant thrombocytopenia and mucositis were observed. The pharmacokinetic parameters AUC of docetaxel and time-period above a concentration of 100 ng/ml (T100) were significantly correlated with the leucocytopenia and thrombocytopenia. Of interest, the T100 turned out to be the pharmacokinetic parameter with the highest correlation coefficient with pharmacodynamic parameters. Previous studies with docetaxel have not identified this parameter. Also, the AUC of unbound platinum was significantly correlated with the granulocytopenia, leucocytopenia and thrombocytopenia, however the correlation coefficients were slightly lower than of docetaxel. The AUC, and in particular T100 of docetaxel, as well as the AUC of unbound platinum could adequately be fitted to the % decrease leucocytes and thrombocytes, applying the sigmoid Emax model (Hill equation). The correlation between the AUC or T100 of docetaxel and the AUC of unbound platinum was low. Therefore, AUC or T100 of docetaxel and AUC of unbound platinum were used in combination as two independent pharmacokinetic parameters. Results of fits revealed that the % decrease granulocytes and leucocytes could best be described using the combination of T100 and AUC of unbound platinum than any of the single parameters in one Emax model. The root mean squared error % (RMSE%) was used as a measure of precison (8). The pharmacokinetic-toxicity relationships in the two sequences of administration were completely overlapping. The DNA-adduct

formation of cisplatin in WBC was significantly inhibited when docetaxel preceded cisplatin. The maximum adduct level at one hour after the end of the cisplatin infusion was 1.1 ± 0.37 without and 0.50 ± 0.41 (pg Pt/μg DNA) with prior docetaxel ($p<0.01$). There was a trend towards more pronounced leucocytopenia in the sequence cisplatin followed by docetaxel ($p=0.09$), however it did not reach statistical significance. Additional studies in our laboratory have revealed that the pharmacodynamic interaction did not occur in two human tumor cell lines IGROV-1 and WIDR. Up to now, no data are available on the uptake kinetics of cisplatin in tumors in patients when co-administered with docetaxel. Further clinical pharmacologic studies are needed to unravel whether the observed pharmacodynamic interaction between cisplatin and docetaxel has therapeutic implications. The interaction has also been observed for paclitaxel (see Chapter 6).

In Chapter 6 the mechanism of the interaction between cisplatin and the taxanes docetaxel and paclitaxel is described. In a clinical and pharmacologic phase I/II study, which is described in Chapter 5, a pharmacodynamic interaction between docetaxel and cisplatin became apparent. The formation of DNA-adducts of cisplatin in WBC was very much reduced when docetaxel preceded cisplatin. In Chapter 6 the interaction was simulated *in vitro* using docetaxel as well as paclitaxel as model substrates. Incubation of whole blood of healthy subjects and patients with solid tumors with docetaxel or paclitaxel inhibited the uptake of cisplatin by 46%. This interaction explains the reduced DNA-adduct formation which was observed in the clinical study.

The pharmacokinetic-dynamic relationship of cisplatin has been studied *in vitro* using a human ovarian cancer cell line IGROV-1 (Chapter 7). The aim was to establish whether different rates of input of cisplatin affected the DNA-adduct kinetics and cell survival. An "i.v. bolus, 3-hour and 20-hour infusion" schedule were simulated, because in the clinic the optimal schedule of administration has not been defined. The different schedules were simulated using human albumin additions to bind active cisplatin in the cell culture medium. The doses and albumin additions were constructed in such a way that the area under the curve of unbound, i.e. active, cisplatin (measured as platinum) was equal in all schedules. Cell survial and DNA-adduct levels were used as end-points. The results reveal that the 3 different "infusion" schedules had a significant influence on the shape of the DNA-adduct-time curve. The "i.v. bolus" schedule had the highest and the "20-hour infusion" schedule the lowest maximum DNA-adduct level. However, the area under the DNA-adduct-time curve (AUA) was not significantly different between the 3 schedules. In addition, the cell survival curves were completely overlapping. There was a linear relationship between the AUA and AUC of unbound cisplatin when

higher doses were applied. The results indicate that the magnitude of the AUC of unbound cisplatin and not the rate of input is correlated to the cell survival. In addition, linear relationships were observed between the AUC and the level of DNA-adduct formation over a wide concentration-range. These results may guide design of clinical administration schedules of cisplatin.

In experiments described in Chapter 8 the pharmacodynamics of cisplatin were determined in a cisplatin resistant stable subline of the cisplatin sensitive human ovarian cancer cell line IGROV-1. The subline, denoted $IGROV_{CDDP}$, was developed through intermittent incubation with increasing concentrations of cisplatin. After 9 months a stable cell line had developed with a resistance factor of 24 in the clonogenic assay. The cellular accumulation in $IGROV_{CDDP}$ was approximately 65 % lower than in the parental cell line at equal incubation conditions. Also, the reduction in the level of the DNA-adducts in the resistant cell line compared to IGROV-1 was of the same magnitude. $IGROV_{CDDP}$ was moderately to significantly cross-resistant to carboplatin, doxorubicin, paclitaxel, docetaxel, VP16 and topotecan, indicating that no unique selective mechanism of resistance had developed. Cell survival, DNA-adduct peak height and area under the DNA-adduct-time curve (AUA) were determined every 4 passages during development of the resistant subline. Highly significant linear relationships could be established between the passage number of cisplatin and the DNA-adduct peak height and AUA as well as the log cell survival. Also, linear relationships were found between the log cell survival and DNA-adduct peak height ($R=0.97$) or the AUA ($R=0.96$). These results support the observation that reduced intracellular accumulation of cisplatin is a major mechanism of resistance in this cell line. Glutathion concentrations were not increased in $IGROV_{CDDP}$.

The influence of ATP-depletion, reduction of temperature and addition of ouabaine, a Na^+, K^+-ATPase inhibitor on the uptake and efflux of cisplatin over the cellular membrane were evaluated in the sensitive IGROV-1 and resistant $IGROV_{CDDP}$ cell lines. ATP-depletion was pursued by using glucose-free medium containing 10 mM sodiumazide (NaN_3). It resulted in an approximately 70 % decrease of the intracellular accumulation of cisplatin in the sensitive cell line. However, no significant change was observed in the resistant cell line. Under the condition of ATP-depletion the intracellular accumulation of cisplatin was not significantly different between IGROV-1 and $IGROV_{CDDP}$. Reduction of the temperature to 0°C resulted in a substantial decrease in the cisplatin accumulation in both sensitive and resistant cell lines and the levels of accumulation between the cell lines were no longer significantly different. Co-incubation with ouabain revealed that the reduction of cisplatin accumulation was more pronounced in the sensitive than in the resistant cell line (44% vs 26%). A small, but significantly higher

efflux of platinum was observed in the resistant compared to the parental cells (0 vs 17%). The small difference in efflux kinetics most likely does not account for the 3-fold reduction in the accumulation of cisplatin in resistant cells. The outlined results reveal that the uptake of cisplatin in IGROV-1 is partly mediated through energy- and temperature-dependent processes. This energy-dependent process appears to be abrogated in the resistant $IGROV_{CDDP}$, resulting in reduced cisplatin accumulation.

In Chapter 9 the cytotoxic activity was studied of the combination of cisplatin and the topo I inhibitors topotecan and SN38. SN38 is the active metabolite of the topo I inhibitor irinotecan. The cell line panel consisted of IGROV-1, MCF7 breast cancer (estrogen and progesteron receptor +), EVSAT breast cancer (receptor -), M19 melanoma, WiDR colon cancer, A498 renal cell cancer and H226 undifferentiated non-small cell lung cancer. End-points were cell survival, total DNA-adducts and interstrand cross-links (ISC's) of cisplatin as well as the cell cycle distribution under the influence of cisplatin and topotecan. Applying median effect analysis (9), significant synergistic cytotoxicity of topotecan or SN38 in combination with cisplatin was observed in 7 out of 8 tested cell lines. In IGROV-1 and $IGROV_{CDDP}$, co-incubation with topotecan resulted in a consistently higher level of ISC's which reached statistical significance at 48 h post-incubation ($p=0.008$ in IGROV-1 and $p=0.02$ in $IGROV_{CDDP}$). Total DNA-adducts determined with atomic spectroscopy were not significantly different when cisplatin was compared with cisplatin plus topotecan. Reduced repair of ISC's may well contribute to the observed synergy. Besides simultaneous administration, the sequences of cisplatin followed by topotecan 2 days later (C→T) and topotecan followed with cisplatin 2 days later (T→C) were evaluated in the same cell panel. Also, SN38 was applied as model substrate in combination with cisplatin. In IGROV-1, $IGROV_{CDDP}$ and MCF7 significant schedule-dependency of the cytotoxicity was observed. C→T (and C→SN38) was significantly more cytotoxic than any other schedule. In all other cell lines, except H226, the same trend was found. A498 did not show significant synergy in any schedule. Cell cycle distribution after cisplatin administration at the IC_{50} resulted in accumulation of cells in G_2/M phase and corresponding decrease of cells in G_1 in all cell lines, which is in line with previous reports. After exposure to topotecan, the cell cycle distribution was cell type specific. Only in H226 an arrest in S phase was observed. In A498 almost all cells arrested in G_2/M following treatment with topotecan. In the other cell lines an accumulation of cells in G_2/M was found, with a conconitant decrease of the number of cells in G_1. The pattern of cell cycle distribution after exposure to cisplatin, topotecan and the combination did not provide an explanation for the observed schedule-dependent

synergy in the 3 outlined cell lines. Further studies are needed to unravel the schedule-dependency of the synergy.

In Chapter 10 the development and characterization are described of a subline of IGROV-1 which is stable and resistant to the topo I inhibitors topotecan and SN38. The subline, denoted $IGROV_{T100R}$ was 30-fold resistant to topotecan and 51-fold to SN38, but hardly cross-resistant to camptothecin (resistance factor = 3). The intracellular accumulation of topotecan in $IGROV_{T100R}$ was 85 % and of SN38 65 % lower compared with IGROV-1. The reduced accumulation was not mediated by the multidrug resistance P-glycoprotein or the multidrug resistance-associated protein MRP. Rapid efflux of topotecan was observed with a half-life of 3-4 minutes in both sensitive and resistant cell lines. ATP-depletion experiments revealed that the uptake of topotecan in the sensitive cell line is partly mediated through energy-dependent processes, which mechanism is abrogated in $IGROV_{T100R}$. No significant differences were observed in topo I and topo II catalytic activities.

FUTURE PERSPECTIVES

Results of clinical studies presented in this thesis clearly illustrate the need for extended pharmacologic studies in order to characterize concentration-response relationships quantitatively. The wide interpatient variability in the dose-AUC relationship of unbound cisplatin, coupled with the significant relationship between the AUC and the likelihood of tumor response forms a strong basis for individualized treatment with cisplatin. Standard dosing using the body surface area is inadequate to reduce pharmacokinetic variability. Presently, a large phase II study with targeting of the AUC on the arbitrary chosen level of 3 (μg.h/ml) is ongoing. In addition, limited sampling and estimation of pharmacokinetic parameters using a Bayesian algorithm is currently applied to increase the feasibility of wide-scale application of pharmacologic guided dosing of cisplatin. Future studies should be designed to determine whether individualized dosing increases progression-free and overall survival and/or the quality of life and is coupled with acceptable toxicity.

Most often combination chemotherapy is applied instead of therapy with anticancer agents given as single agent. In order to investigate overlapping toxicities, concentration-effect relationships should be investigated where representative pharmacokinetic parameters of all administered drugs are combined and treated as independent parameters. This approach is particularly indicated when drugs are known to have overlapping toxicities and have low correlation coefficients between their main pharmacokinetic parameters.

Other dynamic end-points should be developed in clinical studies. The relationships between standard pharmacokinetic parameters, such as AUC, Cl and time-period above a threshold concentration and for example % decrease granulocytes, leucocytes and thrombocytes determined at the nadir do not sufficiently explain the observed interpatient concentration-response variability. Assessment of the AUC of the granulocytopenia, leucopenia or thrombocytopenia with time following chemotherapy was an attempt described in Chapter 5, which should also be evaluated in future studies to determine its usefulnes. Also, other more specific parameters of bone marrow toxicity should be evaluated in future studies, because myelosuppression is the main dose-limiting toxicity of currently applied anticancer agents.

Besides parameters which are related to the toxicity of anticancer therapy new pharmacodynamic end-points should be explored which correlate with the antitumor effect of drugs. An example which was explored in the present thesis is the DNA-adduct formation in WBC. Results of clinical studies revealed high correlation coefficients between DNA-adduct levels and likelihood of tumor response and toxicity. However, the additional value of the DNA-adducts in WBC is limited, because the parameter was highly correlated with the AUC of unbound platinum. Additional clinical studies should reveal whether DNA-adducts in other healthy tissue of which the cells have the potency to divide, for example buccal smears or rectal biopsies are correlated with the pharmacokinetics cisplatin in plasma. New assays with at least 10-fold higher sensitivity should be developed for this aim. The ^{32}P-postlabeling assay is promising in this respect (10). Preliminary experiments in our laboratory indicate a linear relationship between the incubation concentration of cisplatin and GG and AG adducts over a wide concentration range and high correlation coefficients with total DNA-adducts measured with atomic spectroscopy (AAS). The lower limit of quantitation is at least 10-fold better than of AAS and presumably lower than can be obtained with competitive ELISA (11).

Other dynamic end-points are related to the supposed mechanism of action of the anticancer agent. For example, measurement of the activity of topo I activity in tumor tissue and in healthy tissues in the case of the novel group of topo I inhibitors should give further insight into the relationship between the magnitude of exposure and the pharmacologic effect (i.e. likelihood of tumor response and toxicity). Also, measurement of spindle bundle activity may give useful information about the interpatient pharmacodynamic variability after administration of taxane drugs.

Useful amounts of tumor tissue in vivo in patients are difficult to obtain, in particular serial sampling may be extremely inconvenient for a

patient. For this, and other reasons mechanistic studies *in vitro* should be carried out in order to characterize 1) useful surrogate dynamic end-points (DNA-adducts and others as presented in Chapter 7), quantitative concentration-effect relationships (as presented in Chapter 7), mechanisms of interaction (as presented in Chapter 9) and mechanisms of resistance (as presented in Chapters 8 and 10).

Hopefully, these studies will result in a better understanding of concentration-effect relationships of presently available and novel agents and will lead to "tailor-made" anticancer therapy.

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CHAPTER 12

DUTCH SUMMARY - NEDERLANDSE SAMENVATTING (voor niet ingewijden)

Tussen patiënten onderling bestaan er grote verschillen in de respons op chemotherapie. Met andere woorden, de relatie tussen de gegeven dosis en het optredende farmacologische effect is in de regel tussen patiënten sterk verschillend. Het farmacologische effect betreft gewenste effecten, dus tumorrespons (kleiner worden of verdwijnen van de tumor) en ongewenste effecten, de bijwerkingen. De relatie tussen de dosis en het effect kan onderverdeeld worden in twee grote begrippen : farmacokinetiek en farmacodynamiek. De farmacokinetiek beschrijft processen als absorptie van de plaats van toediening van een geneesmiddel, verdeling over het lichaam en eliminatie uit het lichaam. Farmacodynamiek beschrijft de relatie tussen de mate van blootstelling aan een geneesmiddel en de optredende farmacologische effecten. Effecten bij antikanker therapie zijn de al dan niet optredende respons van het kwaadaardige gezwel en het patroon aan bijwerkingen.

In dit proefschrift staat het onderzoek aan drie antikanker middelen centraal, te weten cisplatinum, topotecan en docetaxel. Cisplatinum is een van de belangrijkste geregistreerde antikanker middelen. Het middel wordt succesvol toegepast bij de behandeling van uitgezaaide zaadbalkanker. Ook zijn andere tumoren goed of redelijk goed gevoelig voor cisplatinum, zoals eierstokkanker, baarmoederhalskanker, blaaskanker, niet-kleincellige longkanker en kanker van het hoofd-halsgebied. Topotecan is een geheel nieuw antikankermiddel met een uniek werkingsmechanisme. Topotecan is momenteel in een ver gevorderde fase van klinisch onderzoek. Het middel remt de functie van het kerneiwit topoisomerase I (topo I), dat een vitale rol speelt bij de ruimtelijk heroriëntatie van het grote DNA-molecuul in de celkern. Diverse tumoren zijn gebleken goed gevoelig te zijn voor topotecan en andere topo I remmers, evenals eierstokkanker, niet-kleincellige en kleincellige longkanker en borstkanker. Docetaxel (Taxotere®) is ook een nieuw antikanker middel met een hoge mate van activiteit tegen diverse tumoren, zoals borstkanker, niet-kleincellige longkanker, maagkanker en kanker van het hoofd-halsgebied. Het werkingsmechanisme is uniek. Docetaxel, evenals paclitaxel (Taxol®), grijpt aan op de delingsbundel tijdens de celdelingsfase. Het remt de functie van de delingsspoel en dit leidt uiteindelijk tot celdood.

Voorafgaande aan het farmacologisch onderzoek zijn er kwantitatieve meetmethoden opgezet waarmee concentraties van cisplatinum in bloedplasma eenvoudig, nauwkeurig en reproduceerbaar kunnen worden gemeten (hoofdstuk 3). Met behulp van toevoeging van zeer koude ethanol aan plasma worden plasma eiwitten neergeslagen en vervolgens wordt na centrifuge de bovenlaag verzameld. De gemeten concentratie is de concentratie van niet-eiwitgebonden, het zgn. "vrije" (farmacologische

actieve) cisplatinum. De metingen worden uitgevoerd met atoomabsorptiespectrofotometrie en omdat hiermee het atoom platinum wordt gemeten en niet het molecuul cisplatinum wordt gesproken over platinum-concentraties na toediening van cisplatinum. In hoofdstuk 2 wordt uiteengezet dat een eerder gepubliceerde methode voor het opwerken van DNA uit witte bloedcellen leidt tot een overschatting van de binding van cisplatinum aan DNA in witte bloedcellen kort na het einde van het cisplatinuminfuus. Met een in dit proefschrift beschreven verbeterde monstervoorbewerkingsmethode is de reproduceerbaarheid en de betrouwbaarheid van de bepaling sterk toegenomen. Bovendien leidt de verbeterde methode tot een fundamenteel andere interpretatie van de reparatiecapaciteit na DNA-schade in de witte bloedcel.

In een grote klinische studie waarin hoge doses van cisplatinum werden toegediend in een wekelijks schema werd de relatie onderzocht tussen de mate van blootstelling en de kans op tumorrespons en bijwerkingen (hoofdstuk 4). Tevens werd de mate van binding onderzocht van cisplatinum aan het DNA in witte bloedcellen in het perifere bloed. De werking van cisplatinum is gekoppeld aan de binding aan DNA in tumorcellen. De vraagstelling was of de binding aan DNA in witte bloedcellen een voorspellende waarde heeft ten aanzien van de kans op tumorrespons. De resultaten van het onderzoek laten zien dat er tussen patiënten onderling een grote variatie is in de relatie tussen de gegeven dosis, gerelateerd aan de grootte van het lichaamsoppervlak, en de mate van blootstelling in plasma. Tevens werd gevonden dat de mate van blootstelling sterk gecorreleerd was aan de kans op tumorrespons. Er werd ook een lineaire relatie met hoge correlatie gevonden tussen de mate van blootstelling en de mate van binding van cisplatinum aan DNA (DNA-adducten) in witte bloedcellen. Hieruit vloeit direct voort dat ook de hoogte van de DNA-adducten goed gecorreleerd was aan de kans op het optreden van een gunstige tumorrespons. Tevens was er een significante correlatie tussen de hoogte van de blootstelling en de kans op bijwerkingen, met name de mate van daling van de bloedplaatjes in het perifere bloed. Deze uitkomsten vormen een sterke basis om af te stappen van de gestandaardiseerde dosering van cisplatinum en de therapie per patiënt te individualiseren. Hierbij krijgen patiënten een dosis toegediend die leidt tot een vooraf gedefinieerde hoogte van de blootstelling. Dit vervolgonderzoek is momenteel gaande. Zeer voorlopige resultaten tonen aan dat deze benadering praktisch haalbaar is en leidt tot hoge responspercentages.

In een tweede grote klinische studie werd de combinatie cisplatinum en docetaxel toegediend aan patiënten met zeer uiteenlopende vormen van kwaadaardige tumoren. Het doel van het klinische gedeelte van het

onderzoek was om de maximale tolerabele dosis van de combinatie vast te stellen. Het doel van het farmacologisch onderzoek was om na te gaan of de volgorde van toediening van docetaxel en cisplatinum een invloed zou hebben op de farmacokinetiek en farmacodynamiek van beide middelen en of er relaties konden worden vastgesteld tussen de farmacokinetiek en de optredende toxiciteit. In totaal werden 64 patiënten in deze studie behandeld. De mate van blootstelling aan docetaxel en cisplatinum, gemeten aan de hand van concentraties in het plasma, na toediening van een standaard dosis gerelateerd aan de grootte van het lichaamsoppervlak varieerde zeer sterk tussen patiënten. Er was geen significante correlatie tussen de dosis van docetaxel en de mate van blootstelling aan docetaxel, maar wel tussen de dosis van cisplatinum en de mate van blootstelling aan vrij platinum. De farmacokinetiek van docetaxel en cisplatinum was tussen de patiënten die werden behandeld met de volgorde docetaxel-cisplatinum (49 evalueerbare patiënten) en de volgorde cisplatinum-docetaxel (15 evalueerbare patiënten) niet verschillend. De mate van blootstelling aan docetaxel was significant gecorreleerd aan de mate van beenmergtoxiciteit (daling van de witte bloedcellen en bloedplaatjes). Een nieuwe bevinding in deze studie was dat de tijdsduur dat de plasmaconcentratie van docetaxel zich boven een gekozen drempelwaarde bevond van 100 ng/ml als farmacokinetische parameter beter correleerde met de beenmergtoxiciteit dan de mate van blootstelling (uitgedrukt als de oppervlakte onder de plasmaconcentratie-tijd curve). Een ander interessant gegeven was dat de combinatie van blootstelling aan cisplatinum en tijdsduur van docetaxel boven 100 ng/ml beter correleerde met de beenmergtoxiciteit dan een van deze farmacokinetische parameters afzonderlijk. Bij de patiënten die werden behandeld met het schema docetaxel gevolgd door cisplatinum bleek dat de DNA-adduct niveaus in de witte bloedcellen veel lager waren dan in de patiënten in het omgekeerde schema. Er was een sterke trend dat de beenmergtoxiciteit (met name de daling van de witte bloedcellen) in het schema docetaxel-cisplatinum minder was dan in het omgekeerde schema. Het is nog niet opgehelderd of de farmacodynamische interactie op het niveau van de DNA-adductvorming therapeutische betekenis heeft. Dit moet worden onderzocht in vervolgstudies.

De invloed van docetaxel op de DNA-adductvorming door cisplatinum in witte bloedcellen is in detail onderzocht en beschreven in hoofdstuk 6. Incubatie van witte bloedcellen van gezonde vrijwilligers en van patiënten buiten het lichaam met cisplatinum of met de combinatie docetaxel-cisplatinum heeft de eerder beschreven farmaco-dynamische interactie bevestigd. Het onderzoek toonde aan dat ook paclitaxel de DNA-adductvorming door cisplatinum remt. Het mechanisme is dat taxanen de

opname van cisplatinum in de witte bloedcellen met bijna 50% verminderen. Aanvullend onderzoek heeft opgeleverd dat de inhibitie van de opname van cisplatinum in twee van menselijke tumoren afgeleide cellijnen niet optreedt. Of de interactie in tumoren in de mens en in zgn. voorlopercellen in het beenmerg optreedt is momenteel niet bekend.

In de kliniek is het niet duidelijk of de snelheid van intraveneuze toediening van cisplatinum van invloed is op de effectiviteit van cisplatinum. In door anderen uitgevoerd onderzoek is duidelijk geworden dat een intraveneuze bolus van cisplatinum leidt tot meer toxiciteit, met name nierschade, dan geleidelijke toediening in een langer durend infuus. In hoofdstuk 7 worden experimenten beschreven waarin drie infuusschema's worden nagebootst, die in het verleden in de kliniek zijn toegepast of momenteel nog worden toegepast. Het model was een kweekmedium met een van een menselijke eierstokkanker afgeleide cellijn IGROV-1. Eindpunten in dit onderzoek waren de celoverleving, de DNA-adductvorming en de mate van blootstelling aan actief (dus niet-eiwitgebonden) cisplatinum. De drie "infusie-schema's", te weten een intraveneuze bolus, een 3- en een 20-uurs infuus zijn nagebootst door op gezette tijden albumine en cisplatinum aan het celkweekmedium toe te voegen. Het albumine bindt cisplatinum dat daardoor wordt geïnactiveerd. De drie schema's werden zo ontworpen dat de mate van de blootstelling (oppervlakte onder de curve aan vrij platinum in het kweekmedium) bij alle schema's gelijk was. Het resultaat van het onderzoek was dat de celoverleving tussen de schema's niet significant verschilde. Tevens was er geen verschil in de grootte van de oppervlakte onder de DNA-adduct-tijd-curve in de cellijn. Het maximale gehalte aan de DNA-adducten was het grootst na de "intraveneuze bolus", gevolgd door het "3-uurs" en het "20-uurs infuus". De conclusie is dat de snelheid van toediening geen invloed heeft op de DNA-adductvorming in de tijd of op de celoverleving. Deze resultaten kunnen niet zonder meer worden geëxtrapoleerd naar de kliniek. Het is echter op grond van dit onderzoek aannemelijk dat de infusieduur in de kliniek geen belangrijke determinant is van de kans op respons van de tumor.

In hoofdstuk 8 is de ontwikkeling en karakterisering beschreven van een cellijn die is afgeleid van de beschreven IGROV-1. De IGROV-1 cellijn werd intermitterend blootgesteld aan opklimmende concentraties van cisplatinum met als doel een nieuwe cellijn te maken die ongevoelig is voor cisplatinum. De sublijn, genoemd $IGROV_{CDDP}$, was stabiel na ongeveer 9 maanden incubatie. De cellijn is 24 maal ongevoeliger voor cisplatinum dan de uitgangscellijn IGROV-1. De ophoping van cisplatinum in de $IGROV_{CDDP}$ cellijn bleek zeer veel (ongeveer 70 %) lager te zijn dan in IGROV-1. Hieraan gekoppeld bleek dat ook de DNA-adductvorming sterk verlaagd was ten opzichte van IGROV-1 onder

dezelfde incubatiecondities met cisplatinum. Aanvullend onderzoek heeft opgeleverd dat de opname van cisplatinum in IGROV-1 deels verloopt via een energie-afhankelijk proces en deels via passieve diffusie. In $IGROV_{CDDP}$ bleek dat de actieve, dus energie-afhankelijke, opname van cisplatinum in de cel was uitgeschakeld. Dit is waarschijnlijk het belangrijkste resistentiemechanisme. Het onderzoek heeft een model opgeleverd voor verder onderzoek aan de kinetiek van opname van cisplatinum in tumorcellen. Een belangrijke conclusie is dat de opname van cisplatinum in tumorcellen deels via een energie-afhankelijk proces kan verlopen en deels via passieve diffusie.

Zoals boven uiteengezet zijn topo I remmers belangrijke nieuwe antikanker middelen. Het werkingsmechanisme is anders dan dat van cisplatinum en ook het profiel van de bijwerkingen in de kliniek overlapt vrijwel niet met dat van cisplatinum. Deze eigenschappen tezamen vormen een sterke basis voor toepassing van de combinatie van cisplatinum en een topo I remmer in de kliniek. Dit onderzoek wordt momenteel voorbereid. In hoofdstuk 9 is onderzoek beschreven met de combinatie van cisplatinum en topotecan of SN38 in een goed gedefinieerd panel van 8 van menselijke tumoren afgeleide cellijnen. Cisplatinum en topotecan of SN38 werden afzonderlijk en in combinatie toegediend aan de celkweken. De resultaten lieten zien dat de combinatie van cisplatinum met een topo I remmer synergistisch is ten aanzien van de optredende celdood. Onderzoek naar het effect van de topo I remmer op de DNA-adductvorming door cisplatinum bracht aan het licht dat de DNA-adducten (met name de zgn. interstrand crosslinks) in hogere mate aanwezig bleven na blootstelling van IGROV-1 en $IGROV_{CDDP}$ aan cisplatinum gecombineerd met topotecan. Mogelijk draagt deze interactie bij aan de beschreven synergie tussen deze middelen. Het onderzoek heeft eveneens opgeleverd dat de synergie in 3 van de 8 cellijnen het sterkst was als topotecan niet gelijktijdig met, maar 2 dagen na cisplatinum werd toegediend aan het celkweeksysteem. Dit resultaat heeft mogelijk betekenis voor de ontwikkeling van het optimale schema van toediening van deze combinatie in de kliniek.

In hoofdstuk 10 wordt de ontwikkeling en karakterisering beschreven van een andere sublijn van IGROV-1 die resistent is tegen de topo I remmers topotecan en SN38. Deze cellijn is gemaakt door een celcultuur van IGROV-1 continu bloot te stellen aan topotecan in opklimmende concentratie. De cellijn, genaamd $IGROV_{T100R}$, bleek stabiel te zijn na ongeveer driekwart jaar incubatie. De sublijn was een factor 30 resistent tegen topotecan. Er was een volledige kruisresistentie met SN38, maar niet met een andere topo I remmer camptothecine. Aanvullend onderzoek heeft opgeleverd dat de intracellulaire accumulatie van topotecan en SN38 ongeveer 70 % lager was dan in de uitgangscellijn IGROV-1. Experimenten zijn beschreven waarin de opname en

afgifte van topotecan en SN38 werden bestudeerd. De resultaten laten zien dat de topo I remmers in IGROV-1 deels via een actief, energie-afhankelijk proces en deels via passieve diffusie wordt opgenomen. In de resistente sublijn $IGROV_{T100R}$ is de energie-afhankelijke opname volledig afwezig. Waarschijnlijk is deze aanpassing het belangrijkste mechanisme achter de resistentie. De uitstroomsnelheid van topotecan uit de twee cellijnen was zeer hoog, met een halfwaardetijd van minder dan 4 minuten. Er bestond geen significant verschil in de uitstroomsnelheid tussen de twee cellijnen. De energie-afhankelijke opname van topotecan in de uitgangscellijn IGROV-1 en de afwezigheid van dit proces in de resistente cellijn is een nieuwe niet eerder gerapporteerde bevinding. Toekomstig onderzoek zal erop gericht zijn om het mechanisme verder te ontrafelen.

De resultaten beschreven in dit proefschrift geven aanleiding tot vervolgonderzoek. Het wordt belangrijk geacht om verder onderzoek te doen aan de therapie-individualisatie met cisplatinum. Tevens is het van klinisch belang om de interactie tussen cisplatinum en taxanen nader te karakteriseren in onderzoek in de kliniek en in het laboratorium. Het is relevant voor de praktijk om het mechanisme van de synergie tussen cisplatinum en topo I remmers nader te karakteriseren. Ook is het van belang om onderzoek te doen naar het beschreven mechanisme van resistentie tegen topo I remmers en te onderzoeken of de afwezige energie-afhankelijk opname van topotecan en SN38 in de resistente cellijn klinische betekenis heeft.

De hoop is dat het in dit proefschrift beschreven onderzoek bijdraagt aan een beter inzicht in de werking van de beschreven antikanker middelen, dat het een aanzet betekent voor verder farmacologisch onderzoek aan concentratie-effect relaties in de kliniek en naar het mechanisme van werking van de beschreven middelen en dat dit vervolgens leidt tot een optimale klinische toepassing van deze middelen bij patiënten met kanker.

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Curriculum vitae

Jianguo Ma was born on 19 July, 1957, Tianjin, P.R. China. He studied pharmacy from 1978 until 1982 at the West China University of Medical Sciences, Chengdu, Sichuan, P.R. China. After his graduation, he was appointed as assistant researcher at the Department of Pharmacology, Institute of Hematology, Chinese Academy of Medical Sciences/Beijing Union Medical University and started the study of experimental chemotherapy under the supervision of Prof. Dr. Chunzheng Yang. In December 1987 followed the appointment as Research Associate. In January 1992 he started his Ph.D. training at the Laboratory of Experimental Chemotherapy and Pharmacology, Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek)/University Hospital Rotterdam, The Netherlands under the supervision of Prof. Dr. Gerrit Stoter and Dr. Jan H. M. Schellens. Initially, this was sponsored by the World Health Organization and later by the Department of Medical Oncology.

Abbreviations

AAS	atomic absorption spectroscopy
9-AC	9-amino-20(S)-camptothecin
ATP	adenosine triphosphate
AUA	area under curve of DNA adducts
AUC	area under the curve
BCS	bovine calf serum
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CBDCA	carboplatin, cis-diamminecyclobutane-1,1-dicarboxylato platinum(II)
CDDP	cisplatin, cis-diamminedichloroplatinum(II)
CI	combination index
CPT	camptothecin
CPT-11	7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonycamptothecin
CS	Cockayne's syndrome
CUP	cancer of unknown primary site
DMSO	dimethyl sulfoxide
DNase I	deoxyribonuclease I
DRP	damage recognition protein
DTT	dithiothreitol
EB	ethidium bromide
EDTA	ethylene diammine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERCC	excision repair cross complementing
FCM	flow cytometry
5-FU	5-fluorouracil
GGR	global genome repair
GSH	glutathion
GSSH	glutathion disulfide
GST	glutathion s-transferase
HMG	high-mobility group proteins
H/N	head and neck cancer
IC_{50}	concentration of drug inhibiting cell survival by 50%
ID_{50}	dose of drug inhibiting cell survival by 50%
ISC	interstrand cross-links
MDR	multidrug resistance
MRP	multidrug resistance-associated protein
MT	metallothioneins

MTT	3-(4.5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
MTX	methotrexate
MX	mitoxantrone
NER	nucleotide excision repair
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
Pgp	P-glycoprotein
PMSF	phenyl methyl sulphonyl fluoride
Pt	platinum
Pt-AG	intrastrand cross-link at neighbouring A and G nucleotides
Pt-GG	intrastrand cross-link at neighbouring G and G nucleotides
Rf	resistance factor
RNase A	ribonuclease A
SN38	7-ethyl-10-hydroxycamptothecin
SD	standard deviation
SDS	sodium dodecyl sulfate
SRB	sulforhodamine B
TCR	transcription-coupled repair
TFIIH	transcription factor IIH
TOPO I	topoisomerase I
TOPO II	topoisomerase II
TPT	topotecan
VP16	etoposide
WBC	white blood cells
XP	xeroderma pigmentosum