UNIVERSITY OF
CALGARY

UNIVERSITY OF CALGARY

Interaction of Mesna with Platinum Chemotherapy Agents Cisplatin and Carboplatin: Chemistry, *in vitro*, *in vivo* and Clinical Pharmacokinetics

by

Shahbal B. Kangarloo

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

OCTOBER, 2001

ABSTRACT

Sodium 2-mercapethanesulfonate (mesna) often comes into contact with platinum drugs (Pt-drugs) cisplatin and carboplatin in combination protocols involving oxazaphosphorines and Pt-drugs. This co-administration might be unfavorable based on the inactivation of Pt-drugs by thiol groups *in vitro*. To elucidate whether mesna reduces effectiveness of Pt-drugs, the chemical reaction, *in vitro* and *in vivo* interactions, and clinical pharmacokinetics of Pt-drugs in combination with mesna were investigated. Mesna reacted with cisplatin in a fast 2 to 1 reaction as opposed to a slow 1 to 1 reaction with carboplatin. In glioma cells, mesna significantly reduced platinum accumulation and DNA binding of cisplatin but not that of carboplatin. Tumors and various tissues in SCID mice did not show a reduction in platinum levels and platinum-DNA adducts in presence of mesna. Pharmacokinetics of cisplatin and carboplatin were not affected by the co-administration of mesna in pediatric patients. Thus this thesis led to the reassurance that it is safe to combine mesna with Pt-drugs in combination chemotherapy protocols, provided that the agents are not mixed in solution prior to infusion into the patients.

ACKNOWLEDGMENTS

I wish to express my deepest gratitude to my supervisors: Dr. Johannes Wolff, and Dr. Stefan Glück. To Dr. Wolff, who introduced me to the field of pharmacokinetics, for his patience and encouragement throughout this thesis, and for obtaining the funding and giving me the opportunity to perform this study in his laboratory. I owe my sincere thanks to my co-supervisor Dr. Glück, for his guidance and constant interest in this project and in particular his support in providing me with the environment to finish this thesis in addition to his careful reading of this manuscript during its preparation.

I am most grateful to Dr. Suman Gangopadhyay for her valuable advice and unlimited help with the scientific rationale and design of my experiments from day one to the end of this thesis.

My warmest thanks goes to Shadi Fattahi, for her encouragement, enthusiasm, her genuine interest in my research, and her patience throughout this project. I specifically like to thank Shadi for her skillful help in formatting this thesis.

I would like to thank the members of my supervisory committee, Dr. R. N. Johnston, and Dr. M. Hollenberg for their advice on several aspects of this project. I would like to thank Dr. G.G. Miller for his contribution as an external examiner.

I also would like to thank my co-workers Sandra Bitner and Sharon Clark for their support and unforgettable moments spent during completion of this thesis. In particular I thank Sandra for her assistance with the animal work involved in this thesis.

Finally, I would like to thank my family, my parents, and my brothers who have been the pillar of support throughout this degree. I wish to thank them all for their invaluable support and encouragement.

This project was supported financially by the Alberta Cancer Board, the Alberta Children's Hospital Foundation, and the Steve Fonyo Foundation, all of which are gratefully acknowledged.

DEDICATION

I dedicate this thesis to Mr. N. Shafiee, my lifelong teacher, who always believed in me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

[ ]	concentration
µg	microgram
µL	microliter
µM	micromolar
µmol	micromole
Ae	amount excreted
AAS	atomic absorption spectrometry
ABC	adenosine triphosphate-binding cassette
AG	deoxyadenosine-deoxyguanosine
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the serum concentration versus time curve
BSA	bovine serum albumin
°C	degree centigrade
carboplatin	*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)
CBDCA	*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)
CDDP	*cis*-diamminedichloroplatinum(II)
cisplatin	*cis*-diamminedichloroplatinum(II)
CL_R	renal clearance
CL_t	total clearance
Cmax	maximum plasma concentration
cMOAT	canalicular multispecific organic anion transporter
CV	coefficient of variance
Da	dalton
ddH2O	double distilled deionized water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
FCS	fetal calf serum
fmol	femtomole
g	gram
GFR	glomerular filtration rate
GG	deoxyguanosine-deoxyguanosine
GSH	glutathione
GS-X	glutathione-s-conjugate
h	hour
HMG	high mobility group
HNO_3	nitric acid
HPLC	high performance liquid chromatography
IF	Ifosfamide
i.v.	intravenous
i.p.	intraperitoneal

KCl	potassium chloride
kg	kilogram
L	liter
M	moles per liter (molar)
m^2	meter squared
mesna	sodium 2-Mercaptoethanesulfonate
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MRP2	multidrug resistance protein-2
MRT	mean residence time
MT	methallothionein
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N	nitrogen
NaCl	sodium chloride
ng	nanogram
nm	nanometer
nmol	nanomole
NMR	nuclear magnetic resonance
OAP	oxazaphosphorines
O.D.	optical density
PBS	phosphate buffer saline
PK	pharmacokinetics
pmol	picomole
Pt	platinum
Pt-DNA	platinum bound to DNA
Pt-drugs	platinum drugs
s	second
s.c.	subcutaneous
SCID/NOD	severe combined immunodeficient / non-obese diabetic
SD	standard deviation
SEM	standard error of the mean
SH	sulfhydryl group
$t_{1/2}$	half-life
thiol	sulfur containing
Tris	tris-hydroxymethyl-amino methane
UF	ultrafiltrate
U/L	units per liter
UV	ultraviolet
V	volume of distribution
Vc	volume of central compartment
Vss	volume of distribution at steady state

Chapter One: Background

Introduction

Platinum (Pt) chemotherapeutic agents (cisplatin and carboplatin) (Pt-drugs) and oxazaphosphorines (OAP) ifosfamide and cyclophosphamide are increasingly used simultaneously in cancer treatment. Hemorrhagic cystitis, a side effect of OAP, is routinely prevented by co-administration of mesna. Mesna was developed as a specific chemoprotective agent against acrolein induced bladder toxicity of OAP. It is a thiol compound, which inactivates OAP metabolites in the bladder by forming an inert thioether, and their subsequent elimination in the urine (fig. 1.1). However, when OAP and Pt-drugs are given simultaneously, mesna also comes into contact with the Pt-agents. This might be unfavorable since mesna reduces the efficacy of cisplatin and carboplatin *in vitro* (Sauter et al., 1986; Williams and Lokich, 1992; Wolff et al., 1998). However the chemical reaction, the cellular mechanism of this inactivation, and the *in vivo* implications of this interaction are not fully clarified yet.

Cisplatin

History

The platinum co-ordination complex ***cis*-diamminedichloroplatinum(II)** (CDDP) or cisplatin was first synthesized in 1845 by Peyrone (Basolo and Pearson, 1962). The useful biological effects of the compound, however, were not discovered for more than a century. In 1965, biophysicist Barnett Rosenberg was examining the effects of electrical fields on the bacterium *Escherichia coli* (Rosenberg et al., 1965). It was noted that alternating current delivered through platinum electrodes into a bacterial culture inhibited cell division and caused the bacteria to grow into long filaments. The same result was seen when attempts

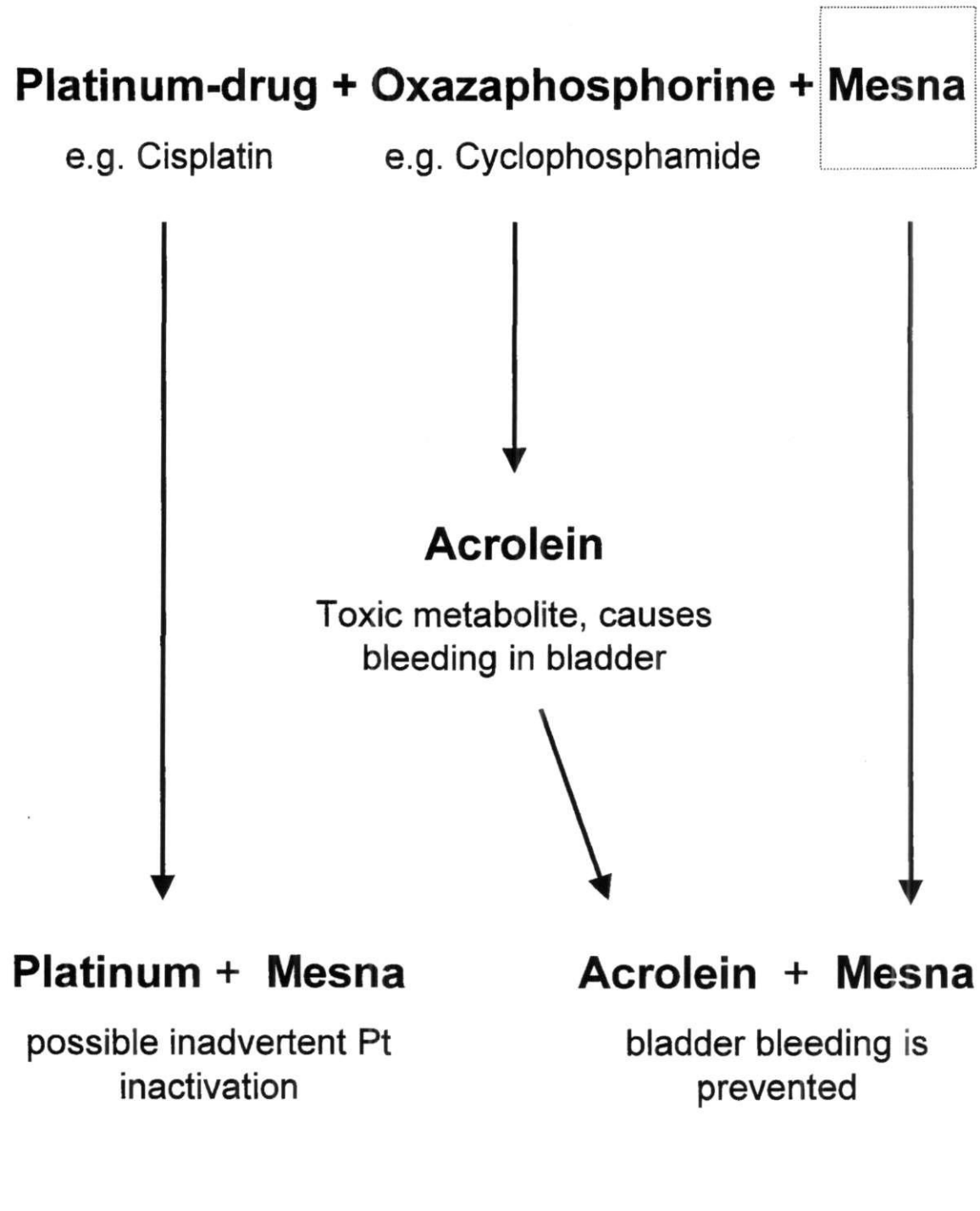


Figure 1.1. Interaction of Pt-drugs with mesna in combination chemotherapy involving oxazaphosphorines.

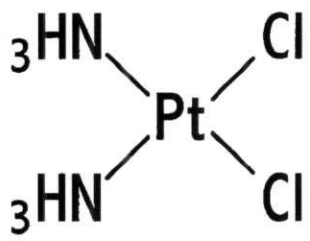
were made to grow the bacteria in medium that had previously been subjected to the electric current. The result was notable because similar effects on bacteria were known to be produced by a variety of DNA damaging agents, including radiation and alkylating agents. Subsequent studies showed that platinum atoms from the electrodes entered the medium in the form of hexachloroplatinate anions that, in the presence of light, reacted with ammonia ions in the medium to form complexes containing both chloride and ammonia. Examination of several complexes of this type showed that bacterial growth into filaments was effectively produced by the *cis* isomer of $Pt(NH_3)_2Cl_2$ (Rosenberg et al., 1967). In 1969 Rosenberg et al. (Rosenberg et al., 1969) reported that the *cis* isomer had potent antitumor activity against sarcoma 180 and leukemia L1210 in mice. The *trans* isomer however, was inactive (Rosenberg et al., 1969).

Cisplatin was approved by the United States Food and Drug Administration in 1979, and is now one of the most widely used chemotherapeutic agents for the treatment of human cancers (Trimmer and Essigmann, 1999). Cisplatin demonstrates significant activity against tumors of the ovary, bladder, lung, head and neck, but it is most strikingly effective against testicular cancer (Trimmer and Essigmann, 1999).

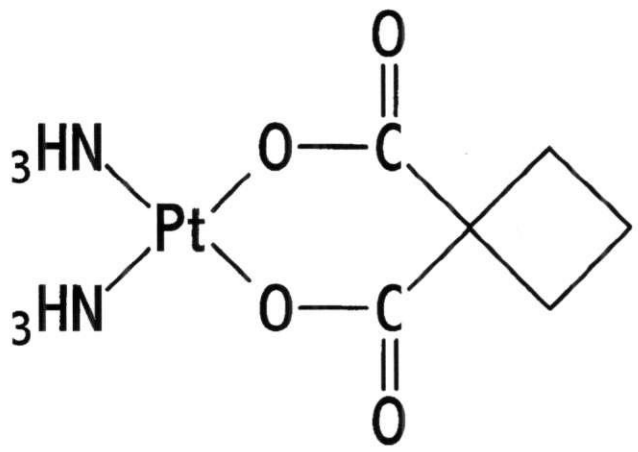
Chemistry

Platinum is in the third row of transition metals in the periodic table and has eight electrons in the outer *d* shell. Since platinum has a large number of total electrons, the orbitals of its outer electrons are polarizable, hence bonds involving these orbitals have a highly covalent character (Reed and Kohn, 1990). Covalent bond character provides two essential properties: (1) stereospecificity and; (2) an energy barrier to exchange reactions (Reed and Kohn, 1990). The steric arrangement of the bonds depends on the oxidation state, which can be +2 or +4. In the +2 oxidation state the metal has four bonds that point to the corners of a square with a metal atom at the center (like cisplatin fig.

A. *cis*-diamminedichloroplatinum(II) (cisplatin), molecular weight 300.05 (g/mol)



B. *cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II) (carboplatin), molecular weight 371.25 (g/mol)



C. Sodium 2-mercaptoethanesulfonate (mesna), molecular weight 164.2 (g/mol)

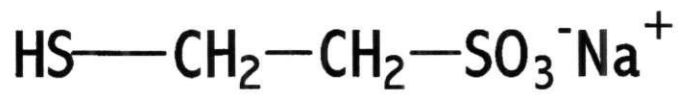


Figure 1.2. Chemical structures, A. cisplatin, **B.** carboplatin, and **C.** mesna

1.2 a). In the +4 state there are an additional two bonds directly 90° above and below the planer molecule. Although some Pt(IV) (oxidation state +4) complexes have antitumor activities, this may be due to their reduction *in vivo* to the active Pt(II) state (Blatter et al., 1984). Pt complexes are stereospecific in that bound ligands remain fixed in position, thereby producing distinct *cis* and *trans* $Pt(NH_3)_2Cl_2$ isomers.

Pt(II) reactions of greatest interest from the chemotherapeutic viewpoint involve the replacement of one ligand by another. The important considerations are the stability of a given ligand bound to the Pt atom and the leaving ability of ligands bound to Pt. The order of stability from highest to lowest for binding strength of some selected ligands to Pt has been determined as:
$CN^- > RS^- > OH^- > NH_3 > SCN^- > I^- > Br^- > Cl^- > H_2O$, and in general the stability increases with the polarizability of the ligand (Thomson et al., 1972). However, NH_3 and OH^- form strong bonds with Pt despite having low polarizability (Thomson et al., 1972). Also the kinetic entry rate, (i.e. the most likely ligand to react with Pt) of some ligands from highest reactive to lowest reactive toward Pt(II) complexes has been determined as:
$RS^- > I^- > Br^- > NO_2^- > NH_3 > Cl^- > RO^-$, where polarizability completely dominates this list (Thomson et al., 1972). It is noteworthy that thio groups rank highest in this list; hence the high likelihood of mesna, a thiol compound, in reacting with Pt-drugs.

Studies have shown that minor variations in the structure of these ligands can have a profound effect on the antitumor activity and toxicity of platinum complexes. Almost all *trans*-compounds tested are ineffective, while the *cis*-counterparts are quite the opposite (Reed and Kohn, 1990). It appears that the *cis*-conformation is required for a complex to be an effective agent. Both *cis*- and *trans*- isomers exchange chloride ions for such nucleophilic groups as RS^- to form links that can be very stable (Reed and Kohn, 1990). The substitution of ligands of planar platinum(II) compounds, such as cisplatin, may follow one of two pathways in aqueous solution: (fig. 1.3).

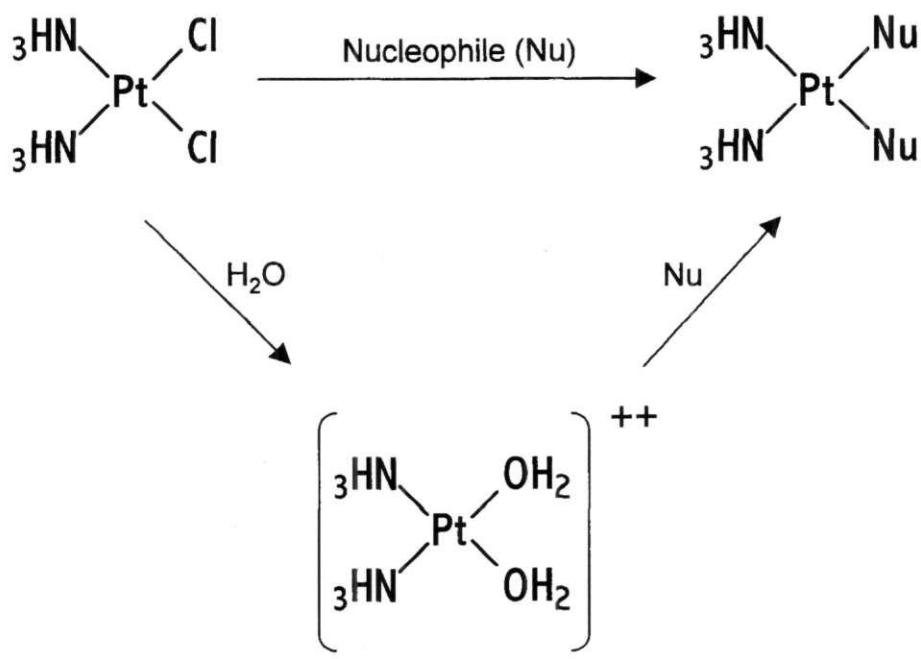


Figure 1.3. Ligand substitution pathways for cisplatin. Strong nucleophiles such as thiols proceed through the direct pathway, whereas weaker nucleophiles such as the nitrogen-7 on guanosine, proceed through the aqua intermediate.

As shown in figure (1.3), a chloride ion may be replaced by water to produce an aqua intermediate, the solvent molecule being subsequently eliminated by an incoming nucleophile. Alternatively, there may be direct replacement of the leaving group without the participation of the solvent. This direct pathway is only possible with strong thiol nucleophiles (Reed and Kohn, 1990).

Since cisplatin is administered as an aqueous solution, it is therefore, essential that ligand substitution be minimized before it reaches the tumor. This is achieved by using isotonic saline which has a relatively high chloride ion concentration (0.9% NaCl or 0.15 M NaCl), thus keeping the substitution equilibrium to the left (fig. 1.3). This ensures that the "inactive" cisplatin complex will predominate, reducing the amount being converted to the "active" aqua form prior to administration. Once in the body, the high chloride ion concentrations present in plasma and extracellular fluid (>100mM), maintains the persistence of the electroneutral complex and prevents any premature activation or unwanted direct ligand substitutions (Reed and Kohn, 1990). Being uncharged, the molecule is able to cross cell membranes and thus into cancer cells (Reed and Kohn, 1990). The relatively low chloride concentration of the cytosol favors the formation of the active aquated species, which goes on to react with nucleophilic groups (Reed and Kohn, 1990).

Mechanism of action

Cisplatin is a well known DNA damaging agent. The current thinking is that DNA platination is an essential first step in anti-cancer activity of the drug (Perez, 1998). One potentially important way by which Pt-DNA adducts may kill cells is by induction of programmed cell death or apoptosis (Eastman, 1990). However, a recent growing body of evidence suggests that cisplatin-induced cell death does not always arise from "classic" apoptosis (Gonzalez et al., 2001).

DNA as the critical target for Pt-drugs

The discovery in 1965 by Barnett Rosenberg that cisplatin caused filamentous growth of *E. coli* was the prelude to testing cisplatin as an anticancer agent (Rosenberg et al., 1969). Evidence for DNA as the target of cisplatin's antitumor effects is provided by the following observations: (1) The original observation by Rosenberg (Rosenberg et al., 1965), the filamentous growth of *E. coli* induced by cisplatin is characteristic of DNA-damaging agents such as UV radiation, ionizing radiation and hydroxyurea (Jamieson et al., 1999); (2) cells from patients with diseases in which DNA repair processes are deficient (e.g. xeroderma pigmentosum) (Fraval et al., 1978), and DNA repair-deficient mutants of Chinese hamster ovary cells (Sorenson and Eastman, 1988), are all hypersensitive to cisplatin; (3) correlations have been shown between levels of Pt-DNA adducts in peripheral blood lymphocytes and disease (or toxicity) response in patients receiving Pt-drugs (Reed et al., 1987); and (4) the 1,2-intrastrand Pt-DNA adducts which have been implicated as the anti-cancer Pt adduct (discussed later) are not formed by the inactive *trans*-isomer of cisplatin (transplatin) (Eastman and Barry, 1987).

Many cellular components that have nucleophilic sites such as DNA (including mitochondrial DNA), RNA, proteins, membrane phospholipids, cytoskeletal microfilaments, and thiol-containing molecules react with cisplatin (Gonzalez et al., 2001). However, based on the evidence listed above, genomic DNA is generally accepted as the critical pharmacological target of Pt-drug induced cytotoxicity.

Only approximately 1% of the intracellular cisplatin reacts with nuclear DNA to yield a variety of adducts that include interstrand and intrastrand DNA cross-links and DNA-protein cross-links (Gonzalez et al., 2001) (fig. 1.4). The major site of platination in double-stranded DNA (65%) derives from interstrand cross-links between two neighboring deoxyguanosines (GG) (fig. 1.4b) (Fichtinger-Schepman et al., 1985). About 20% of the DNA platination derives

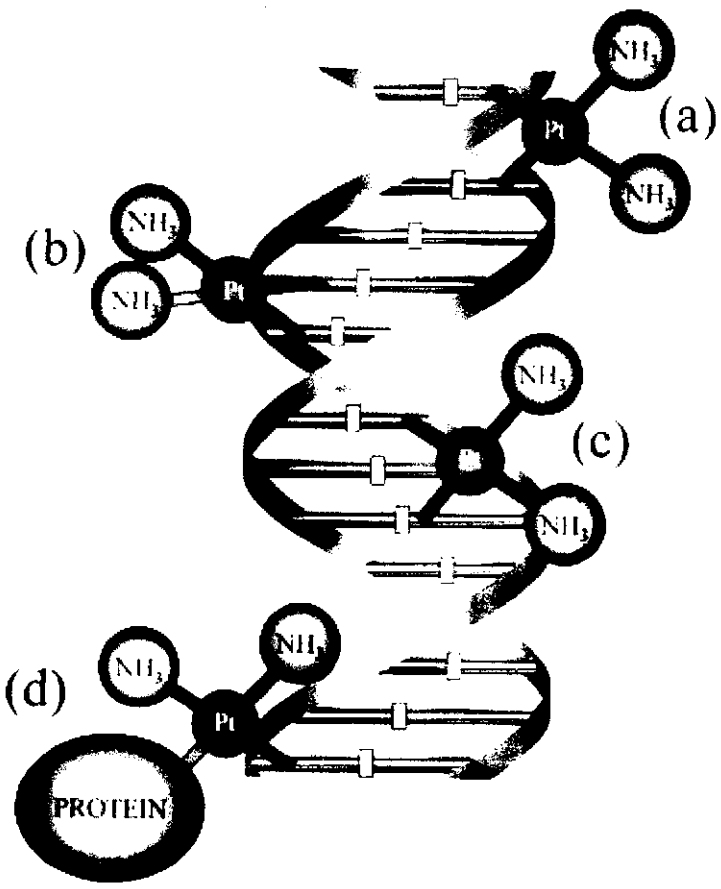


Figure 1.4. Major adducts formed in the interaction of cisplatin with DNA. (a) interstrand cross-link, (b) 1,2-intrastrand cross-link, (c) 1,3-intrastrand cross-link, and (d) protein-DNA cross-link (from Gonzalez et al., 2001).

from intrastrand cross-links at an deoxyadenosine-deoxyguanosines (AG) (fig. 1.4b) sequence (Fichtinger-Schepman et al., 1985), however no adducts are detected when these two nucleosides are in reverse order (i.e., GA) (Fichtinger-Schepman et al., 1985). Another 9% of the platination derives from a cross link between two deoxyguanosines separated by a third nucleoside (i.e., in a GNG sequence where N is any nucleoside) (fig. 1.4c) (Fichtinger-Schepman et al., 1985). All of these modifications are through the N(7) position on the purine ring (Eastman, 1999). Another 2-3% of the Pt-DNA adducts are the result of mono-functional binding to guanosine (fig. 1.4d), with the second Pt site being bound to GSH or proteins (Fichtinger-Schepman et al., 1987). DNA interstrand cross-links have also been purified and found to be formed between two deoxyguanosines (fig. 1.4a), but occur only at less than 1% of the total platination of DNA (Fichtinger-Schepman et al., 1985).

Cellular proteins that bind to Pt-DNA adducts

The mechanism of cisplatin-induced DNA damage toward cytotoxicity is beginning to be disentangled. In the past it was thought that cisplatin cytotoxicity was the result of inhibition of DNA synthesis. However, DNA repair-deficient cells die at concentrations of cisplatin that do not inhibit DNA synthesis (Fraval et al., 1978; Sorenson and Eastman, 1988). Moreover, DNA repair-proficient cells survive at concentrations of cisplatin high enough to inhibit DNA synthesis and cell arrest in the S-phase (Sorenson and Eastman, 1988). Thus, cisplatin-induced cell death does not always correlate with inhibition of DNA synthesis. To help understand the initial events that link cisplatin-induced DNA damage to the cell death pathway, considerable attention has recently focused on identification and characterization of proteins that recognize cisplatin-induced DNA damage. At present, several families of proteins are implicated as important: 1) nucleotide excision repair (NER) proteins, 2) mismatch repair (MMR) proteins, 3) DNA-dependent protein kinase (DNA-PK), and 4) high-mobility group (HMG) proteins.

It is becoming clear that the NER pathway is responsible for the repair of cisplatin-DNA adducts (Zamble et al., 1996). This system recognizes Pt-DNA adducts at adjacent GG intrastrand sites and excises the damaged DNA site (Mu et al., 1996).

MMR is a postreplication repair system that corrects unpaired or mispaired nucleotides (Kartalou and Essigmann, 2001). Human mismatch repair complex hMutS- detects, but does not remove cisplatin-DNA adducts (Yamada et al., 1997). This protein has been shown to specifically recognize a single cisplatin intrastrand adduct between two adjacent guanosines within a double-strand oligonucleotide (Yamada et al., 1997). The current thinking is that MMR proteins would try to insert the "correct" nucleotide on the non-damaged strand opposite the intrastrand adduct between two adjacent guanosines and that this "futile" repair cycle might then induce apoptosis (Yamada et al., 1997).

Human Ku autoantigen, a component of the DNA-dependent protein kinase (DNA-PK), is a repair system that is required for the elimination of DNA double-strand breaks that are induced by ionizing radiation, and also reportedly cisplatin-DNA lesions (Turchi and Henkels, 1996). Normally, Ku binds to DNA, and the Ku-DNA complex activates DNA-PK activity (Turchi and Henkels, 1996). The activated DNA-PK complex can phosphorylate a number of different proteins, including p53, and other transcription factors (Turchi and Henkels, 1996). When Ku binds to cisplatin-modified DNA, DNA-PK is not activated, potentially interfering with the regulation of transcription factors through phosphorylation (Turchi and Henkels, 1996).

High mobility group (HMG) domain proteins are a large family of proteins that bind specifically to cisplatin-modified DNA (Jamieson et al., 1999). An HMG protein called structure specific recognition protein-1 (SSRP-1) binds to cisplatin but not transplatin adducts (Jamieson et al., 1999). This protein specifically binds to 1,2-intrastrand adducts, but not to 1,3-intrastrand or to trans-DDP modified DNA (Jamieson et al., 1999). In the "repair shielding model", HMG proteins could

protect cisplatin-DNA adducts from recognition by DNA repair enzymes, hence leading the cell into apoptosis (Jamieson et al., 1999).

In summary, in a particular cell line, cisplatin cytotoxicity might be determined by a "dynamic contest" between proteins that repair DNA and proteins that interfere with DNA repair and trigger apoptosis (Gonzalez et al., 2001).

Resistance

Several mechanisms of Pt-drugs resistance have been identified by the various cellular model systems (fig. 1.5). These include: (1) reduced drug accumulation; (2) increased drug inactivation by sulfur-containing compounds, such as glutathione (GSH) and metallothionein (MT) proteins; (3) enhanced repair of Pt-DNA adducts; (4) increased tolerance of Pt damage; and (5) altered expression of regulatory proteins. It is emphasized that development of resistance is a multifactorial process and therefore a given tumor may become resistant by one or more of these mechanisms.

Drug uptake/efflux

Reduced intracellular accumulation of cisplatin, which may arise because of decreased uptake or increased efflux, is frequently observed in cisplatin resistant cell lines (Kartalou and Essigmann, 2001). To date the exact mechanism by which cisplatin is taken up by the cells is not fully understood. The rate limiting factor for cisplatin uptake is its concentration (Gale et al., 1973). Also, cisplatin uptake is not inhibited by structural analogues (Andrews et al., 1988), and its uptake is not saturable (Gale et al., 1973), therefore it has been suggested that cisplatin enters the cells by passive diffusion.

There is, however, some evidence that indicates that cisplatin uptake is mediated by membrane proteins. For example, a variety of pharmacological

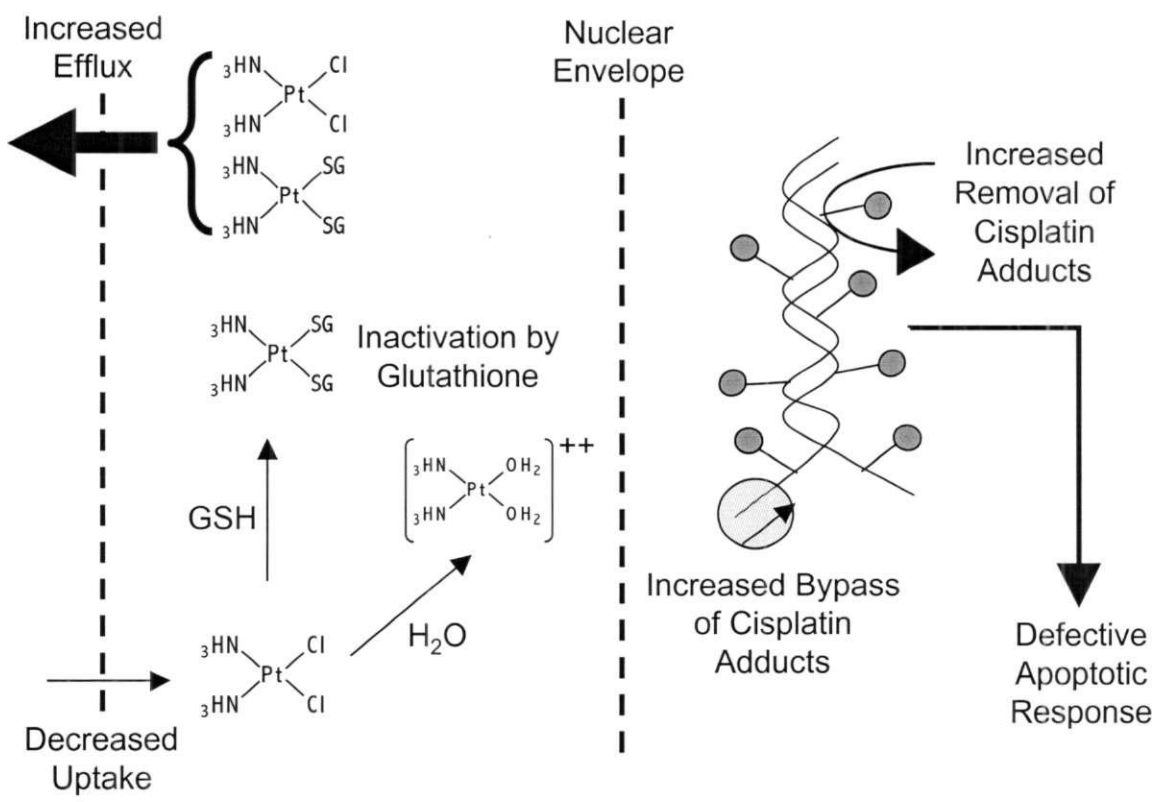


Figure 1.5. Possible mechanisms of resistance to cisplatin. Reduced intracellular accumulation of cisplatin which may arise because of decreased uptake or increased efflux; increased inactivation by cellular thiols; increased repair of Pt-DNA adducts; increased ability to replicate past Pt-DNA adducts; and defects in the apoptotic response pathway (adapted from Kartalou & Essigmann, 2001). Mesna might also be synergistic at decreasing uptake; inactivation by thiols & hence increasing efflux, thereby reducing Pt-DNA adducts.

agents that do not alter the permeability of the membrane inhibit cisplatin uptake. The sodium–potassium ATPase inhibitor ouabain inhibits cisplatin uptake by 50% (Andrews et al., 1991). Moreover, several aldehydes inhibit Pt-drug uptake, presumably by forming Schiff bases with membrane proteins (Dornish and Pettersen, 1985). These results suggest a carrier mediated transport system for the cellular uptake of cisplatin. Interestingly, a 48 kDa membrane protein is expressed in lower levels in cisplatin resistant cells that show decreased cellular accumulation of cisplatin, indicating that this protein might be involved in cisplatin uptake (Bernal et al., 1990). Recently, evidence was provided for uptake of cisplatin by the organic cation transport system in renal epithelial cells (Endo et al., 2000).

One model that accommodates most of the existing evidence is that approximately one-half of the initial drug uptake rate is due to passive diffusion and that the other half is due to facilitated diffusion through an as yet unidentified pump (Gately and Howell, 1993).

In addition to the mechanisms described above, decreased cellular accumulation of cisplatin might occur by increased efflux of the drug from the cells. Cisplatin is not a substrate for the P-glycoprotein (the product of the *mdr* gene), which is over-expressed in multi-drug resistant cells and functions as a drug efflux pump (Kool et al., 1997). However an ATP-dependent export pump, the glutathione-s-conjugate (GS-X) export pump, notably the canalicular multispecific organic anion transporter (cMOAT), also known as the multidrug resistance associated protein-2 (MRP2), might contribute to reduced cellular accumulation of Pt-drugs via their increased efflux (Borst et al., 2000; Ishikawa and Ali-Osman, 1993; Kool et al., 1997). Evidence has been provided that MRP2 could contribute to cisplatin resistance by exporting the cisplatin-glutathione complex out of the cell and therefore reducing cisplatin accumulation inside the cell (Borst et al., 2000).

Inactivation of cisplatin by cellular thiols

A second mechanism by which cells may limit the number of cisplatin adducts formed is by increasing the concentration of sulfur-containing molecules that can react with the drug before it reaches the DNA. Such molecules include glutathione, the most abundant thiol in the cell (Anderson, 1998), and metallothioneins, small cysteine-rich proteins involved in detoxification of heavy metals (Pattanaik et al., 1992).

Many Pt-resistant cell lines exhibit relatively high levels of intracellular GSH compared to their Pt-sensitive counterparts (Kelland, 2000). Cisplatin can be covalently linked to GSH after nucleophilic attack of the glutathione thiolate anion (Ishikawa and Ali-Osman, 1993), and this complex can be transported out of the cell through the aforementioned MRP2 pump. Additionally, conjugation with GSH inhibits the conversion of Pt-DNA mono-adducts to bi-functional adducts, thereby reducing the cytotoxic potential of the Pt-DNA adducts (Eastman, 1987).

Metallothioneins bind to cisplatin in a protein:drug ratio of 1:10 and may affect sensitivity to the drug (Pattanaik et al., 1992). Overexpression of MT can sometimes cause resistance to cisplatin, and at least some cell lines that have acquired resistance to cisplatin overexpress MT (Kasahara et al., 1991). Additionally, cadmium resistant cell lines overexpress MT and are cross resistant to cisplatin (Koropatnick and Pearson, 1993).

Post DNA-binding mechanisms

It is now clear, at least from cell-line studies, that even if sufficient Pt forms adducts on the DNA of tumor cells, cell death may still not follow. These mechanisms include, but are not limited to: increased DNA repair of adducts (increased NER), increased tolerance to Pt-DNA adducts (possibly due to replication bypass as a result of loss of MMR mechanism), and decreased

apoptosis (alterations in expression of oncogenes such as H-ras, c-myc and tumor suppressor genes such as p53, to name a few). Detailed discussions of these mechanisms are beyond the scope of this thesis and have been reviewed in detail previously (Gosland et al., 1996; Kartalou and Essigmann, 2001; Kelland, 2000; Perez, 1998).

Clinical Pharmacology

Activity

Cisplatin is one of the most active drugs in the treatment of solid tumors. It has become the foundation of curative protocols in testicular and ovarian cancers. It also demonstrates significant activity against cancers of lung, head and neck, esophagus, bladder, and cervix (Go and Adjei, 1999).

Since the introduction of cisplatin, 70% to 80% of patients with advanced germ cell tumors (testicular) can now be cured (Go and Adjei, 1999). With the use of aggressive chemotherapy (bleomycin, etoposide, cisplatin, (BEP)) along with surgery and radiation therapy, over 90% of the patients are now expected to be cured (Horwich et al., 1997). There is general agreement that the most important contribution to this success has been the development of cisplatin and its incorporation into combination chemotherapy for treatment of this disease (O'Dwyer et al., 2000).

Cisplatin is an integral part of standard regimens for the treatment of ovarian cancer. Compared to other solid tumors, ovarian cancer is relatively responsive to chemotherapy, but unlike testicular cancer, cure is not common for patients with advanced disease (Hoskins et al., 1992). One of the largest randomized trials by the Gynecologic Oncology group (GOC), randomized 227 patients to receive either cyclophosphamide/doxorubicin or cyclophosphamide/doxorubicin/cisplatin. The response rates (26% vs. 51%),

medium response duration ratio (8.8 vs. 14.6 months), and survival (9.7 vs. 15.7 months) all favored the cisplatin containing protocol (Omura et al., 1986).

Platinum-based combination chemotherapy has become the cornerstone of therapy for both non-small-cell lung cancer (Giaccone et al., 1998; Le Chevalier et al., 1994) and small-cell lung cancers (Lassen et al., 1996; Skarlos et al., 1994). As of yet, no clear consensus regarding the superiority of a single regimen has been reached, but it is clear that Pt based combinations can improve the survival and quality of life in patients with advanced lung cancer (O'Dwyer et al., 2000).

Advanced stage, recurrent, or metastatic head and neck cancers have a poor prognosis, with a median survival time of approximately 6 months (Go and Adjei, 1999). The most widely used combination chemotherapy is the fluorouracil/cisplatin regimen (De Andres et al., 1995). Overall chemotherapy administered concurrent with radiation has produced the most promising results (Taylor et al., 1994) and cisplatin has been found to be superior to carboplatin (De Andres et al., 1995).

Toxicity

Nephrotoxicity was the dose-limiting toxicity for cisplatin in early clinical trials, with effects ranging from reversible azotemia to irreversible renal failure requiring dialysis (Daugaard and Abildgaard, 1989). Hydration with normal saline, hypertonic saline infusion, and diuretics have been used to effectively reduce this toxicity (Al-Sarraf et al., 1982).

Neurotoxicity is now the major dose-limiting toxicity of cisplatin. This includes: hearing loss, Lhermitte's sign (electric shock-like sensation transmitted down the spine upon neck flexion), seizures, and encephalopathy (Cersosimo, 1989). It has been reported that neurotoxicity occurs in 85% of patients with cumulative dose greater than 300 mg/m^2 (Cersosimo, 1989).

Nausea and vomiting are the most common and dreaded side effects of cisplatin-based chemotherapy (Marty et al., 1990). Development of 5-hydroxytryptamine receptor antagonists have significantly reduced cisplatin-related acute emesis (Marty et al., 1990).

Clinical Administration

The standard method of administering cisplatin is as a slow intravenous infusion every three to four weeks. Since cisplatin is administered as an aqueous solution, it is therefore essential that ligand substitution be minimized before it reaches the tumor. This is achieved by using isotonic saline which has a relatively high chloride ion concentration (0.9% NaCl = 0.15 M NaCl), thus keeping the chloride ligands on the Pt complex.

Cisplatin is supplied for clinical use as a lyophilized powder in vials that contain 10mg of the drug, a diuretic, usually mannitol, and salt. Reconstitution of the powder is performed with sterile water to a concentration of 1 mg/mL, followed by further dilution with saline (usually 500 mL of 0.9% NaCl, USP) for intravenous (i.v.) administration over 1 to 24 h. Once reconstituted, the solution is kept at room temperature. Patients are hydrated regularly before, during and after cisplatin administration. A minimum of one to two liters of normal saline, and hypertonic saline must be infused, during, pre- and post- administration. Typically, a total dose of 12.5 to 25g of mannitol with or without furosemide (20 mg), is given to increase urine flow (Al-Sarraf et al., 1982), and an antiemetic (5-HT3, antagonists) to reduce the severity of the nausea caused by cisplatin (Marty et al., 1990). Hydration and diuresis can reduce the urinary concentration of cisplatin whereas forced chlorouresis provides high chloride levels in the kidney, thus minimizing aquation of cisplatin in renal tubules. Specific dose of cisplatin will vary from patient to patient (ranging from 20 mg/m^2 to 120 mg/m^2), depending on a number of criteria, which include the disease being treated and the patient's size (usually based on body surface area in m^2).

Pharmacokinetics

The pharmacokinetics of cisplatin are complex and have been studied mainly by using assays for elemental platinum (by atomic absorption spectrometry) or by using preparations of the drug containing radioactive platinum.

Following i.v. infusion of cisplatin peak plasma platinum concentrations occur immediately after the end of the infusion (Vermorken et al., 1986). Following a 1hr i.v. infusion of 50 and 70 mg/m^2 of cisplatin to patients with normal renal function, peak plasma total platinum concentrations of 2.26-2.45 and 4.25-7.02 µg/mL respectively have been reported (McEvoy, 1999). When cisplatin is administered by i.v. infusion over 6 or 24 hours, plasma concentrations of total Pt increase gradually during the infusion (McEvoy, 1999). When equal doses of cisplatin are administered by rapid i.v. infusion or infusions over 2-24 hours, the AUC for non-protein bound Pt appears to be equivalent, suggesting that the AUC is not dependent on the infusion schedule (Reece et al., 1989).

Cisplatin is widely distributed into body fluids and tissue. Highest concentrations are found in the kidney, liver and prostate (Stewart et al., 1982). Lower concentrations are found in bladder, muscle, testes, pancreas, spleen, leukocytes and erythrocytes (Stewart et al., 1982). Platinum accumulates in body tissues following administration of cisplatin and has been detected in many tissues for up to 6 months after the last dose of the drug (Stewart et al., 1982). The volume of distribution for total platinum has been reported to range from 20-80 L/m^2 (McEvoy, 1999). Cisplatin rapidly and extensively binds to tissue and plasma proteins including albumin and gamma-globulins. Binding to tissue and plasma protein appears to be irreversible (van der Vijgh and Klein, 1986). Protein binding increases with time and less than 2-10% of Pt in blood remains unbound several hours after i.v. cisplatin (Vermorken et al., 1986).

Total Pt and non-protein bound Pt have been reported to decline in a bi-phasic manner. In adults the following plasma elimination half-lives have been reported: Total Pt 8.1-49 minutes in the initial phase and 30.5-107 hours in the terminal phase (McEvoy, 1999). Non-protein bound: 2.7-30 minutes in the initial phase and 32-54 hours in the terminal phase (McEvoy, 1999). In children, total Pt elimination of 18.3 minutes in the initial phase and 81.9 hours in the terminal phase and for non-protein bound Pt 16.9 minutes in the initial phase and 59.0 hours in the terminal phase has been reported (Dominici et al., 1989).

The metabolic fate of cisplatin has not been completely elucidated. There is no evidence to suggest that the drug undergoes enzymatic biotransformation (McEvoy, 1999). The activated platinum drug reacts with nucleophilic sites such as cysteine amino acid and GSH. Intact cisplatin and its Pt containing products are excreted mainly in urine (Andersson et al., 1996). There is no evidence of intestinal secretion or fecal elimination of the drug (McEvoy, 1999). Renal excretion of cisplatin appears to occur predominately via glomerular filtration, but there is evidence that secretion and reabsorption of the drug also occurs (McEvoy, 1999).

Carboplatin

***Cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)**, sometimes abbreviated as CBDCA and more commonly known by the generic name carboplatin, an analogue of cisplatin was introduced into clinical trials in 1981 to help overcome some of the toxicities associated with cisplatin (van der Vijgh, 1991). While cisplatin has two chloride "leaving" groups, carboplatin possesses a cyclobutane moiety as its leaving group (fig 1.2b). This leaving group attributes to carboplatin's slower dissociation/activation and hence slower reaction with biological molecules including DNA, however the end product of activation is the same, resulting in identical Pt-DNA adducts as cisplatin (Blommaert et al., 1996; Blommaert et al., 1995; Knox et al., 1986).

The rate constant for aquation of carboplatin in phosphate buffer (pH 7.0) at 37 °C was 100 fold slower than that observed for cisplatin (Knox et al., 1986). Consistent with this finding, 100 fold more carboplatin was required to produce the same amount of Pt-DNA adducts as cisplatin, on isolated plasmid DNA (Knox et al., 1986). However in cells, only a 20 to 40 fold larger dose of carboplatin was needed to produce levels of Pt-DNA adducts equivalent to cisplatin (Knox et al., 1986). When the same amount of Pt-DNA binding was obtained in cells, cytotoxicity was the same for the two compounds, thus indicating the same Pt-DNA adducts are formed by the two drugs. The relatively stronger activity of carboplatin in cells as opposed to that in isolated DNA, may be due to enzymatic activation of carboplatin inside the cell (Blommaert et al., 1995) or activation by cellular thiols (Barnham et al., 1996; Natarajan et al., 1999) such as GSH and MT.

The major site of platination on double-stranded DNA for carboplatin has been identified as interstrand cross-links between two neighboring deoxyguanosines (GG) at 58% of total DNA platination (Blommaert et al., 1995). Similarly, Hongo and colleagues demonstrated that carboplatin induced the same Pt-DNA adducts as those induced by cisplatin (Hongo et al., 1994). Also, the mutational outcome in the Chinese hamster *aprt* gene after exposure to cisplatin and carboplatin has been bound to be identical (de Boer and Glickman, 1992). Additionally, antibodies reacting with Pt-DNA adducts formed by cisplatin recognized adducts formed by carboplatin with similar affinity (Sundquist et al., 1987). This is only a fraction of the evidence supporting the idea that carboplatin has similar mechanisms of action as cisplatin, but with slower activation and reactivity.

Cellular resistance in carboplatin is less well studied. Because carboplatin and cisplatin share the same "activated" intermediate, lead to the same DNA lesions, and hence have the same mechanism of action, they are cross-resistant in most instances (Gore et al., 1989; Lebwohl and Canetta, 1998; Rixe et al.,

1996). It is assumed that similar mechanisms of resistance to cisplatin are involved in carboplatin resistance.

Clinical Pharmacology

Activity

The side effects associated with cisplatin therapy (nephrotoxicity, neurotoxicity) prompted efforts to design a less toxic Pt analogue. It was hypothesized that modification of cisplatin to contain a less labile leaving group could alter toxicity. The search for a less toxic Pt-agent was pursued at the Institute for Cancer Research in the U.K., which led to the development of carboplatin (Harrap, 1995). Using a murine screen for nephrotoxicity, it was found that substitution of more stable ligands for the chloride leaving groups did indeed diminish renal effects, while antitumor activity was retained (Harrap, 1985).

Although carboplatin has a similar mechanism of action and preclinical spectrum of activity to cisplatin, it does not have the same clinical efficacy in all Pt-sensitive tumors. Carboplatin can substitute for cisplatin in the treatment of ovarian cancer (Swenerton et al., 1992). Carboplatin can also be substituted for cisplatin in the treatment of non-small-cell and extensive-stage small-cell lung cancers (Go and Adjei, 1999). Carboplatin is inferior to cisplatin in germ-cell, head and neck, and esophageal cancers and should not be substituted in place of cisplatin for treatment of these cancers (Go and Adjei, 1999).

Toxicity

The major advantage of carboplatin over cisplatin is with respect to toxicity, since carboplatin has reduced nephrotoxicity and reduced ototoxicity compared to cisplatin (Calvert et al., 1989; Egorin et al., 1985; Obermair et al.,

1998). Myelosuppression is the dose-limiting toxicity of carboplatin (Evans et al., 1983). The drug is most toxic to the platelet precursors, but neutropenia and anemia are frequently observed (Evans et al., 1983). The other toxicities of carboplatin, nausea and vomiting, are milder and shorter in duration than those for cisplatin (O'Dwyer et al., 2000).

Clinical Administration

Another advantage of carboplatin over cisplatin is with respect to ease of carboplatin administration, since pre and post-infusion hydration are not required because of the lack of nephrotoxicity at the standard doses of carboplatin. According to the manufacturer, carboplatin powder for injection may be reconstituted with 0.9% NaCl or 5% dextrose to provide solutions containing 10 mg/mL carboplatin. The reconstituted solution may be infused directly or further diluted with 0.9% NaCl injection or 5% dextrose injection to a concentration as low as 0.5 mg/mL. Carboplatin is administered by i.v. infusion over a period of 15 minutes or by continuous infusion up to 24 hours.

Pharmacokinetics

Pharmacokinetics of carboplatin are simpler and more predictable than those for cisplatin. A smaller percentage of carboplatin is protein bound, 20-40% initially with greater portions of the drug bound to plasma proteins at later times (Murry, 1997). Elimination of ultrafilterable platinum (protein free) from plasma is bi-phasic with distribution and elimination half-lives of 6 to 25 minutes and 1.6 to 4.2 hours respectively (Calvert et al., 1982; Harland et al., 1984; Van Echo et al., 1984). Highest concentrations of Pt after administration of carboplatin are found in the kidney, liver, skin and tumor tissue and lower concentrations are found in fat and brain (Boven et al., 1985). Carboplatin undergoes more extensive renal excretion, 71% within 24 hours (van der Vijgh, 1991). There in no evidence of

enzymatic biotransformation of the drug, instead dicarboxylate ligands of the drug are displaced by water forming a charged Pt complex which then reacts with nucleophilic sites on DNA or SH groups of proteins (van der Vijgh, 1991).

The efficacy of carboplatin is dose dependent and is most closely related to the drug exposure in a patient as measured by the area under the plasma concentration – time curve (AUC) (Calvert et al., 1989). Also the clearance of carboplatin is linearly related to GFR (Calvert et al., 1989). Therefore Calvert and colleagues developed dosing formulas to target exposure to carboplatin as follows: $\text{Dose(mg)} = \text{target AUC(mg.min/mL)} \times [\text{GFR(mL/min)} + 25]$ (Calvert et al., 1989). Also a corresponding formula for children has been proposed: $\text{Dose(mg)} = \text{target AUC(mg.min/mL)} + [\text{GFR(mL/min)} + 0.36 \times \text{BW(kg)}]$ (Newell et al., 1993). Both of these formulas were derived using GFR measured directly and accurately with radioactively labeled EDTA. Results using GFR estimation based on creatinine clearance are more variable, but still superior to empirical dosing (Newell et al., 1993). Chatelut and colleagues have provided an alternative formula that does not require GFR or creatinine clearance measurements:

$$\text{Dose (mg)} = \text{target AUC (mg.min/mL)} \times \text{carboplatin clearance (mL/min)},$$

where carboplatin clearance is calculated as follows:

$$= \frac{(0.134 \times \text{weight}) + [218 \times \text{weight} \times (1 - (0.00457 \times \text{age}))] \times [1 - (0.314 \times \text{gender})]}{\text{serum creatinine } (\mu\text{M})},$$

where weight is in kg, age is in years and gender is 0 for males and 1 for females (Chatelut et al., 1995).

Mesna

Sodium 2-Mercaptoethanesulfonate (mesna) (fig. 1.2c) is a synthetic sulfhydryl (thiol) compound that acts as a sulfhydryl donor (Brock et al., 1982). It was developed as a specific chemoprotective agent against urotoxic metabolites

of oxazaphosphorine derivatives (ifosfamide, cyclophosphamide) to prevent or decrease the frequencey and severity of bladder toxicity such as hemorrhagic cystitis and hematuria induced by these drugs (Brock et al., 1982). In the bladder, mesna reacts chemically with the urotoxic metabolites of oxazaphosphorines by binding to the double bond of acrolein (fig. 1.6) and resulting in detoxification of these toxic metabolites (Brock et al., 1982).

The efficacy of mesna as an uroprotective agent has been attributed to its distinctive pharmacokinetic profile (fig. 1.6). Analogous to the physiological cysteine-cystine system, mesna is rapidly oxidized to the chemically stable and pharmacologically inert disulfide metabolite dimesna (disodium-2,2'-dithio-bis-ethane sulfonate) in the systemic circulation (Brock et al., 1982). Subsequently dimesna is reduced to the active drug mesna in the kidney by the enzymes thiol transferase and glutathione reductase (Brock et al., 1982). Mesna is then delivered to the bladder, whereupon the free sulfhydryl group inactivates acrolein (Brock et al., 1982). Because the activity of mesna is restricted to the urinary tract, the systemic activity and the non-urological toxicity of the oxazaphosphorines are not affected, and thus it is possible to administer mesna and ifosfamide or cyclophosphamide simultaneously (Links and Lewis, 1999).

Following an i.v. infusion of mesna, the elimination half-life of mesna in adults is approximately 9 to 11 minutes (Goren et al., 1998). Most of the dose of mesna is eliminated in urine within 4 hours (Goren et al., 1998). Mesna and dimesna are hydrophilic so they remain mainly in the intravascular compartments (Shaw et al., 1986). Studies in the rat indicate approximately 10% of circulating mesna/dimesna bound to plasma proteins (Ormstad et al., 1983). Both mesna and dimesna pass unchanged through the hepatic vasculature. They are not taken up into the liver cells or excreted in bile (Shaw et al., 1986). In the kidney, dimesna is subject to glomerular filtration and is subsequently re-absorbed (Ormstad and Uehara, 1982). Reduction to the active thiol takes place in the renal tubular epithelium (Ormstad and Uehara, 1982).

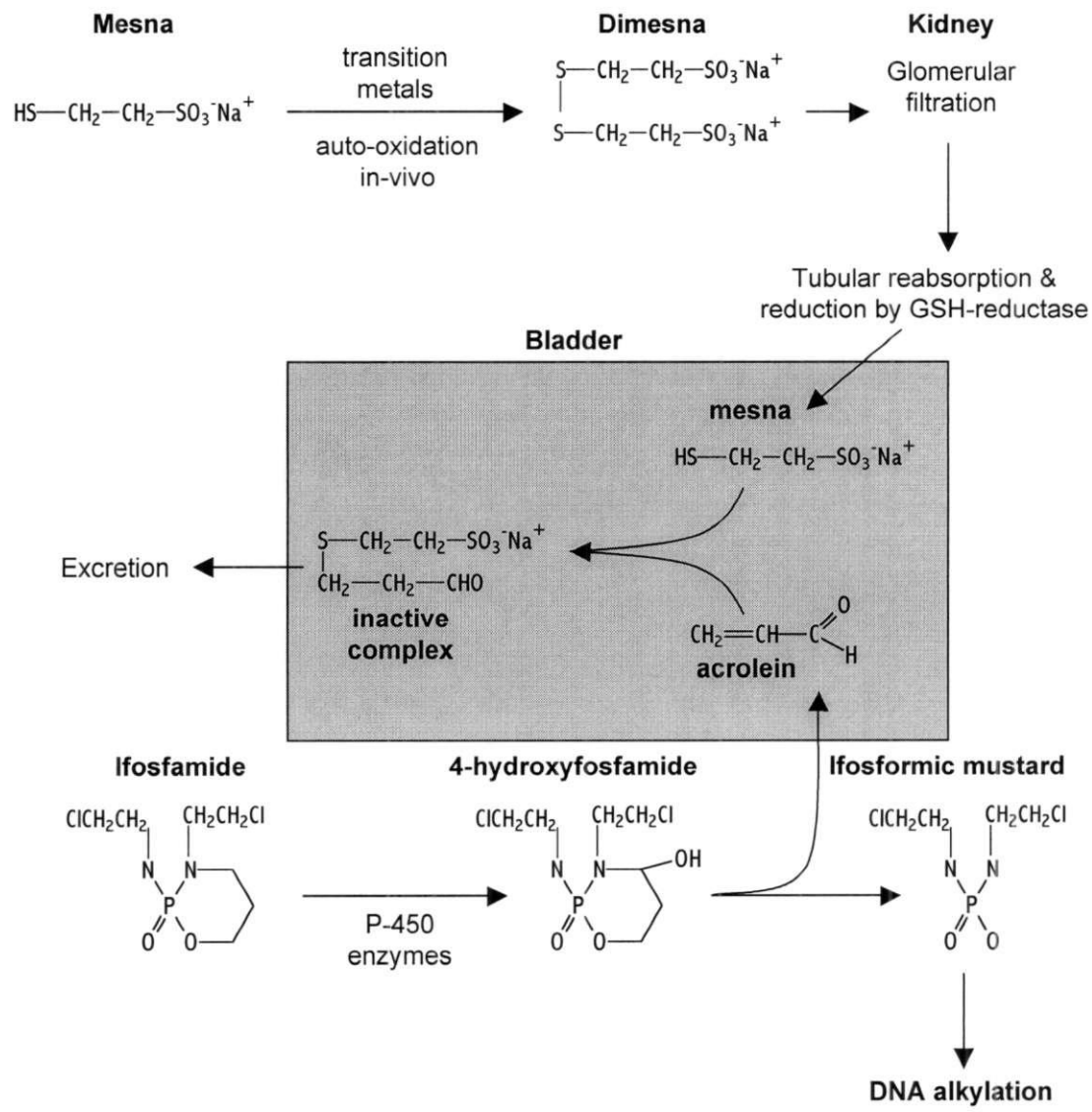


Figure 1.6. Mesna distribution and inactivation of ifosfamide toxic metabolite in the bladder (modified from Dorr, 1991).

In patients receiving oxazaphosphorines the total daily i.v. dosage of mesna is generally equivalent to 60-160% of the total daily dosage of the oxazaphosphorine agent, depending on the OAP agent (Dorr, 1991; Goren et al., 1998; James et al., 1987). The manufacturer recommends to divide this into three equivalent doses with the first dose given 15 minutes before the oxazaphosphorine and the other mesna doses at four and eight hours after the oxazaphosphorine agent.

Cyclophosphamide

2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide monohydrate (cyclophosphamide) is a nitrogen-mustard derivative, a chemotherapeutic alkylating agent. Cyclophosphamide is a prodrug and requires activation by the P-450 enzyme in the liver to the active compound 4-hydroxy-cyclophosphamide (Moore, 1991). The activated drug functions as an alkylating agent, interfering with DNA replication and transcription (Moore, 1991). Important side effects of the drug include myelotoxicity, gastrointestinal toxicity and hemorrhagic cystitis (Moore, 1991). The agent is given together with mesna, which antagonizes the hemorrhagic cystitis.

Ifosfamide

3-(2-chloroethyl)-2 [(2-chloroethyl) amino] tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide (ifosfamide), is another oxazaphosphorine alkylating agent, structurally related to cyclophosphamide (Kerbusch et al., 2001). Ifosfamide also requires biotransformation in the liver by the cytochrome P-450 system before it can exert its cytotoxic effects (Kerbusch et al., 2001). By controlling the severe hemorrhagic cystitis observe with ifosfamide, mesna has enabled widespread use of this alkylating agent (Kerbusch et al., 2001).

Combinations of Platinum-drugs with oxazaphosphorines

Combinations of cisplatin with cyclophosphamide are used increasingly in chemotherapeutic protocols for ovarian cancer (Shapiro et al., 1998; Wils et al., 1999), non-small cell lung carcinoma (Ueoka et al., 1998; Urban et al., 1999), and testicular tumors (Culine et al., 1997; Logothetis et al., 1987). Alberts and colleagues reported that patients with stage III ovarian cancer who received intraperitoneal cisplatin and i.v. cyclophosphamide appeared to have improved survival and fewer toxic effects than those receiving i.v. cisplatin and i.v. cyclophosphamide (Alberts et al., 1996). This could be due to higher inactivation of cisplatin by mesna not taken into account with respect to cisplatin via the i.v. route as compared to the IP route.

Cisplatin and ifosfamide, and hence mesna are also commonly used in combination chemotherapeutic protocols. Some of these include: ovarian cancer (Araujo et al., 1991; Polyzos et al., 1999), cervical carcinoma (Cervellino et al., 1995; Leone et al., 1996), and non-small cell lung carcinoma (Graziano et al., 1996; Miller et al., 1995). Advantages of combination protocols over cisplatin alone protocols could not be found in non-small lung cancer by Graziano and colleagues (Graziano et al., 1996). A potential reason for this observation might have been due to the inactivation of cisplatin by mesna, which is always given with ifosfamide but not taken into account with respect to cisplatin.

Carboplatin has been used alone or in combination with other chemotherapeutic agents. Combinations of carboplatin with ifosfamide (Fetscher et al., 1999; Gerke et al., 2000; Margolin et al., 1996), and combinations of carboplatin with cyclophosphamide (Edmonson et al., 2001; Polyzos et al., 1999; van Warmerdam et al., 1996) have become popular and are also used in children (Kreissman et al., 1997; Meyer et al., 2001). However, the interaction of carboplatin with mesna in these combination protocols has not been addressed.

Interactions of Platinum-drugs with mesna

Thiols such as mesna exhibit a high nucleophilic reactivity toward Pt (II) complexes (Howe-Grant and Lippard, 1980). In aqueous solution mesna and dimesna react with cisplatin and its aquated form (Leeuwenkamp et al., 1991). Cisplatin concentration of 0.2 mM was incubated with typical plasma concentrations of mesna and dimesna (5 mM & 3 mM respectively) at pH 7.4. The half-lives for cisplatin under these conditions were found to be 150 and 629 minutes as a result of reacting with mesna and dimesna respectively (Leeuwenkamp et al., 1991). When the same experiment was conducted with 0.2 mM of the cisplatin monoaqua, the half-life for this species was found to be 18 and 60 minutes when it reacted with mesna and dimesna respectively (Leeuwenkamp et al., 1991). A cisplatin concentration of 0.2 mM was incubated with typical urine concentrations of mesna and dimesna (500 mM each) at pH 5.3. The half-lives for cisplatin under these conditions were found to be 5.3 and 5.8 minutes when it reacted with mesna and dimesna respectively (Leeuwenkamp et al., 1991). The same experiment was conducted with 0.2 mM of monoaqua cisplatin, the half-life for this species was found to be less than 1 minute in each case (Leeuwenkamp et al., 1991).

Williams and Lokich reported that a dilution of 1 mg/mL carboplatin or 0.02 mg/mL cisplatin with 1 mg/mL mesna resulted in the rapid degradation of cisplatin and carboplatin (Williams and Lokich, 1992). The results of this study indicated that carboplatin appears to exhibit instability and compatibility patterns similar to those of cisplatin, and therefore should not be stored in the presence of mesna like nucleophilic compounds (Williams and Lokich, 1992).

Obrocea and colleagues compared *in vitro* interaction of mesna with carboplatin in aqueous solution, human plasma, and urine (Obrocea et al., 1998). Contrary to the data of Williams and Lockich (Williams and Lokich, 1992) Obrocea and colleagues reported that incubation of carboplatin with mesna in human plasma up to eight days did not result in a statistically significant

interaction (Obrocea et al., 1998). They therefore concluded that it is unlikely that mesna would substantially affect the pharmacokinetics of carboplatin when both agents are given together to patients as part of combination chemotherapeutic regimens (Obrocea et al., 1998). However this conclusion might be incorrect since carboplatin needs to be activated intracellularly, therefore this interaction needs to be investigated in a living system such as cells.

Mesna reduced the cytopathogenic effect of cisplatin in BT-20 human mammary carcinoma cells (Sauter et al., 1986). Wolff and colleagues investigated the interaction of mesna with cisplatin and carboplatin in cell culture (Wolff et al., 1998). Mesna protected human malignant glioma cells from cytotoxic effects of both cisplatin and carboplatin (Wolff et al., 1998), however the mechanism of this protection was not investigated.

Animal models have been used previously for understanding the interaction between cisplatin and mesna. The researchers have tried to answer three questions: (1) Does mesna reduce cisplatin induced toxicity (nephrotoxicity and gastrointestinal toxicity)?; (2) Does mesna protect against lethal concentrations of cisplatin?; (3) Does mesna protect tumors from cisplatin?

The idea to use mesna as a chemoprotective agent against cisplatin comes from the chemical reactivity of mesna toward cisplatin. However the possibility of tumor protection is the most important adverse outcome associated with this idea and therefore it needs to be investigated.

Millar and colleagues (Millar et al., 1985) investigated mesna's ability to reduce cisplatin induced nephrotoxicity in healthy (non-tumor bearing) rats. The animals received the maximally tolerated dose of cisplatin (6.5 mg/kg) i.v. via the tail. A single dose of mesna (300 mg/kg) was given immediately after cisplatin i.p. or the same dose i.p. on three occasions at 0,2, and 4 hours after cisplatin administration. On day 4, rats where anesthetized and blood (2-5 mL) was removed via cardiac puncture. Urea levels in the plasma were used for determination of nephrotoxicity. There was no evidence for protection against

nephrotoxicity when this drug combination was examined in the rat (Millar et al., 1985).

Allan and colleagues (Allan et al., 1986) investigated protective effect of mesna on the gastrointestinal toxicity, lethality, and tumor protection of cisplatin in CBA mice. To examine mesna's effect on preventing gastrointestinal toxicity, cisplatin was administered at maximally tolerated doses 10 mg/kg i.p. and mesna was given at 400 mg/kg at –2, 0, and +2 hours after cisplatin. Mice were sacrificed by cervical dislocation on days 1,3,5,7, and 10. A section of ileum was taken and examined for gastrointestinal toxicity. Mesna caused a significant reduction in the gastrointestinal toxicity of cisplatin (Allan et al., 1986).

Lethality of cisplatin was investigated in mice given a lethal dose of cisplatin, 15 mg/kg i.p. alone or cisplatin plus mesna (mesna doses as before) prior to or immediately after cisplatin (Allan et al., 1986). Administration of mesna prior to or immediately following this 67% lethal dose of cisplatin protected 87-100% of the animals from the lethal effects (Allan et al., 1986).

To test whether mesna influences the therapeutic efficacy of cisplatin, $1x10^6$ L1210 cells (mouse leukemia cells) were injected into mice and drugs administered (doses as above) the following day. Survival was assessed by the 30^{th} day. The antitumor efficacy of cisplatin in L1210 leukemia bearing mice was not affected by co-administration of mesna (Allan et al., 1986).

Dorr and Lagel investigated the interaction between cisplatin and mesna in mice with respect to lethal concentrations and tumor efficacy of cisplatin (Dorr and Lagel, 1989). CD-1 adult male mice were observed for survival over a 60 day period after a lethal dose of cisplatin, and compared to a cisplatin plus mesna group. Mesna was found to protect CD-1 mice from high dose cisplatin lethality (Dorr and Lagel, 1989). DBA/2J mice bearing the P-388 leukemia (10^6 cells i.p. 24 h before cisplatin) were observed for survival over a 60 day period after appropriate drug treatments in each group. Mesna mixed directly with cisplatin reduced cisplatin's antitumor effects in DBA/2J mice, but when mesna was

administered 5 minutes after cisplatin it did not reduce cisplatin's antitumor efficacy (Dorr and Lagel, 1989).

Wagner and colleagues also investigated effects of mesna on the lethality and antitumor activity of cisplatin (Wagner et al., 1988). They used the lethality model in healthy non-tumor bearing female NMRI mice, DBA2 mice, and female Wistar rats. For antitumor activity of cisplatin they used the L-1210 leukemia model (10^5 cells injected into DBA2 mice 24 hours before drug treatments). Again, end points for both assays were survival in days. Mesna did not protect L-1210 leukemia from the antitumor effect of cisplatin, but it did protect against lethal concentrations of cisplatin (Wagner et al., 1988).

In summary of these animal models, in one study mesna did not offer protection against nephrotoxicity of cisplatin, but in a different study it did offer protection. These conflicting results could be due to the different assays chosen to measure nephrotoxicity, plasma urea levels vs. plasma creatinine levels. In all of the studies mesna protected the animals from lethal concentrations of cisplatin. In all of the studies mesna did not reduce cisplatin's antitumor efficacy, except in one study where mesna was mixed with cisplatin in the same solution.

It is well known that cisplatin is not particularly useful in the treatment of leukemia (Dorr and Lagel, 1989), yet all of these studies used leukemia as a model for efficacy of cisplatin combined with mesna. I think the interaction between mesna and cisplatin should be investigated in an experimental solid tumor model *in vivo*.

Thiosulfate (an agent similar to mesna) has been successfully used in reducing renal toxicity associated with cisplatin (Howell et al., 1983). The direct binding of cisplatin to the sulfhydryl group of thiosulfate in renal tissue (Elferink et al., 1986) accounts for this protection and also explain why the area under the serum-concentration vs. time curve (AUC) for cisplatin is decreased when thiosulfate is co-administered (Goel et al., 1989; Howell et al., 1983). This phenomenon could also be possible with mesna, since like thiosulfate mesna

also exhibits a reactive sulfhydryl group toward Pt-drugs, however, such clinical pharmacokinetic interactions have never been investigated.

Rationale and Hypotheses

Among many mechanisms of drug resistance described *in vitro*, at levels of resistance >40-fold over baseline, increased levels of cellular thiols appear to take primary importance (Godwin et al., 1992). A number of studies have suggested that competing reactions can change the reactivity of Pt-drugs in the cellular environment based on the capture of the reactive Pt species by sulfhydryl compounds (Bose et al., 1997; Dedon and Borch, 1987; Natarajan et al., 1999). The effects of such competing reactions with mesna as the sulfhydryl donor has not been investigated, and the outcome could be different for cisplatin and carboplatin. Mesna is a sulfhydryl containing compound that is highly reactive toward cisplatin (Leeuwenkamp et al., 1991) and less reactive toward carboplatin (Obrocea et al., 1998). The presence of mesna in cells at the same time as Pt-drugs might be synergistic to mechanisms of resistance by cellular thiols. It is not known if mesna, similar to cellular thiols, leads to reduced Pt-DNA binding. This result could be important since it was shown that mesna protected glioma cells from the cytotoxicity of Pt-agents (Wolff et al., 1998).

Considering the background information presented in this chapter I **hypothesize** that :

(a) Sodium 2-Mercaptoethanesulfonate (mesna), because of its sulfhydryl group and based on the two chloride leaving groups on cisplatin, should react with *cis*-diamminedichloroplatinum(II) (cisplatin) in a molar ratio of 2 to 1. Also, mesna should react with *cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II) (carboplatin) in a 2 to 1 molar ratio, since the end product of carboplatin's reaction has been shown to be the same as cisplatin's;

(b) based on mesna's fast reaction rate with cisplatin but slow reaction rate with carboplatin, mesna may reduce Pt-DNA binding of cisplatin but not that of carboplatin; and

(c) Similarly, based on mesna's reaction rate with the Pt-drugs, and mesna's short half-life in patient's serum, mesna may reduce serum Pt levels of cisplatin but should not affect carboplatin's pharmacokinetics in patients.

Specific Aims

In order to address the above hypotheses, this thesis investigated the following questions:

(1) What is the stoichiometry of the reaction between mesna and the Pt-drugs?

(2) Does mesna reduce the Pt-DNA binding of Pt-drugs in cells? and if so, does this reduction hold true *in vivo*?

(3) Does mesna influence the pharmacokinetics of Pt-drugs in patients?

The overall recommendation based on the answers to these questions will define the drug delivery kinetics and physical administration of mesna with respect to Pt-drugs (as opposed to only in relation to oxazaphosphorines).

Chapter Two: Chemical Reaction Between Mesna and the Platinum Drugs

Methods

Reagents

Drugs; cisplatin injection 1 mg/mL, Faluìding Canada Inc. (Vaudreuil, Quebec), carboplatin injection 10 mg/mL, Novopharm Ltd. (Toronto, ON), and Uromitexan (mesna) injection 100 mg/mL, Bristol-Myers Squibb Canada Inc. (Montreal, Quebec), were kindly provided by Oncology pharmacy, Southern Alberta Children's Hospital (Calgary, AB). Stock cisplatin and carboplatin were stored at room temperature and protected from light. Diluted samples of cisplatin and carboplatin were made fresh daily in 0.15 M NaCl. Mesna stock was stored at 4°C, and its diluted samples were made fresh daily in 0.15 M NaCl. Chemical agents were purchased from Sigma-Aldrich (Canada) unless otherwise specified. MTT dye was dissolved in PBS (0.2 M NaCl, 0.027 M KCl, 0.010 M Na_2HPO_4, 0.0018 M KH_2PO_4, pH 7.4) as a stock solution of 5 mg/mL and stored at 4°C, protected from light. All water used was de-ionized by Millipore Milli-Q system (Millipore Corporation, Nepean ON).

Colorimetric determination of mesna

The concentration of mesna was determined by an oxidation/reduction reaction between mesna and (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; (MTT) dye as previously described (Oprea et al., 2001).

Briefly, in this reaction mesna is oxidized to dimesna and yellow MTT dye is reduced to a bluish-purple product which can be measured spectrophotometrically at 600 nm. Mesna calibration curves were generated by adding 100 µL aliquots/ well for various mesna concentrations to a 96-well plate

containing 100 µL of MTT/ well. Absorbance was read 20 minutes after addition of mesna samples to MTT dye, upon development of bluish-purple precipitates, using a Beckman Biomek 1000 automated workstation with a 600 nm photometer plate reader, (Beckman Instrument Inc., Fullerton CA). The calibration range was 0.4 – 7.1 mM mesna, and the curves were linear (r^2 = 0.98 to 0.99) between 0.7 to 7.1 mM mesna. The lower limit of detection was 0.7 mM mesna (fig. 2.1).

Determination of Drug Kinetics

Reaction kinetics between mesna and the Pt-drugs were determined based on loss of mesna vs. time. The 10.0 mL incubation solutions contained either cisplatin 3.3 mM or carboplatin 3.3 mM and mesna (0.7,1.4, 2.8, 3.5, & 7.1 mM) in 0.15 M NaCl, pH 7.4. These are the concentrations present in infusion bags/lines for cisplatin and carboplatin respectively (Elferink et al., 1986) and in plasma to infusion bag range for mesna (Leeuwenkamp et al., 1991; Obrocea et al., 1998).

Mesna was added to the Pt-drug at time zero. All samples were incubated in the dark at room temperature (22 °C ± 2 °C). At t = 5.0 s and pre-selected time intervals, 100 µl aliquots were taken from the reaction mixture and immediately analyzed using the colorimetric assay. All measurements were performed at least in quadruplicate.

Analysis of reaction kinetics

If the disappearance of mesna obeys the first order rate law (1):

$$\frac{-d[mesna]}{dt} = k_{obs} \cdot [mesna] \qquad (1)$$

$$\ln[mesna] = \ln[mesna]_{t0} - k_{obs} \cdot t \qquad (2)$$

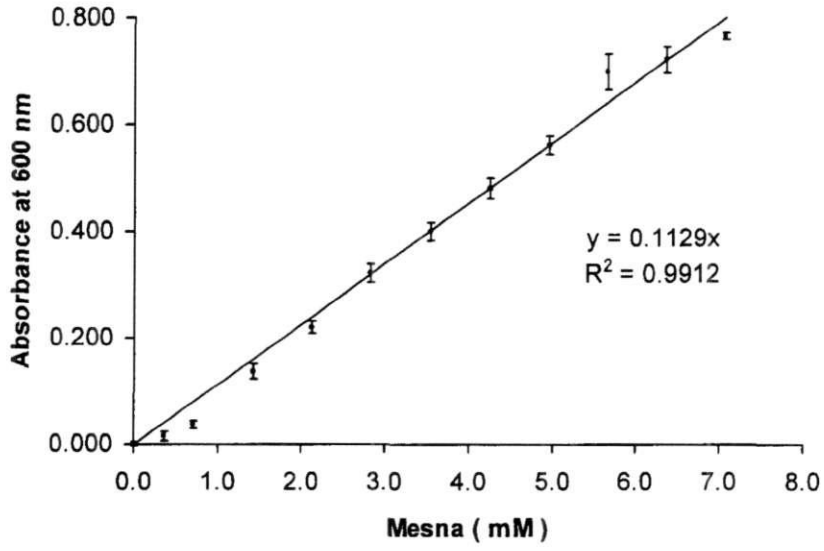


Figure 2.1. Mesna calibration curve. Absorbances were read 20 minutes after addition of mesna calibrators to MTT dye. Each point is the mean of 8 replicates with error bars as SDs.

where k_{obs} is the observed (pseudo) first-order constant for loss of mesna, and integration yields equation (2), then according to equation (2), semi-logarithmic plots of measured mesna concentration vs. time will be linear. Half-life of mesna was calculated by: $t_{1/2} = \ln 2/k_{obs}$, where k_{obs} was obtained from slope of the line. The plots were fitted using the linear least-squares method in Microsoft Excel.

Mesna – cisplatin reaction rates were bi-exponential and were analyzed as follows: The slope of the second slower process yielded a pseudo first-order rate constant. This line was extrapolated back to the y-axis, mesna concentration of the faster process subtracted from this line and the resultant differences were plotted on the same semi-logarithmic mesna concentration vs. time curve. The slope of the resultant line defined the initial faster pseudo first-order rate constant of the reaction.

Stoichiometry of mesna binding Pt-drugs

Four different concentrations of mesna (0.7, 1.4, 3.5, 7.1 mM), each in an independent experiment were added to eight different concentrations of cisplatin (range: 0, 0.07 mM to 3.3 mM) in 10.0 mL reaction mixtures at 22 °C. The reactions were followed to completion for more than seven half-lives as determined from kinetics studies. Controls included, mesna alone, and cisplatin alone, for the entire duration of the experiment. All of the samples were measured at least in triplicate, and in at least three independent experiments.

For mesna - carboplatin stoichiometry however, this method did not produce reliable and reproducible results. This was because mesna reacted very slowly with carboplatin, and the rate of this reaction was dependent on mesna concentration. It was discovered that 20% dimethylsulfoxide (DMSO) speeds up the rate of reaction between mesna and Pt compounds. Hence a single excess concentration of mesna at 14 mM was reacted with 3.3 mM of carboplatin and loss of mesna followed over time until no more mesna reacted with carboplatin or there was no more carboplatin left (since mesna was in excess).

Controls included, mesna plus 20% DMSO, carboplatin plus 20% DMSO, and all of the calibrators made in 20% DMSO. Mesna concentrations were determined by the colorimetric assay. Absorbance was read at 600 nm, 10 minutes after addition of MTT dye, upon development of bluish-purple precipitates.

Statistical Analysis

The data were summarized as means ± standard deviations (SD) where appropriate. Results among the half-lives of reactions at various concentrations of mesna were evaluated by the one-way analysis of variance (ANOVA). The level of significance was taken as $P \leq 0.05$ in all of the tests.

Results

Reaction kinetics of mesna and cisplatin

Before titration reactions for determination of stoichiometry of reaction between mesna and the Pt-drugs were performed, basic kinetic experiments were conducted to provide some estimates of reaction times required for complete reaction of mesna with each of the Pt-drugs.

Figure 2.2 represents a typical semi-logarithmic plot of mesna concentration versus time. As indicated from this plot, the reaction of mesna with cisplatin was bi-exponential, and two kinetic steps were resolved (as described under methods) which obeyed pseudo first order kinetics.

Table 2.1 summarizes the kinetics for reaction of 3.3 mM cisplatin with 1.4 mM to 7.1 mM mesna. The faster initial phase of mesna disappearance was independent of the mesna concentration (ANOVA, P = 0.2609), with an average half-life of: $t_{1/2\ initial}$ = 16.7 ± 7.4 minutes. The slower second phase was best described by the power function (r^2 = 0.91): Half-life = 6916/[mesna]^2, hence

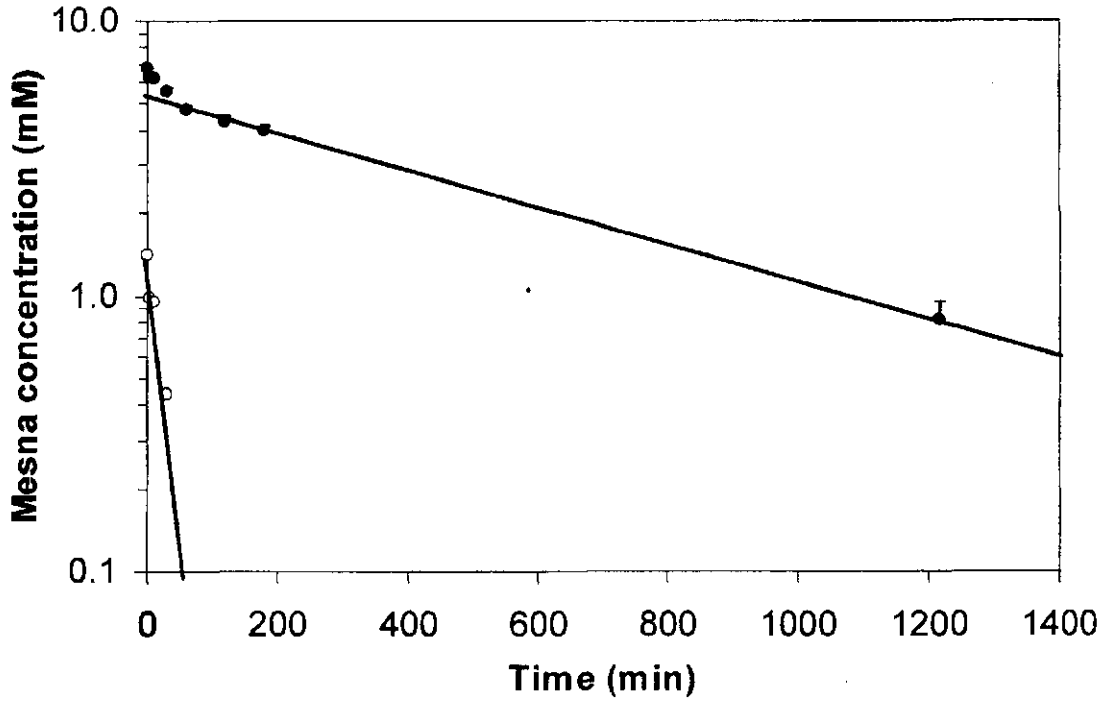


Figure 2.2. Typical bi-exponential kinetics of mesna in reaction with cisplatin. In this case, during the reaction of 7.1 mM mesna with 3.3 mM cisplatin, pH 7.4, 22 °C, in 0.15 M NaCl, 10.0 mL total reaction volume. Solid circle, terminal phase of mesna's disappearance. Open circles, initial phase of mesna's disappearance, determined from the resultant difference of the actual data points and the extrapolated terminal phase. Mean and SDs of 4 replicates in one experiment is shown. Where error bars can't be seen, they were too small to appear on the log-scale.

Table 2.1. Reaction kinetics of various mesna concentrations with 3.3 mM cisplatin

Mesna (mM)	$t_{1/2\ initial}$ (min) *	$t_{1/2\ terminal}$ (min) *	$t_{1/2\ terminal}$(min.mM^2) ‡
1.4	6.5 ± 3.5	4621 ± 3267	1.3 ± 0.9
2.8	22.5 ± 7.8	1362 ± 525	1.5 ± 0.6
3.5	22.0 ± 18.4	495 ± 223	0.9 ± 0.4
7.1	16.0 ± 0.1	325 ± 153	2.4 ± 1.1
mean ± SD †	16.7 ± 7.4	1700 ± 1999	1.5 ± 0.6
P-value	0.2609	0.0428	0.2078

The reaction conditions were: pH 7.4, 22 °C, in 0.15 M NaCl. P-values based on one-way ANOVA.

* Mean and SD of three independent experiments

†Mean and SD of the four mesna concentrations

‡Obtained using the relationship: $t_{1/2\ terminal} = 6916/[Mesna]^2$

mesna's half-life appeared to be inversely proportional to the square of the mesna concentration (ANOVA, P = 0.0428). The average normalized half-life of mesna using this relationship was $t_{1/2\ terminal}$ = 1.5 min.mM^2. However this was misleading, due to the fact that at lower mesna concentrations all of the mesna had already reacted with cisplatin, therefore there was no more mesna left for the subsequent phase of the reaction.

In summary, the overall rate of the reaction in these series of experiments were pseudo first-order. The reaction rates were zero-order with respect to mesna. An average half-life of 400 minutes was estimated based on the average terminal half-lives of 3.5 mM and 7.1 mM mesna reactions with 3.3 mM cisplatin. Subsequent titration experiments were conducted over a minimum of seven half-lives of this value (i.e. 7 X 400 min = 2800 min).

Reaction kinetics of mesna and carboplatin

Linear semi-logarithmic plots of mesna absorbance vs. time were obtained (r^2 = 0.78 to 0.90) for the reaction of 0.7 mM to 7.1 mM mesna with 3.3 mM carboplatin (fig. 2.3). Loss of mesna was mono-exponential and appeared to be dependent on mesna concentration (P < 0.0001). The relationship between mesna's half-life and mesna's concentration was best described by the power function (r^2 = 0.97): Half-life = 150[mesna]^2, hence mesna's half-life appeared to be directly dependent on the square of the mesna concentration. The average normalized half-life of mesna using this relationship was $t_{1/2\ terminal}$= 1.0 min/mM^2. Table 2.2 summarizes the kinetic parameters of these reactions.

Stoichiometry of mesna binding cisplatin

Four different concentrations of mesna (0.7, 1.4, 3.5, 7.1 mM), each in an independent experiment were added to eight different concentrations of cisplatin

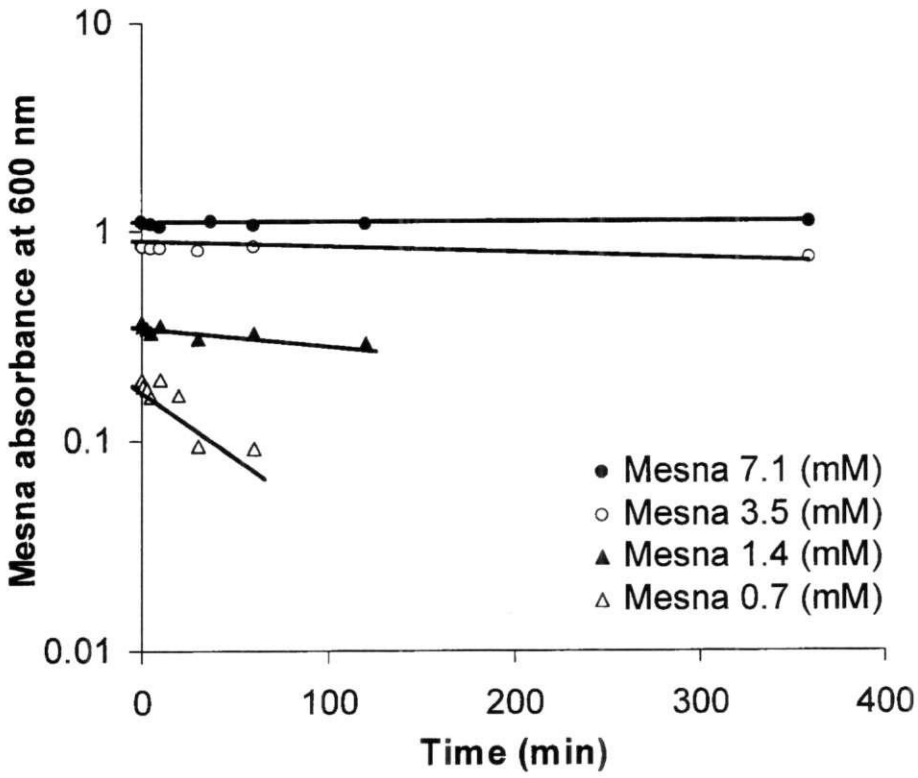


Figure 2.3. Semi-logarithmic plot of mesna abosrbance vs. time. Obtained during the reaction of four different mesna concentrations with 3.3 mM carboplatin, pH 7.4, 22 ºC, in 0.15 M NaCl. Each point is the mean of 4 replicates in one experiment. Error bars were too small to appear on the logarithmic scale.

Table 2.2. **Reaction kinetics of various mesna concentrations with 3.3 mM carboplatin**

Mesna (mM)	$t_{1/2\ terminal}$ **(min)** *	$t_{1/2\ terminal}$ **(min/mM^2)** ‡
0.7	52 ± 1.4	0.7 ± 0.1
1.4	443 ± 67.2	1.5 ± 0.2
3.5	2094 ± 419	1.1 ± 0.2
7.1	5614 ± 1683	0.7 ± 0.2
mean ± SD †	2051 ± 2535	1.0 ± 0.4
P-value	< 0.0001	< 0.0001

The reaction conditions were: pH 7.4, 22 °C, in 0.15 M NaCl. P-values based on one-way ANOVA.

* Mean and SD of four independent experiments

†Mean and SD of the four mesna concentrations

‡Obtained using the relationship: $t_{1/2\ terminal} = 150[Mesna]^2$

(range: 0, 0.07 mM to 3.3 mM). Figure 2.4a summarizes the results of titration experiments as a plot of mesna absorbance vs. cisplatin concentrations.

The stoichiometry for the mesna/cisplatin reaction determined at molar ratios (mesna/cisplatin) of 0.4 to 7.0 is shown in figure 2.4b. Percent mesna loss decreased with increasing mesna/cisplatin molar ratio. Maximum % mesna loss was maintained at mesna/cisplatin molar ratios below 2. Hence the endpoint of the titration was at the ratio of 2 mesna per mole of cisplatin, indicating that 2 moles of mesna are involved in the reaction with 1 mole of cisplatin.

Stoichiometry of mesna binding carboplatin

As the kinetics results for the reaction between mesna and carboplatin indicated: 1. Mesna reacted very slowly with carboplatin, and 2. The rate of this reaction was dependent on mesna concentration. Due to these experimental difficulties multiple titration reactions as done for mesna and cisplatin could not be done for mesna and carboplatin. Instead a single excess concentration of mesna at 14.2 mM was reacted with 3.3 mM of carboplatin in 20% DMSO, monitored over 48 hours (fig. 2.5).

DMSO increased the rate of the reaction substantially. Within seconds mesna concentration dropped to a plateau, which remained relatively stable by endpoint of data collection at 48 h.

The average value of mesna concentration within this plateau was 11.5 ± 0.2 mM. Based on this the stoichiometry of the reaction was carboplatin/mesna molar ratio = 1, since 3.3 mM carboplatin completely reacted with 2.7 mM mesna.

As a check for stoichiometry reported for mesna/cisplatin titration, 14.2 mM mesna was reacted with 3.3 mM cisplatin in 20% DMSO (fig 2.5). Similar to carboplatin reaction, mesna concentration dropped to a plateau within 30 s, which remained relatively stable by endpoint of data collection at 48 h.

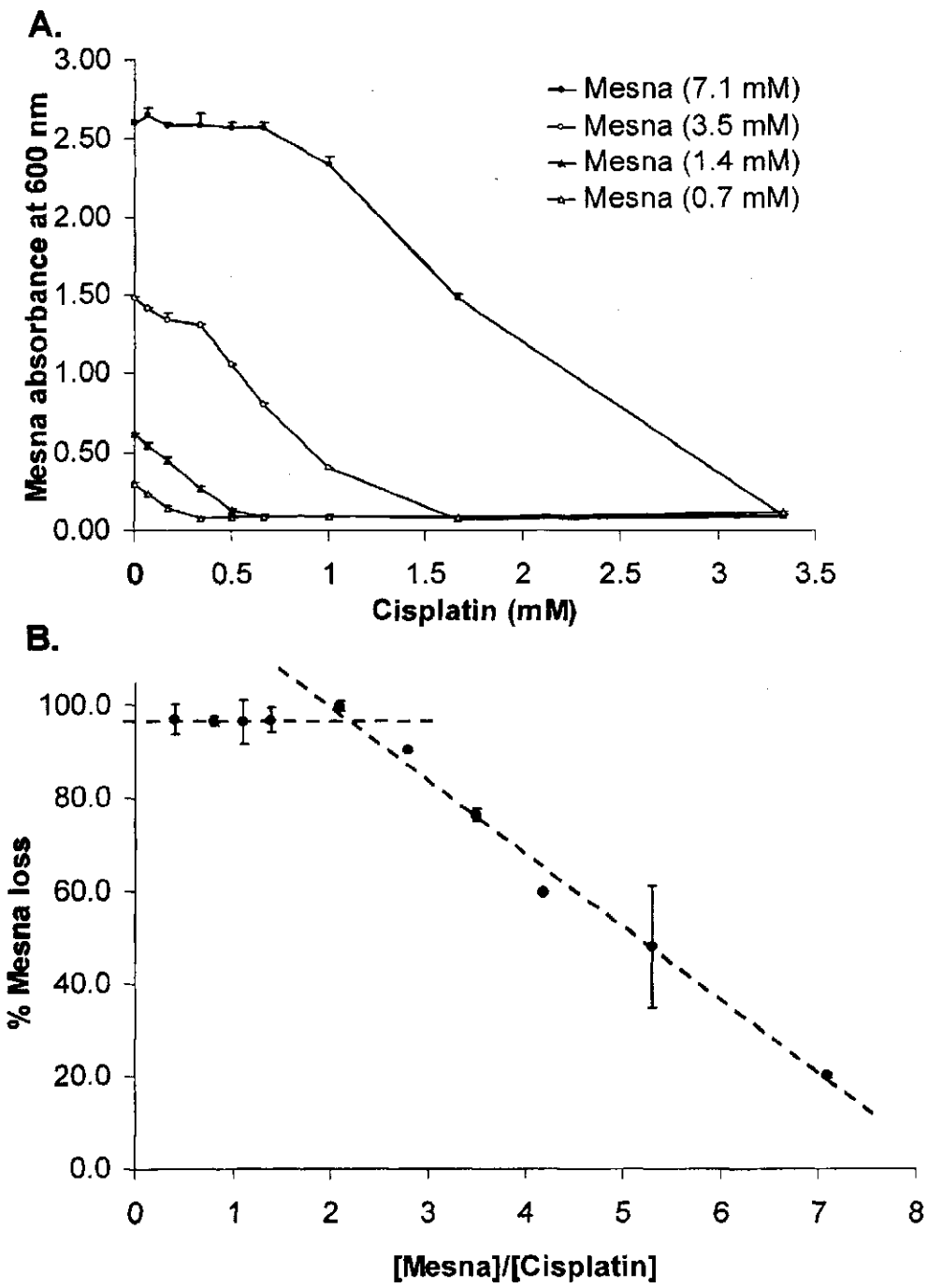


Figure 2.4. Titration of mesna with cisplatin. A. Four different mesna concentrations were titrated with various cisplatin concentrations, pH 7.4, 22 °C, in 0.15 M NaCl, for 72 hours which exceeded seven half-lives of mesna's disappearance. **B.** Molar ratios from the results in A were plotted against % mesna loss, which were determined based on lowest level of mesna detected at each of the mesna concentration levels (taken as 100% at the end of the titration). Mean and SDs are from the four independent experiments, where there are no error bars, they were too small to appear on the scale.

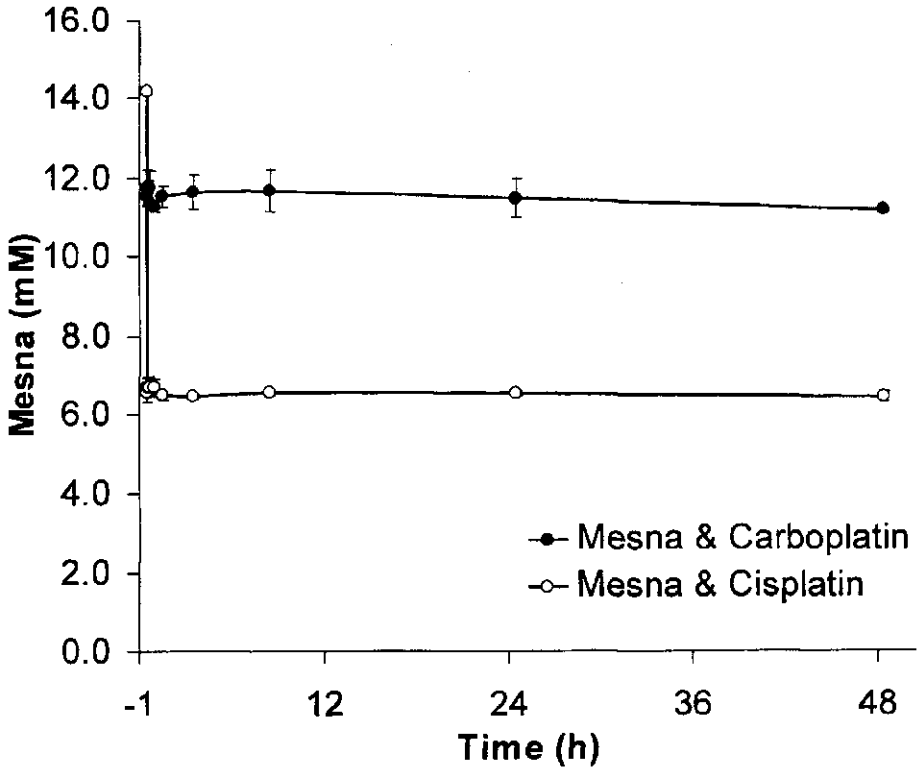
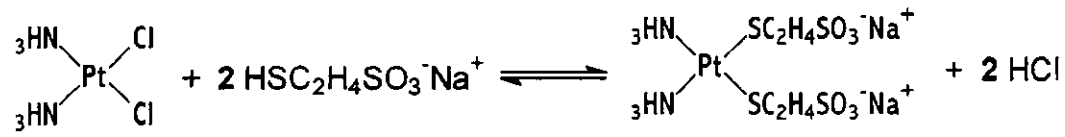


Figure 2.5. Stoichiometry of mesna binding the Pt-drugs determined from time course reactions in 20% DMSO. The amount of mesna which completely depleted all of the Pt-drug was determined based on 14.2 mM mesna at time zero minus the mean of the mesna concentrations at the plateau. Each point represents the mean of 3 independent experiments with error bars as SDs. Reaction conditions were: pH 7.4, 22 °C, 20% DMSO in 0.15 M NaCl, 14.2 mM mesna at time zero and 3 mM of each Pt-drug in a 10.0 mL total reaction volume.

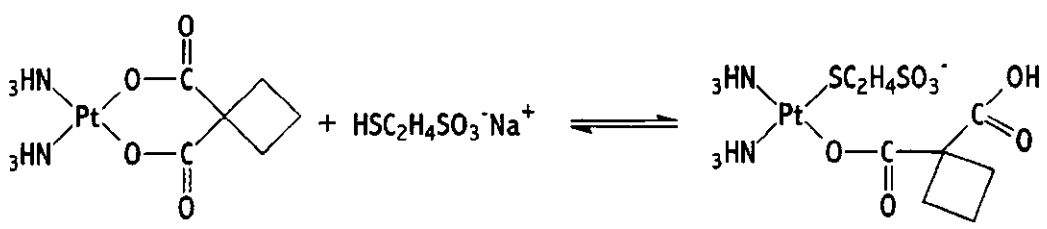
The average value of mesna concentration within this plateau was 6.6 ± 0.1 mM. Based on this the stoichiometry of the reaction was mesna/cisplatin molar ratio = 2, since 3.3 mM cisplatin completely reacted with 7.6 mM mesna, confirming the result from mesna/cisplatin titration reactions in figure 2.4.

Discussion

The purpose of series of experiments in this chapter was to determine stoichiometry of reaction between mesna and Pt-drugs in order to give us a better understanding of inactivation of Pt-drugs by mesna *in vitro*. It was found that at conditions where mesna and cisplatin might come into contact with one another, in an infusion bag or an infusion line, two moles of mesna react with one mole of cisplatin. The proposed balanced chemical equation is (equation 1):



Similarly, the stoichiometry of mesna in reaction with carboplatin was elucidated. It was found that one mole of mesna reacts with one mole of carboplatin. A possible chemical equation for this reaction is (equation 2):



The results presented here support the hypothesis that two moles of mesna react with one mole of cisplatin, but reject the hypothesis that mesna reacts with carboplatin in the similar molar ratio.

Although the proposed cisplatin-mesna product in equation (1) was not isolated and identified, based on the 2:1 mesna/cisplatin stoichiometry obtained

and the reactivity of sulfhydryl groups toward cisplatin, it is a reasonable possibility. Chloride ions are the leaving groups on the cisplatin complex (Howe-Grant and Lippard, 1980). In general a nucleophile may bind cisplatin either by direct chloride displacement or via an aqua-intermediate (Zwelling, 1988). The direct pathway is possible by thiol compounds (Zwelling, 1988) similar to mesna.

Similar cisplatin-thiol metabolites as the one in equation (1) have been identified for the reaction of cisplatin with glutathione (GSH) (Ishikawa and Ali-Osman, 1993) and cisplatin with cysteine (cys) (Bose et al., 1997). Ishikawa and Ali-Osman showed a 2:1 molar ratio of GSH/cisplatin, in the reaction between GSH and cisplatin (Ishikawa and Ali-Osman, 1993). Further, they isolated this reaction product, and by molecular mass analysis using a mass spectrometer they identified it as the Pt-GSH complex $Pt(NH_3)_2(GSH)_2$. Recently the reaction between cisplatin and the sulfur containing amino acid cysteine was investigated (Bose et al., 1997). A rapid reaction product $Pt(NH_3)_2(cys)_2$ was identified using nuclear magnetic resonance (NMR) studies (Bose et al., 1997).

The 1:1 carboplatin to mesna stoichiometry was unexpected. After all carboplatin binds DNA similar to cisplatin (Blommaert et al., 1995; Knox et al., 1986), meaning it has two binding sites after the dicarboxylate leaving group is removed. This might be the mechanism of carboplatin activation *in vivo* in the presence of cellular esterases, but *in vitro* evidence is presented for a 1:1 reaction with mesna (fig. 2.5).

The reaction product proposed in equation (2) for the carboplatin mesna reaction is only one possibility among many. This product was not isolated nor identified. Detailed mass spectrometric analysis or NMR studies are needed for definite identification of this reaction product. However, based on the 1:1 stoichiometry obtained from my results and similar ring-opened structures proposed (Barnham et al., 1996), the product proposed in equation (2) is reasonable. Ring-opened carboplatin adducts with sulfur containing amino acids L-methionine, N-acetyl-L-methionine, N-acetyl-L-cyteine, and glutathione in one

to one carboplatin/sulfur containing compounds have been identified (Barnham et al., 1996).

Surprisingly, ring-opened carboplatin thioether ligands can be substituted by N7 of a guanosine base (Barnham et al., 1996), so conceivably methionine containing proteins could transport and transfer some Pt to DNA. Is GSH also involved in such activation of carboplatin? Is this why giving carboplatin after cisplatin resistance is effective in some tumors? Tumors that have elevated levels of GSH? How about contrary to traditional belief, combining carboplatin with GSH in combination chemotherapy? Can mesna be involved in such activation of carboplatin similar to that observed for methionine? Some of these questions are discussed in chapter 3.

The kinetics studies in this chapter were done to provide preliminary information for design of the stoichiometric experiments, however some information regarding mesna's speed of reaction with Pt-drugs was confirmed. In general mesna reacted much faster with cisplatin than with carboplatin (tables 2.1 & 2.2). The half-life of reaction for 7mM mesna with 3mM cisplatin was 16 minutes and 5.4 hours, for initial and terminal half-lives of mesna respectively. In contrast the half-life of mesna in reaction with carboplatin was 105 hours at similar concentrations. These are clinically relevant concentrations of each of these drugs in infusion bags or infusion lines. Therefore, based on these kinetics data the inactivation of cisplatin by mesna is possible, if these two agents are administered at the same time, but inactivation of carboplatin most likely is too slow to be clinically relevant.

The colorimetric assay described here measured mesna using MTT dye in an oxidation/reduction reaction. A weakness of this method was that it could not measure mesna concentrations in serum, because of interference from other thiols present in serum. If kinetics of mesna in reaction with Pt-drugs in serum was desired, either loss of mesna or the Pt-drug could have been determined using HPLC.

Kinetics of cisplatin with mesna in serum and urine have been investigated in detail by Leeuwenkamp and colleagues (Leeuwenkamp et al., 1991). At relevant concentrations for cisplatin and mesna in serum, half-lives for cisplatin were 223.6 min and 150.7 min at pH 5.3 and pH 7.4 respectively (Leeuwenkamp et al., 1991). At relevant concentrations for cisplatin and mesna in urine, half-lives for cisplatin were 5.3 min and 3.3 min at pH 5.3 and pH 7.4 respectively (Leeuwenkamp et al., 1991).

The kinetics of carboplatin in reaction with mesna in human plasma and urine have been investigated (Obrocea et al., 1998). At relevant plasma concentrations for carboplatin and mesna,the half-life for carboplatin was 1.62 days (Obrocea et al., 1998), and at relevant urine concentrations for both agents, carboplatin had a half-life of 2.78 days (Obrocea et al., 1998).

The reaction kinetics of mesna with cisplatin were bi-phasic (fig. 2.2). Possibly this is an indication of the reaction mechanism of mesna with cisplatin. Mesna might displace one of the chlorides on cisplatin in a fast reaction within minutes (table 2.1), but displacement of the second chloride by mesna is much slower, a matter of hours (table 2.1). The slower second phase of elimination for mesna was misleading for mesna concentrations of 1.4 mM and 2.8 mM. In these concentration ranges most of the mesna had already reacted in the initial faster phase, therefore the second phase of mesna disappearance was exaggerated based on the bi-exponential model fit.

The rate of reaction between mesna and carboplatin was dependent on mesna concentration (table 2.2). Theoretically to fully react mesna in the concentration range of 0.7 mM to 7.1 mM with carboplatin would have required 5 half-lives ranging from 4 hours to 20 days. At longer incubation times mesna would have been oxidized to dimesna in air (Goren et al., 1991), hence the titration experiments would have not been as reliable as the ones obtained for mesna/cisplatin titrations. This is why DMSO was used to speed-up the reaction between mesna and carboplatin. DMSO is commonly used to dissolve Pt-compounds. In testing some of our novel Pt-drugs which are dissolved in 20%

DMSO, we discovered that DMSO speeds-up the reaction between Pt-drugs and mesna. A possible explanation of this phenomenon could be that DMSO continuously replenishes un-reactive oxidized dimesna back to reduced and very reactive mesna. Also DMSO decreases the pKa of mesna (Oprea et al., 2001), making mesna a better nucleophile and thus more reactive at pH 7.4. Control samples of 20% DMSO with MTT and 20% DMSO with Pt-drug and MTT showed no interference/reaction in the colorimetric assay.

In summary in these experiments, the stoichiometry of mesna in reaction with cisplatin and carboplatin was elucidated. This information was lacking from previous studies which only determined reaction rates of cisplatin and carboplatin with mesna under physiological conditions. What remains to be found are the exact structures of cisplatin-mesna, and carboplatin-mesna complexes, using mass spectrometry and NMR studies.

The significance of this work is that mesna can possibly inactivate cisplatin but not carboplatin if these agents are mixed together in an infusion bag or infused together through the same infusion line. The next logical step was to see whether this inactivation has any biological consequences, therefore as the first step, Pt-DNA binding of Pt-drugs were investigated with or without mesna in glioma cells.

Chapter Three: Influence of Mesna on Platinum Cellular Accumulation, and Platinum DNA Binding of Cisplatin and Carboplatin in Malignant Glioma Cells

Methods

Reagents

Drugs; cisplatin injection 1 mg/mL, Falulding Canada Inc. (Vaudreuil, Quebec), carboplatin injection 10 mg/mL, Novopharm Ltd. (Toronto, ON), and mesna injection 100 mg/mL, Bristol-Myers Squibb Canada Inc. (Montreal, Quebec). All drugs were kindly provided by Oncology pharmacy, Southern Alberta Children's Hospital (Calgary, AB), and were used as pre-made aqueous solutions suitable for human use. Stock cisplatin and carboplatin were stored at room temperature and protected from light. Diluted samples of cisplatin and carboplatin were made fresh daily. Mesna stock was stored at 4°C, and its diluted samples were made fresh daily. Chemical agents were purchased from Sigma-Aldrich (Canada) unless specified otherwise, cell culture agents were obtained from Gibco BRL, Life Technologies Inc. (Canada). MTT dye was dissolved in PBS (0.2 M NaCl, 0.027 M KCl, 0.010 M Na_2HPO_4, 0.0018 M KH_2PO_4, pH 7.4) as a stock solution of 5 mg/mL and stored at 4°C, protected from light. Stock proteinase-K was 20 mg/mL in water stored at –20 °C, 100 % ethanol (Commercial Alcohols Inc., Brampton, ON), concentrated nitric acid, AnalaR 68-70% (BDH Inc. Toronto, ON), atomic absorption Pt standard, Pt solution 970 μg Pt/mL in 5% HCl. All water used was de-ionized by Millipore Milli-Q system (Millipore Corporation, Nepean ON).

Brain tumor cell lines

The human malignant glioma cell line U-87MG was purchased from the American Type Culture Collection (Rockville MD, USA), and the human

malignant glioma cell line U-251MG was kindly provided by Dr. P. Forsyth of the Cancer Biology Research group, University of Calgary (Calgary, AB). Cells were grown in monolayer cultures in RPMI growth medium [RPMI-1640 medium with L-glutamine pH 7.2 supplemented with 100 U/L penicillin, 100 μg/mL streptomycin (1% Penicillin-Streptomycin solution), and 10% fetal calf serum] in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

Cytotoxicity testing

The cytotoxicity studies were conducted with the (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; (MTT) colorimetric assay as described previously (Carmichael et al., 1987; Mosmann, 1983; Wolff et al., 1998). Cells were plated into 96-well plates on day 0 and exposed to the drugs on day 1; typically the MTT assay was carried out 0, 24, 48, 72, and 96 hrs after drug exposure. Cells from subconfluent 75 cm^2 flasks were trypsinized and re-suspended in RPMI growth medium, counted using a hemocytometer, 5000 cells/100 μl/well were then plated into 96-well plates. Twenty-four hours later 100 μl of various drug dilutions in RPMI growth medium were added to the wells. Typically seven drug concentrations ranging from 0.01 to 100 μM for cisplatin, 0.1 to 1000 μM for carboplatin, and 0.3 to 10 mM for mesna were used. Controls included medium and solvents. After the desired drug incubation time, treatment medium was aspirated and substituted by 100 μl PBS containing 1mg/mL MTT in each well. After 1h incubation with MTT (95% air, 5% CO_2, 37°C), 100 μl of 50% DMF (Sigma) / 20% SDS (EM Science) in ddH_2O was added to each well, and incubated for another 3-4 hrs or until no precipitates remained. Optical density (OD) was measured at 600 nm using a Beckman Biomek 1000 automated workstation with a 600 nm photometer plate reader, (Beckman Instrument Inc. Fullerton CA). Percentage of survival was calculated in comparison to medium treated controls on the same plate. Eight wells were used per drug concentration.

Platinum DNA binding and cellular accumulation

Cells were plated in 75 cm^2 flasks at 1.0 x 10^6 cells/flask. Twenty-four hours later, drugs in RPMI growth medium were added to the flasks and incubated for the desired treatment time (95% air, 5% CO_2, 37°C). Drug concentrations as determined from cytotoxicity testing were 10 μM, and 100 μM for cisplatin, 1000 μM for carboplatin, and 3 mM for mesna. Drug treatment of the glioma cells was terminated after the desired incubation time by transferring the drug treated medium to 50 mL centrifuge tubes (aliquots were saved from these supernatants for total Pt and protein free Pt measurements), the cells were washed with PBS, trypsinized, and centrifuged at 250 x *g* for 10 minutes. Cell pellets were rinsed twice with PBS to remove any residual drugs. The cell pellets were stored at -70 °C until DNA isolation or total cellular lysates were performed.

Isolation of genomic DNA from glioma cell pellets

Genomic DNA was isolated from the cell pellets using a salting out procedure by modifying the protocol obtained from Miller (Miller et al., 1988). This method was chosen among many others tried in order to avoid phenol extraction and other harsh treatments that might have stripped Pt away from the DNA. Also, EDTA was eliminated from all extraction steps since it might chelate Pt, stripping it away from DNA. One mL of lysis buffer (75 mM NaCl, 10 mM Tris pH 7.6, 1% SDS) was mixed with the cell pellet. Protinase-K was added to a final concentration of 200 μg/mL. The mixture was placed on a rocker at 37°C until all of the cell pellet was digested (usually overnight). Three-hundred μL of 6 M NaCl (1.8 M final concentration) was added to the cellular digest, vortexed for 15 seconds and subsequently centrifuged at 12,000 x *g* in an Eppendorf centrifuge model 5415 C, (Brinkmann Instruments Inc. Westbury NY). The supernatant was

transferred to a 5 mL centrifuge tube leaving behind the protein pellet. Exactly two volumes of room temperature 100% ethanol was added to each tube and inverted a few times until the DNA precipitate was visible. If the DNA was spoolable it was removed by a pipet tip and transferred to a new 1.5 mL eppendorf tube. If the DNA was sheared (low molecular weight) it was recovered by centrifugation at 12,000 x *g* for 30 minutes. DNA samples were washed twice with 70% ethanol, left to dry for two minutes, suspended in 200 – 300 μL of 10 mM Tris pH 7.6 in ddH_2O, and placed on a shaker at 37°C overnight to dissolve.

Quantification of DNA samples was done by measuring the absorbence at 260 nm using a Beckman DU-65 spectrophotometer, (Beckman Instruments Inc. Fullerton CA), and verified to be salt and protein free by the 260/280 nm ratio. Typically 98-360 μg of DNA was recovered from one million U-251MG plated cells and 16-42 μg of DNA from one million U-87MG plated cells. Most DNA samples had absorbance 260/280 ratios between 1.8-2.0. If the 260/280 ratio was below this range the DNA sample was further "cleaned-up" in order to avoid bias of Pt bound to protein in addition to Pt bound to DNA. Equal amounts of DNA were taken for Pt measurements as described in Pt measurement section.

Isolation of total cellular lysates from glioma cell pellets

Each cell pellet was suspended in 500 μL of ddH_2O and freeze-thawed three times to lyse the cells. An aliquot of a 20 X dilution was taken from each lysate for protein quantification. The amount of protein in each sample was determined by the Bradford protein assay (Bradford, 1976), (Bio-Rad Laboratories Hercules, CA). Briefly, a calibration set of 0, 0.5, 1.0, 2.0, and 4.0 μg BSA, 200 μL final volume was set up in a 96 well plate. Each sample was aliquoted in 20, 40, and 80 μL, adjusted to 200 μL final volume in the 96 well plate, 50 μL of Coomassie dye was added to all samples and calibrators, pipetted up and down to dissolve all precipitates, and absorbance was read at 600 nm on

the Biomek plate reader fifteen minutes after addition of the dye. Equal amounts of protein were taken for Pt measurements as described in the Pt measurement section.

In vitro protein binding of cisplatin

Cisplatin was incubated in RPMI growth medium at 37°C, 5% CO2, at 10 μM and 100 μM, with and without mesna at 3 mM, for 0,1, 4, 8, 12, 24, 48, 72, and 96 h. At these intervals, two 400 μL aliquots were subjected to centrifugal ultrafiltration using Amicon centrifree micropartition devices (Millipore Corporation, Nepean, ON, Canada) at 2000 x *g* for 30 min, in an IEC model HN-S centrifuge with a 33° fixed angle rotor (International Equipment Company, Needham Heights, MA). Also a one mL aliquot (not ultrafiltered) was saved for determination of total Pt at each time interval. Controls containing cisplatin in water showed no binding of Pt to the membrane of centrifree devices. Ultrafiltrate (UF) samples were checked for leakage of protein through the centrifree membranes by the Bradford Protein Assay. No protein was detected in UF samples.

Total Pt and protein free Pt from UF samples were measured by AAS as described below. Protein binding of cisplatin was reported as %Pt protein bound by: $\frac{TotalPt - FreePt}{TotalPt} \times 100$.

Platinum measurements

Sample preparation

This method of sample preparation was modified from a method used by Johnsson and colleagues (Johnsson et al., 1995). For DNA samples from glioma

cells typically 30 μg of DNA was suspended in 500 μl of 50% HNO_3. For cell lysate samples typically 1.0 mg of protein was suspended in 50% HNO_3, and for supernatant samples from *in vitro* protein binding of cisplatin at 10 μM and 100 μM, 20 times and 200 times dilutions respectively were placed in 500 μl of 50% HNO_3. All samples were digested at 120°C in 1.5 mL Eppendorf tubes on a heating block (Standard heat block, VWR Scientific Products, VWR Canlab, Mississauga, ON) for one hour or until sample volume was concentrated to 150 – 200 μL. The digests were cooled and made up to one mL with ddH_2O. Matching blanks and calibrators were digested in parallel with the samples.

Instrumentation

Platinum agents were analyzed as elemental Pt by atomic absorption spectrometry (AAS). A Varian model Spectra AA-220Z / GTA-110 (Varian Inc., Mulgrave, Victoria, Australia) with Zeeman background correction was used. The instrument was equipped with a Varian Platinum hollow cathode lamp and Varian graphite partition tubes. Instrument coolant included argon gas (ultra high purity, Praxair, Calgary, AB) flow through the furnace chamber at a rate of 3.0 L/min and cold tap water flow through the furnace housing at a rate of 1.5 L/min. Detailed instrument parameters are given in tables (3.1 and 3.2). Absorbance signal was read at steps 10 and 11 (atomization). Argon gas flow was stopped from steps 9 to 11.

DNA samples were measured using the multiple injection feature of the instrument, with the drying steps (1-4) repeated after each repetition of sample placement into the furnace. Once the desired number of repetitions was completed, ashing, atomization and burn-off would proceed as in table 3.2. DNA samples were hot-injected into the furnace at 85°C three times (multiple injection feature) at a volume of 20 μL per injection. Protein samples and all other samples

Table 3.1. Instrument parameters

Instrument Mode	Absorbance
Measurement Mode	Peak height
Lamp Current (mA)	10
Slit width (nm)	0.5
Wavelength (nm)	265.9
Background correction	ON

Table 3.2. Furnace parameters

Step NO.	Action	Temp (°C)	Ramp time (s)
1	Dry	90	5.0
2	Dry	97	30.0
3	Dry	110	45.0
4	Dry	120	10.0
5	Ash	350	10.0
6	Ash	400	20.0
7	Ash	1150	5.0
8	Ash	1150	10.0
9	Ash	1150	2.0
10	Atomize	2600	0.7
11	Atomize	2600	2.0
12	Burn-off	2800	4.0

were hot-injected at 80°C at a single volume (no multiple injects) of 12 μL per sample.

All samples and calibrators were measured three separate times and were only accepted if a precision ≤ 5% was achieved between the separate measurements.

Calibration

A separate calibration curve was run for every different matrix. The calibration range for DNA, cell lysate, and supernatant samples respectively were: (25 nmol/L Pt to 150 nmol/L Pt), (50 nmol/L Pt to 500 nmol/L Pt) & (250 nmol/L Pt to 1000 nmol/L Pt). The standard curves were consistently linear through the entire range of Pt measured. The limit of detection for elemental Pt was 0.3 pmol Pt as determined by (mean blank + 3 SD). The limit of quantitation for elemental Pt was 0.9 pmol Pt as determined by the lowest sample measured with a CV of ≤ 5%. Quality control samples were run at the start and end of an analysis batch, with a tolerance of ≤ 15% drop for acceptance of results. Due to calibration drift no more than ten samples were analyzed with one standard curve. To maintain instrument sensitivity, graphite furnace tubes were changed after approximately 70 firings. All calculations were based on the molecular weight of elemental Pt, which is 195.08 g/mol.

Statistical Analysis

The data were summarized as means ± standard deviations (SD) where appropriate. Standard deviation was chosen over standard error of the mean (SEM) to show variability in the samples. Groups were statistically evaluated by the un-paired Student's t-test (two-tailed, equal or un-equal variance based on the F-test). Results from *in vitro* protein bind of cisplatin were evaluated by the

two-way analysis of variance. The level of significance was taken as $P < 0.05$ in all of the tests.

Results

Cytotoxicity testing

Before starting Pt-DNA binding experiments relevant drug concentrations and incubation times with respect to the cell lines being used needed to be determined. Hence, cytotoxicity testing with the agents under question was done. These experiments are summarized in figures 3.1 and 3.2.

The difference between solvent treated controls and Pt-drug treated cells indicated that cytotoxicity increased with higher drug concentrations and longer drug incubation times. Average cisplatin concentrations resulting in 50% cell death (LC_{50}) in U251 cells after 24, 48, 72, and 96 hours of drug incubation were: 100 μM, 20 μM, 10 μM, and 4.5 μM respectively (extrapolated from figure 3.1a).

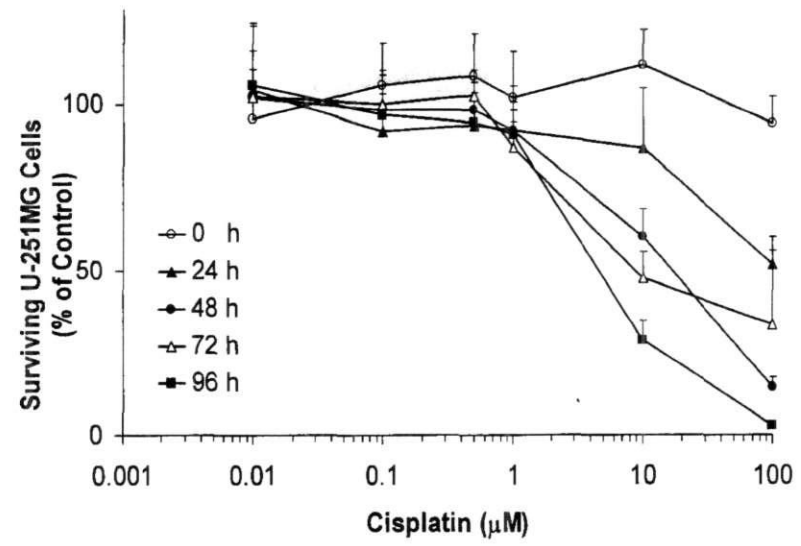
Carboplatin required higher concentrations to create equivalent cytotoxicity. Average LC_{50} values for carboplatin in similar experiments in U251 cells after 24, 48, 72, and 96 hours of drug incubation were: 1000 μM, 250 μM, 50 μM, and 20 μM respectively (extrapolated from figure 3.1b).

The experiments were repeated in U-87MG cell lines with 24 h of drug incubation (fig. 3.1c). This cell line was less sensitive to the Pt-agents as compared to the U-251MG cell line. An LC_{50} value was not achieved by 24 h of drug incubation. Only 40% of the cells were killed by 100 μM cisplatin and 1000 μM carboplatin, concentrations which achieved LC_{50} in the U-251MG cell line. Higher concentrations of carboplatin were required to create equivalent cytotoxicity as those obtained with cisplatin.

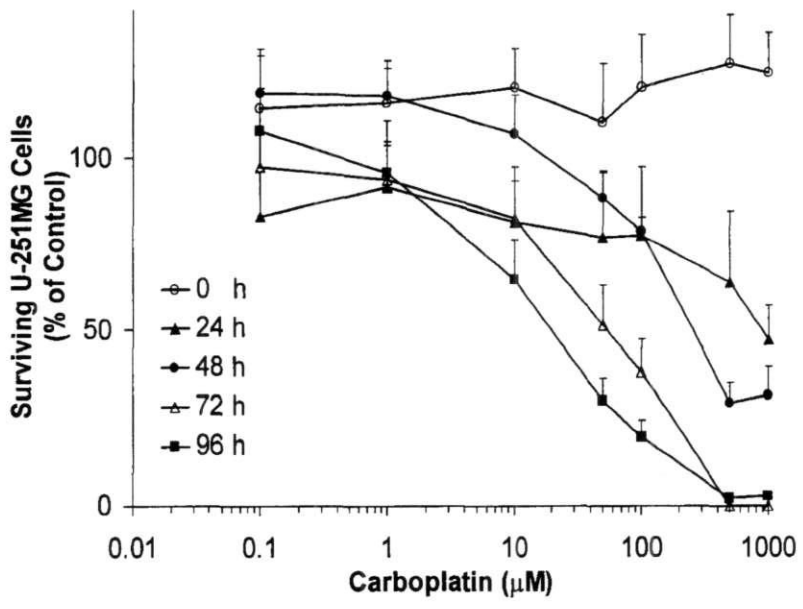
Cytotoxicity of mesna for 24 h in concentration ranges of cellular thiols, cysteine and glutathione (Bose et al., 1997) was evaluated on the cells (fig. 3.2). Mesna was not cytotoxic to the U-251MG cells in the entire concentration range

Figure 3.1. Cytotoxicty of platinum drugs with increasing drug concentration and incubation time. Cell viability was determined by the MTT assay. Each incubation time had its own controls of cells with no drug (=100% survival). **A.** Cisplatin (CDDP) on U-251MG cells. **B.** Carboplatin on U-251MG cells. **C.** 24 hours of drug incubation in U-87MG cells. Error bars represent standard deviation of eight replicates.

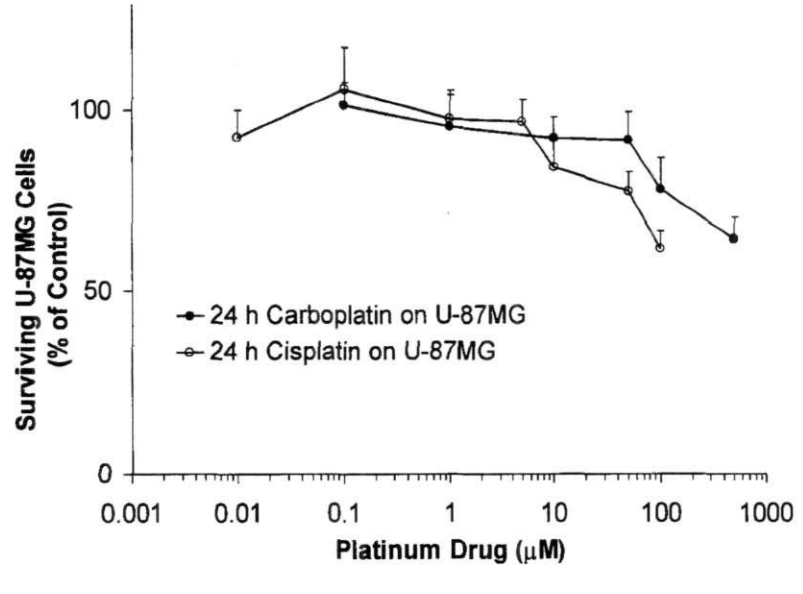
A


Surviving U-251MG Cells
(% of Control)
100
50
0
0 h
24 h
48 h
72 h
96 h
0.001
0.01
0.1
1
10
100
Cisplatin (μM)

B


Surviving U-251MG Cells
(% of Control)
100
50
0
0 h
24 h
48 h
72 h
96 h
0.01
0.1
1
10
100
1000
Carboplatin (μM)

C


Surviving U-87MG Cells
(% of Control)
100
50
0
24 h Carboplatin on U-87MG
24 h Cisplatin on U-87MG
0.001
0.01
0.1
1
10
100
1000
Platinum Drug (μM)

tested (fig. 3.2a). In U-87MG cells mesna concentrations up to 3 mM were not toxic to the cells, however higher concentrations up to 10 mM mesna reduced cell survival by 40%. Therefore the maximum tolerated concentration for mesna was set at 3 mM for all cell culture experiments.

Kinetics of Platinum DNA binding and extracellular protein binding

Preliminary experiments were done to learn about kinetics of Pt binding DNA before evaluating influence of mesna on this phenomenon in more detail. High dose of cisplatin (100 μM), and low dose of cisplatin (10 μM) were chosen based on the cytotoxicity results.

The kinetics of Pt binding DNA in both glioma cell lines are shown in figure 3.3a. The amount of Pt bound to DNA was similar in both cell lines at the two tested concentrations. Platinum DNA binding increased from 1 to 12 h, but past 12 h, Pt-DNA binding reached a plateau. There was a ten-fold difference in the amount of Pt bound to DNA between 100 μM and 10 μM cisplatin concentrations. For U-87MG cells, Pt-DNA binding could not be determined past 48 h at 100 μM cisplatin concentration. This was because the amount of DNA recovered past 48 h was extremely low, most likely due to high extent of damage to DNA and consequent shearing of DNA.

The amount of Pt measured on DNA at the 10 μM cisplatin concentration was slightly above the limit of quantitation for Pt measurement using our method. Therefore, because of this experimental limitation, observations of Pt binding DNA kinetics in which by 24 h Pt-DNA binding was at a plateau, coupled with the cytotoxicity results, 100 μM and 24 h were chosen as dose and incubation time for cisplatin in all subsequent Pt-DNA binding experiments. Since the cytotoxicity results had shown 1000 μM carboplatin to be equivalent to 100 μM cisplatin, 1000 μM was chosen as the test concentration for carboplatin in all subsequent experiments in this section.

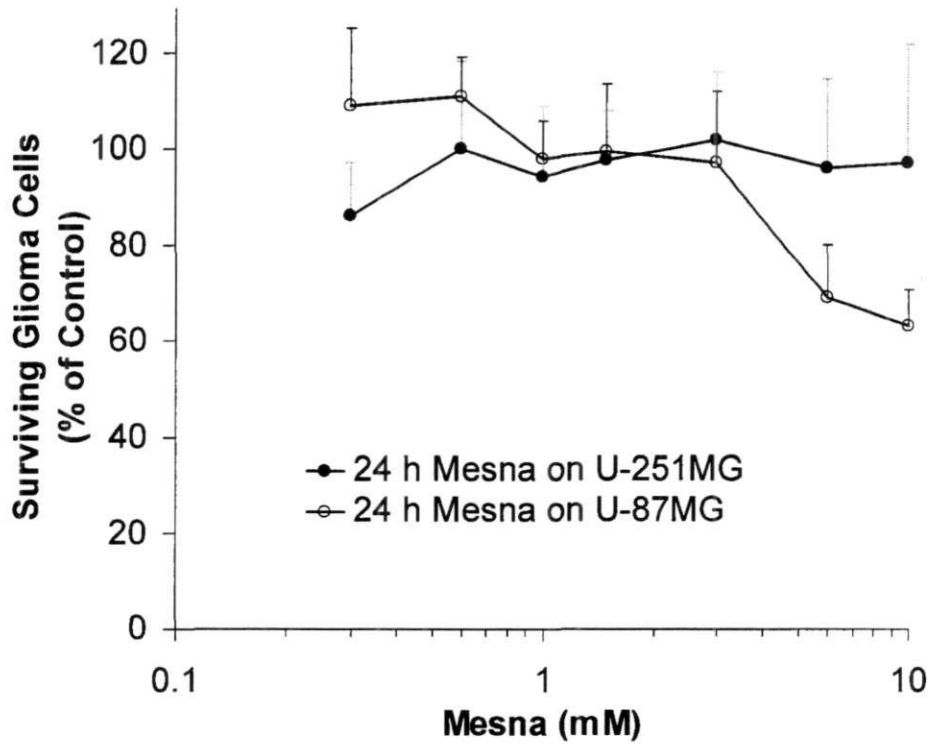


Figure 3.2. Effect of increasing Mesna concentration on glioma cell viability. Cell viability was determined by the MTT assay. Each experiment had its own control of cells with no drug (=100% survival). Error bars represent standard deviation of eight replicates.

Preliminary results on the influence of mesna on kinetics of Pt binding to DNA showed that mesna led to a ten fold reduction in Pt binding to DNA from 1 to 24 hours (fig. 3.3b). This difference was less pronounced by 72 hours. This was an *in vitro* phenomenon since all of the cells were dead by 72 hours and most likely DNA was being released from lysed cells into the growth medium. Mesna also reduced the initial rate of Pt binding DNA for cisplatin (fig. 3.3b). Cisplatin bound to DNA at an initial rate of 0.70 pmol Pt/ μg DNA/ h in contrast to a rate of 0.044 pmol Pt/ μg DNA/ h for cisplatin in the presence of mesna.

The kinetics of protein binding for cisplatin in supernatant of U-251MG cells is shown in figure 3.4. The average concentration of protein in supernatant samples was 3.1 ± 0.31 mg/mL protein. Similar to DNA kinetics, Pt protein binding increased from 1 to 12 h and reached a plateau past 12 hours. Pt protein binding was shifted towards Pt dissociating form protein from 72 to 96 h, P=0.024 for 100 μM cisplatin and P=0.052 for 10 μM cisplatin. There was no significant difference in % Pt protein bound between 100 μM and 10 μM cisplatin, P = 0.568. Therefore the ten-fold difference in Pt binding DNA seen between these two concentrations was not due to extracellular protein binding. Platinum protein binding was reduced in the presence of 3 mM mesna during the equilibrium phase from 12 to 72 h. This difference was significant when 100 μM cisplatin was compared to 100 μm cisplatin in presence of 3 mM mesna, P=0.0021, and strongly significant when 10 μM cisplatin was compared to 10 μM cisplatin plus 3 mM mesna, P=0.0001.

Influence of mesna on DNA binding and cellular accumulation of Pt-agents in glioma cells

Glioma cells were incubated either with cisplatin or carboplatin, with or without mesna for 24 hours. Total cellular lysate or DNA was extracted and Pt measured in each fraction using AAS. When mesna was present concurrently

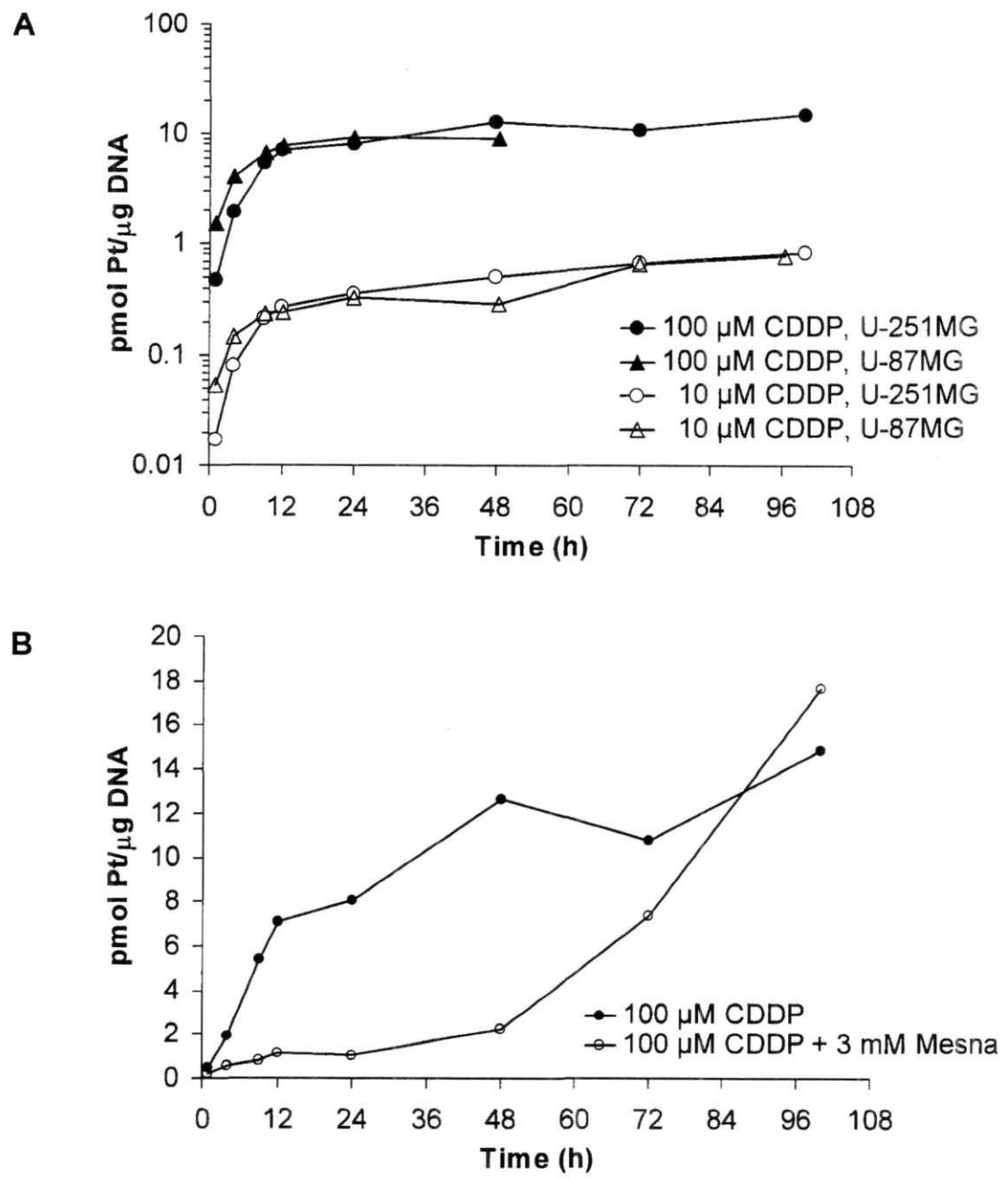


Figure 3.3. Kinetics of platinum binding DNA in glioma cells. Platinum DNA binding for cisplatin was measured after various incubation times. **A.** Kinetics for high dose cisplatin compared to low dose cisplatin in both glioma cell lines. **B.** Influence of mesna on high dose cisplatin in U-251MG cells. Each point represents total genomic DNA isolated from approximately one million plated glioma cells.

with cisplatin, Pt accumulation in U-251MG cells was reduced by a factor of four, $P=2.99 \times 10^{-7}$ (fig. 3.5b), and Pt-DNA binding was reduced by a factor of ten, $P=3.02 \times 10^{-7}$ (fig. 3.5a). However when mesna was present concurrently with carboplatin, Pt-DNA binding was not affected in a statistically significant way, P=0.343 (fig. 3.5c). After this observation no further experiments were done with carboplatin in cell culture.

Experiments were done to simulate a clinical situation where mesna is given one hour after cisplatin. When mesna was given to the glioma cells one hour after cisplatin incubation, Pt-DNA binding in both glioma cell lines was reduced by more than 60% as compared to controls (cisplatin alone) (fig 3.6, mesna 1 h after cisplatin).

The mechanism whereby Pt-DNA binding was reduce was investigated further to determine if the reaction between mesna and cisplatin is an extracellular phenomenon and if mesna is able to enter glioma cells. Glioma cells were incubated with 3mM mesna for 24 h, then mesna was washed off the cells with PBS and the cells were incubated for another 24 h with 100 μM cisplatin. In this experiment there was no statistically significant reduction in Pt-DNA binding as compared to the cisplatin control (fig. 3.6, mesna 24 h before cisplatin).

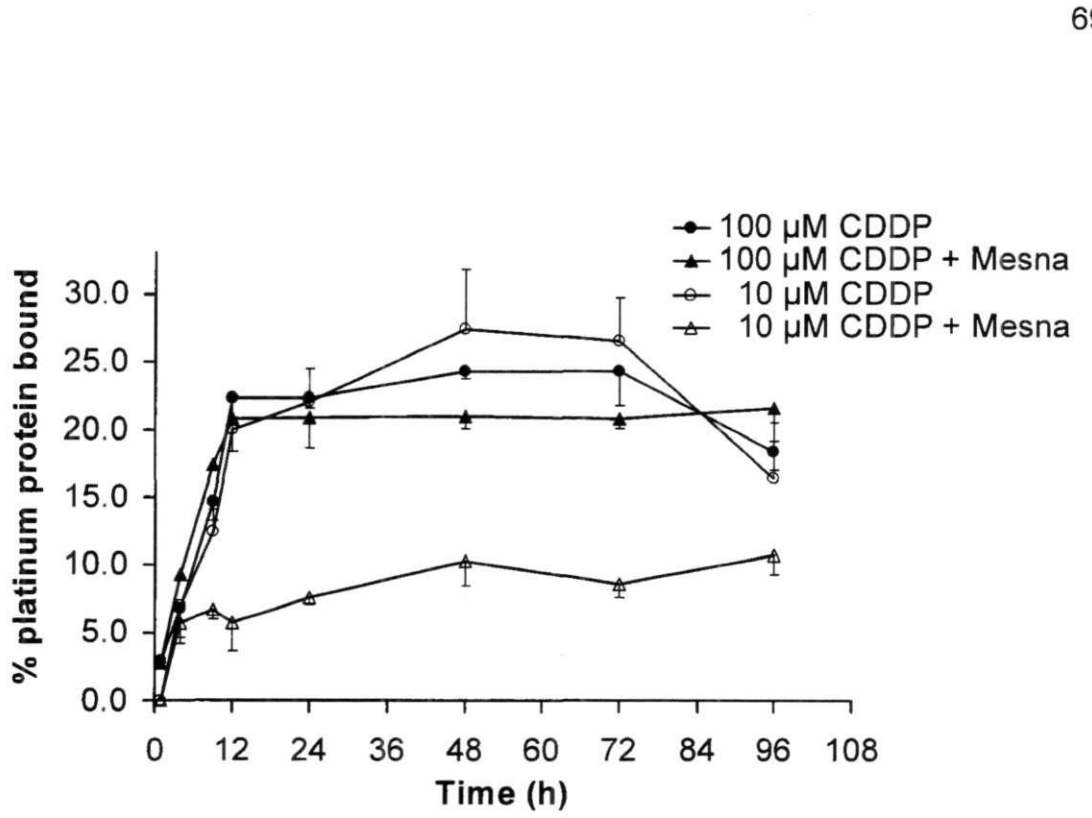


Figure 3.4. Kinetics of extracellular protein binding of cisplatin in RPMI 1640 + 10% Fetal Calf Serum. Protein binding kinetics was determined in the supernatant of U-251MG cells. 100 µM cisplatin (CDDP) was compared to 10 µM CDDP by two-way ANOVA. Similarly, the influence of mesna on protein binding was compared between 100 µM CDDP with or with out mesna and 10 µM CDDP with or with out mesna. Each point is average of three independent experiments with error bars indicating standard deviation.

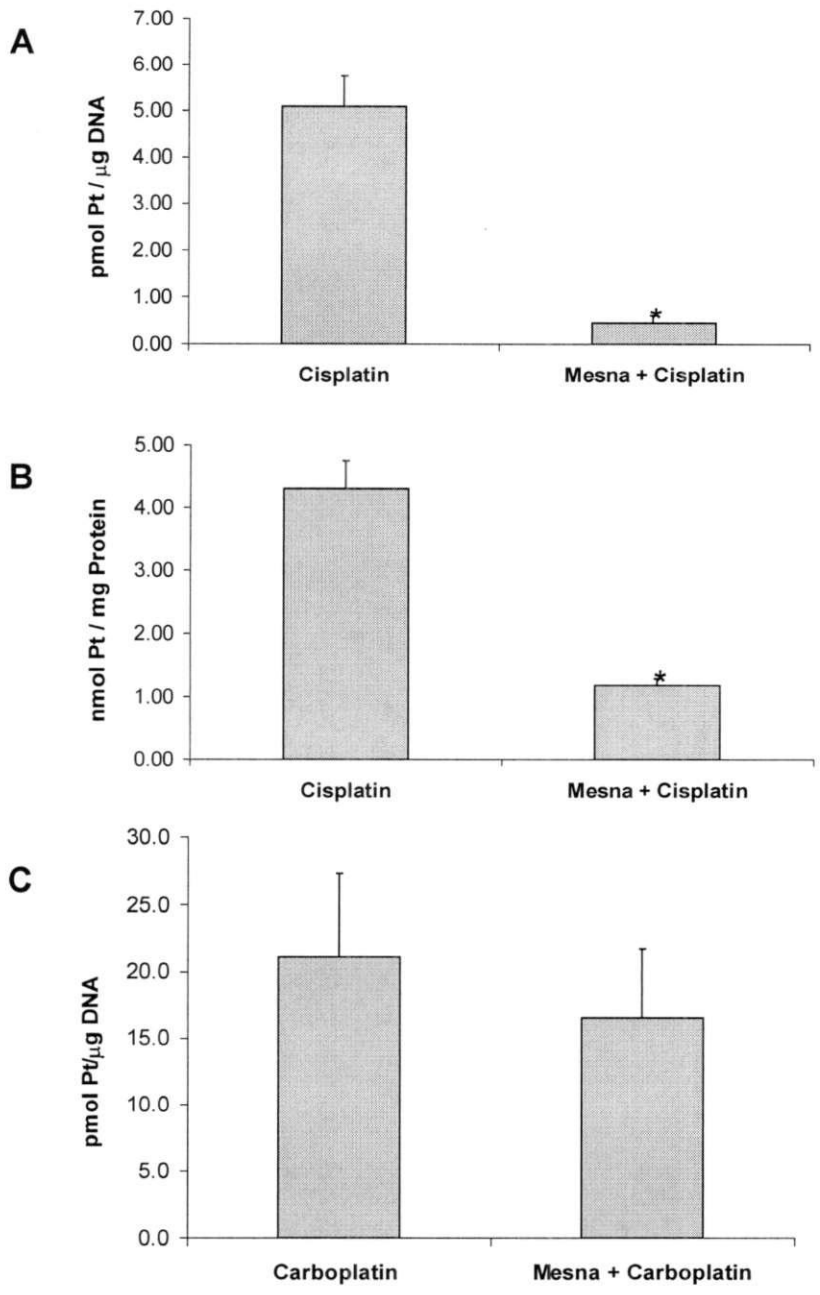


Figure 3.5. Influence of mesna on Platinum DNA binding and cellular accumulation of Pt-Drugs in U-251MG cells. A. Platinum DNA binding is compared between 100 µM cisplatin and 100 µM cisplatin plus 3 mM mesna for 24 hours. **B.** Total cellular accumulation of Pt is compared in similar conditions as in A. **C.** Platinum DNA binding is compared between 1000 µM carboplatin and 1000 µM carboplatin plus 3 mM mesna after 24 hours of drug incubation. Each bar is the average of three to five independent experiments. Error bars indicate standard deviation. Bars marked with * indicate statistically significant difference, $P < 0.05$ as determined by the t-test.

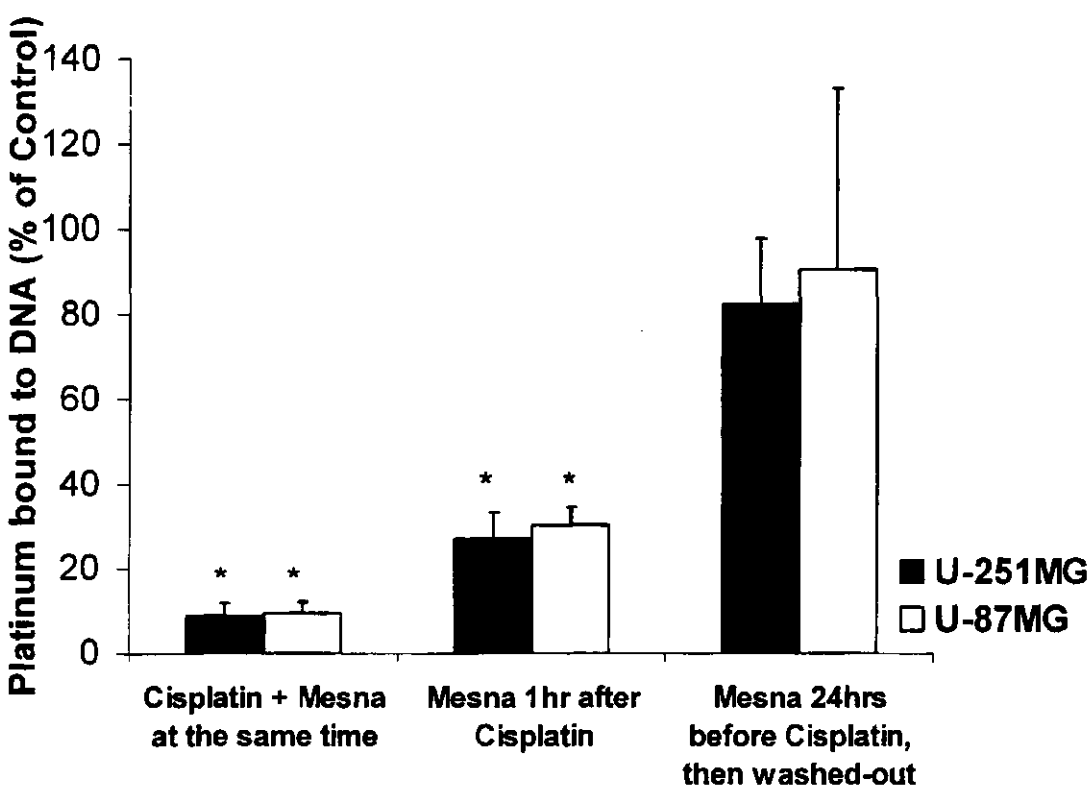


Figure 3.6. Time schedule of mesna incubation in relation to cisplatin incubation and the result in platinum DNA binding in glioma cells. Each experiment (bar) had its own control, 100 μM cisplatin for 24 hours taken as 100% Pt-DNA bound. Each bar is the average of three to five independent experiments, with error bars as SD. Bars marked with * indicate statistically significant difference from their controls.

Discussion

Influence of mesna on cisplatin's Pt-DNA binding and cellular Pt accumulation

These experiments dealt with binding of cisplatin and carboplatin to DNA in the presence and absence of mesna. The results indicated that mesna reduces Pt-DNA binding of cisplatin but not that of carboplatin in glioma cells, therefore providing support for the hypothesis. Mesna, similar to glutathione (GSH), might form complexes with cisplatin, thereby reducing the amount of intracellular cisplatin available for interaction with DNA. These results provide an explanation for the reduction in cytotoxicity of cisplatin in the presence of mesna in glioma cells reported by Wolff et al. (Wolff et al., 1998).

Similar to Pt-DNA binding, total cellular accumulation of cisplatin was reduced in presence of mesna. One possible explanation for this observation is that mesna reacts with cisplatin extracellularly and prevents cisplatin entry into the cell. The fact that mesna is highly reactive toward cisplatin was shown in chapter two and by the work of others (Leeuwenkamp et al., 1991; Leeuwenkamp et al., 1990; Williams and Lokich, 1992)

Another possibility is that a cisplatin-mesna complex enters the cell but is pumped out of the cell via an ATP dependent export pump. One such pump is the glutathione-s-conjugate (GS-X) export pump, notably the canalicular multispecific organic anion transporter (cMOAT), also known as multidrug resistance associated protein-2 (MRP2) (Borst et al., 2000; Ishikawa and Ali-Osman, 1993; Kool et al., 1997). Evidence has been provided that MRP2 could contribute to cisplatin resistance by exporting the cisplatin-glutathione complex out of the cell and therefore reducing cisplatin accumulation inside the cell (Borst et al., 2000). This system might be capable of pumping other cisplatin-S conjugates such as cisplatin-mesna out of the cell. In order to determine if a Pt-mesna complex is pumped out of the cell using the same GS-X pump, an

interesting experiment would be to inhibit this transporter by typical inhibitors of this pump such as DNP-SG [s-(2,4-dinitrophenyl)-glutathione] or leukotriene C_4 and measure Pt accumulation for cisplatin with or without mesna co-incubation.

However inactivation of cisplatin by mesna might be different than that by GSH and other cellular thiols with respect to compartmentalization. Evidence here indicated that cisplatin is inactivated by mesna extracellularly, since mesna does not enter glioma cells (as discussed later), whereas inactivation of cisplatin by GSH is intracellular. It has been reported that almost 1% of cisplatin reacts with genomic DNA whereas the majority of the drug interacts with protein, RNA and small thiol compounds (Eastman, 1990). Here I have shown that mesna in mM concentration significantly reduces Pt-DNA binding of cisplatin. The concentration of cysteine and glutathione in the cell is in the mM range (Bose et al., 1997). Bose and colleagues found that the reaction between cisplatin and cysteine was much faster than that for the corresponding reaction with DNA (Bose et al., 1997). Although cisplatin might react faster with mesna than with DNA in an *in vitro* situation, this is not a possible explanation for these results since they indicate that mesna is acting extracellularly and is not directly in competition with DNA inside the cell. Therefore the observed reduction in Pt binding DNA must be due to reduced Pt accumulation inside the cell.

Mesna entry into glioma cells

Mesna did not influence Pt-DNA binding of cisplatin if it was given to the cells 24 h before cisplatin, and washed away from the cells prior to cisplatin incubation (fig. 3.6). This result could indicate that mesna does not enter U-251MG and U-87MG cells. This conclusion is consistent with the previous findings that mesna was not found in any of the organ cells except intestine and kidney (Brock et al., 1982; Ormstad et al., 1983; Ormstad and Uehara, 1982; Shaw et al., 1986). Therefore, mesna will only interfere with the cytotoxic activity of cisplatin if it comes in contact with cisplatin in the extracellular space. Mesna

does not enter cells due to its highly polar nature (Brock et al., 1982). Mesna undergoes rapid oxidation in plasma and circulates as the chemically inert disulfide (dimesna) (Brock et al., 1982; Ormstad and Uehara, 1982). Thus the various cells and tissues in the body are exposed to dimesna only. Since most cells do not take-up low molecular weight disulfides (Ormstad et al., 1983), dimesna remains in the extracellular space. The ability to take up low molecular weight disulfides and thiols is variably developed among different cell types. For example cysteine is rapidly taken up by kidney cells, but hardly at all by hepatocytes (Ormstad et al., 1983). Specific uptake mechanisms of mesna and dimesna have yet to be clarified. Evidence for the uptake of dimesna through a cystine transporter in the kidney has been rejected (Ormstad and Uehara, 1982), but a relationship to the glutathione transporter in the kidney might be possible (Ormstad et al., 1983).

Contrary to the results reported here, Wolff et al. (Wolff et al., 1998) suggested that mesna might enter U-87MG glioma cells. Present DNA binding data in this cell line did not support this view (fig 3.6). However mesna at concentrations above 3 mM was toxic to this cell line (fig 3.2) and the observations made by Wolff and colleagues used mesna concentrations above 3 mM. Possibly high mesna concentrations in the extracellular space lead to a loss of osmoregulation and membrane integrity in these cells, and thereby entry of mesna into the cells.

Since mesna does not enter glioma cells, reduced Pt-DNA binding of cisplatin is likely to happen extracellularly. The time schedule data (fig 3.6) support this view. Kinetics of Pt binding DNA for cisplatin indicates that it takes twelve hours for Pt to reach maximum DNA binding (fig. 3.3a). Therefore mesna should be kept away from contact with cisplatin for a minimum of twelve hours in an *in vitro* system. Such a precaution most likely in not necessary *in vivo*, since in the circulation mesna is quickly oxidized to dimesna which is not reactive toward Pt-agents. Also, mesna has a very short half-life of 9-11 minutes in the circulation (Goren et al., 1998). The only clinical situation that might mimic the *in vitro*

phenomenon seen in these experiments might occur when mesna and cisplatin are given to the patient through the same infusion line or mixed in the same syringe. This concept was further investigated in SCID mice experiments in chapter four.

Influence of mesna on Pt-DNA binding of carboplatin

Unlike cisplatin, Pt-DNA binding of carboplatin was not reduced upon co-incubation with mesna in U-251MG glioma cells (fig 3.5c). This finding is reasonable based on the slow reactivity between carboplatin and mesna shown in chapter two and by others (Obrocea et al., 1998). The result also explains why Wolff et al. did not see a reduced cytotoxicity for carboplatin (Wolff et al., 1998) using the same concentration that I examined for Pt-DNA binding of carboplatin in presence of mesna. Carboplatin has been shown to react extremely slowly with GSH *in vitro* (Dedon and Borch, 1987), therefore, one expects to find negligible reduction in DNA binding of carboplatin in the presence of thiol groups. However if this result was entirely true, why is carboplatin also cross-resistant to cisplatin resistant cell lines with elevated GSH levels? In defense of reactivity with mesna: In these series of experiments mesna and carboplatin are interacting extracellularly in the growth medium, where carboplatin is in its un-metabolized, un-reactive form. Since mesna does not enter cells it will not influence DNA binding of carboplatin. However when carboplatin is inside the cell, it becomes "biologically activated" giving rise to an as yet unknown platinum species which then binds DNA and cellular thiols such as GSH (Natarajan et al., 1999).

The molecular mechanism of carboplatin activation is still unresolved. Recently stable carboplatin open-ring complexes with sulfur containing amino acids have been observed (Barnham et al., 1996). It has been suggested that open-ringed monodentated sulfur-containing ligands might play a role in the antitumor mechanism of action of carboplatin, in binding DNA as a mono-functional adduct (Barnham et al., 1996). Evidence was provided for a one to one

molar reaction between carboplatin and mesna in chapter two. The 1:1 reaction product might be a similar monodentate sulfur containing (mesna) ligand of carboplatin. Hence, the possibility exists that the reason for no reduction in Pt-DNA binding of carboplatin observed in these experiments is due to a carboplatin-mesna ligand binding to DNA as a mono-functional species. To test this hypothesis one needs to measure specific Pt-DNA adducts such as Pt-GG, Pt-GA, Pt-G—G, and Pt-G. However this hypothesis is weakened based on unlikely probability that a negatively charged carboplatin-mesna ligand will enter the cells. Also only 22% of carboplatin bound to DNA has been reported to be mono-functionally bound Pt-G adduct (Blommaert et al., 1995).

Contrary to the results reported here, Natarajan and colleagues reported a significant increase in formation of Pt-DNA adducts for carboplatin in presence of thiourea (6-fold) and glutathione (3-4) fold as compared to controls in the absence of thiol groups (Natarajan et al., 1999). Remarkably, carboplatin displayed an increase in DNA binding in the presence of the very same S-containing nucleophiles that showed an expected quenching effect in the case of cisplatin (Natarajan et al., 1999). They proposed a thiol activation of carboplatin prior to binding DNA (Natarajan et al., 1999). The experiments by Natarajan and colleagues were done on isolated calf thymus DNA *in vitro* and differ from my results that examined DNA binding of carboplatin inside glioma cells. Mesna might act similar to GSH in activation of carboplatin but would be irrelevant to the results here since mesna can not enter glioma cells.

Since there was no reduction in Pt-DNA binding of carboplatin in co-incubation with mesna, this phenomenon was not pursued further in animals and instead the focus was placed on cisplatin and its interaction with mesna *in vivo*.

Cytotoxicity testing, Pt-DNA, and Pt-protein binding kinetics

These preliminary experiments were done to optimize dose and incubation time for more specific Pt-DNA binding experiments and the logical choices were stated in the results section.

Pt-drugs were cytotoxic to glioma cells. This result confirms earlier *in vitro* reports (Tallen et al., 2000; Wolff et al., 1998). A major barrier to the success of cisplatin is the ability of cells to repair DNA damage, which in turn depends on p53 status and on the integrity of certain cell-cycle check points (Briz et al., 2000). This DNA repair could be a possible explanation for resistance of U-87MG cells to cisplatin as compared to U-251MG cells. U-87MG cells are p53 wild-type whereas U-251MG cells are p53 mutant. Possibly U-87MG cells are able to repair DNA damage by cisplatin (up to a certain concentration), and therefore require higher LC_{50} concentrations of cisplatin as compared to U-251MG cells.

Ten times higher concentrations of carboplatin were required to reach comparable cytotoxicity as cisplatin. This result also confirms earlier reports (Doz et al., 1991; Tallen et al., 2000; Wolff et al., 1998). An interesting side phenomenon was that at comparable cytotoxic concentrations, carboplatin had four times more Pt bound to DNA as compared to cisplatin (fig 3.5a vs. c). This result challenges the view that carboplatin forms similar cytotoxic adducts as cisplatin (Blommaert et al., 1995) and that carboplatin only differs from cisplatin in the kinetics of interaction with DNA (Knox et al., 1986). This finding calls for more investigations into understanding the activation of carboplatin and its interaction with DNA and elucidating which DNA adducts are important in carboplatin's cytotoxic mechanism.

The ten fold difference seen in DNA binding between 100 μM cisplatin vs. 10 μM cisplatin (fig 3.3a) was not due to extracellular protein binding of the drug in supernatant. Surprisingly, protein binding of cisplatin was independent of drug concentration in the medium. Similar protein binding characteristics have been described for Pt-drugs previously (Pendyala and Creaven, 1993). A possible explanation for this phenomenon is that the tested concentrations of cisplatin in these experiments might have been in the maximum velocity (v_{max}) range of

Michaelis-Menten kinetics in their reaction with protein. Therefore, the reaction rate can be described as zero-order with respect to cisplatin concentrations tested here. Indeed the reaction rate might be dependent on the concentration of protein in the medium. Only 20-25% of Pt was protein bound in RPMI 1640/10% FCS (fig 3.4). In contrast protein binding of cisplatin in human serum is greater than 90% (see chapter five). This difference can be attributed to the concentration of protein in 10% FCS vs. in human serum. In 10% FCS the mean protein concentration was 3.1 mg/mL protein, whereas in patient serum the average protein concentration was 72 mg/mL protein.

Mesna reduced protein binding of cisplatin (fig 3.4). This means mesna is a better nucleophile toward cisplatin as compared to cysteine and methionine amino acids in protein. It would be interesting to see if protein binding of cisplatin in the presence of mesna is reduced in the clinical situation. If so, patients who receive mesna concurrently with cisplatin should have increased levels of free Pt in their serum. This phenomenon is dealt with in chapter five.

Mesna also reduced the initial rate of cisplatin's Pt-DNA binding (fig 3.3b). Such an event is important in situations *in vivo* since it could mean that even with mesna's short half-life of 9-12 minutes in the circulation, the Pt-DNA binding of cisplatin or its entry into cells might be compromised.

In summary, for the first time the Pt-DNA binding of cisplatin and carboplatin in presence of mesna has been investigated. Mesna reduced the Pt-DNA binding of cisplatin but not that of carboplatin in glioma cells. This result could mean that patients receiving concurrent cisplatin / oxazaphosphorine / mesna, might not be getting the full benefit of extensive combination chemotherapy treatment. Strengths of cell culture models are that many variables can easily be changed and basic phenomena explored. However in studies such as the one here, the pharmacokinetic characteristics of these agents are missed and therefore would need to be addressed in *in vivo* models. The next logical step was to examine Pt-DNA binding and cellular accumulation of cisplatin in the presence of mesna in an animal model.

Chapter Four: Influence of Mesna on Tissue, Tumor, and DNA Platinum Levels of Cisplatin in Tumor Bearing SCID/NOD Mice

Methods

Animals

Male SCID/NOD mice (Cross Cancer Institute, Edmonton, Alberta), 6-8 weeks of age, weighing 24-33 g were used. Thirty male SCID/NOD mice were implanted with subcutaneous U-87MG malignant glioma cells. Mice were housed in groups of five in plastic isolator cages with filter tops under specific pathogen-free conditions in the mouse barrier facility, Animal Resource Centre (ARC), (Health Sciences, University of Calgary). The animals were maintained at 22 °C with a 12 h light/dark cycle and fed on a standard diet and water *ad libitum*. All animal studies were conducted in accordance with the University of Calgary Animal Care Policy as prescribed by the animal care committee. This study was approved by the ethics committee, protocol #M00023.

Tumor Implantation

The U-87MG human malignant glioma xenograft in SCID/NOD mice has been described previously (Price et al., 1999). For s.c. injection into mice, U-87MG cells were grown to approximately 80% confluence in 1.75 cm^2 flasks (Corning), harvested, counted, rinsed twice in PBS, resuspended at 2.0 X 10^6 cells/50 µL in PBS, and kept on ice until injection. The cells were injected s.c. above the mid-femur region of the left hind limb of the mouse with a 26-gauge, ½ -inch needle. The site of injection was shaved with a small electric clipper (Sunbeam) the day before. For each injection, the cell suspension was drawn up to 200 µL in a one mL syringe, and a volume of 50 µL containing two million cells was injected. Thirty mice were injected in total and six weeks later 24 of the mice (80% tumor take) had tumors with average size of 0.6 cm^2.

Drug treatments

Cisplatin and mesna were diluted to 0.5 mg/mL and 50 mg/mL respectively in 0.9% NaCl saline. Drug doses were: cisplatin, 4 mg/kg i.p. (Vollmer et al., 1999), and mesna, 400 mg/kg i.p. (Allan et al., 1986). The mice with tumors were randomized into three groups of eight. Group A received cisplatin and saline, group B received cisplatin and mesna, using separate back to back injections (max. 30 s delay between injections), group C received a single injection of cisplatin mixed with mesna in the same syringe. Six remaining mice without tumors were used for evaluation of mesna in reducing nephrotoxicity of cisplatin. These mice group D, received cisplatin 4 mg/kg and three separate 400 mg/kg doses of mesna, five min before, 30 min after, and six h after cisplatin injection. The animals were sacrificed 24 h after cisplatin treatment by cervical dislocation while under halothane gas anesthetic.

Tissue preparations

Tumor, kidney, liver, and brain were quickly excised, frozen in liquid nitrogen, and stored at -80 °C until Pt measurements. Blood was collected by cardiac puncture while the animal was under halothane anesthetic. Plasma was separated by centrifugation at 5000 x *g* (Eppendorf microfuge) for ten minutes.

Isolation of genomic DNA from mouse tumor and tissue

DNA isolation was as described in chapter three, with the following modifications. Approximately 100 mg of tissue was taken for DNA extraction. The tissue was suspended in one mL of lysis buffer, and ground using a disposable pellet and pestle in a microtube (Knote, purchased from VWR Scientific). Typically 800 µg of DNA was recovered from 100 mg digested tissue.

Platinum measurements

All platinum measurements were done using AAS as described in chapter three with the following modifications. For DNA isolated from tumor and tissue, typically 500 µg and 300 µg of DNA respectively was digested in 500 µL of 50% HNO_3 by boiling at 120 °C for one hour. For total Pt levels in tissue, samples were weighed on an analytical balance and typically 120 mg for brain and 30 mg for other tissue was digested in 500 µL of concentrated HNO_3 at 120 °C for one hour. Plasma samples did not require nitric acid digestion, they were diluted 1/10 in 0.1% HNO_3. DNA samples were hot-injected into the furnace at 85 °C three times (multiple inject feature) at a volume of 20 µL per injection. Tissue and plasma samples were hot-injected at 80 °C at a single volume of 12 µL. The calibration range for DNA samples and tissue/plasma samples respectively were: (25 nmol/L Pt to 150 nmol/L Pt) and (250 nmol/L Pt to 1000 nmol/L Pt).

Statistical analysis

The data were summarized as means ± standard deviations where appropriate. Groups were statistically evaluated by the un-paired Student's t-test (two-tailed, equal or un-equal variance based on the F-test). The level of significance was taken as $P < 0.05$. Number of animals per group was determined by sample size calculation using the formula:

$n = 2\left[\frac{(Z_\alpha - Z_\beta)(SD)}{d}\right]^2$ (Dawson-Saunders and Trapp, 1994). Where, n = number of animals per group (calculated to be 8), α = type-I error, set at 0.05, β = type-II error, set at 0.1, therefore $Z\alpha$ = two-tailed Z value (1.96), and $Z\beta$ = lower one-tailed Z value (-1.28) (Dawson-Saunders and Trapp, 1994). Standard deviation (SD) was estimated from cell culture data = 1.5 units, and d =

difference worth detecting between control group (cisplatin) and mesna groups, based on cell culture data was 2.5 units.

Results

Influence of Mesna on tumor and tissue platinum concentrations in tumor bearing SCID/NOD mice

Tumor and tissue Pt concentrations in cisplatin (Group A, n = 8) and cisplatin plus mesna (Group B, n = 8) treated mice are compared in figure 4.1. There was no significant difference in Pt concentrations in tumors between the two groups 24 hours after drug treatment (4.1 ± 1.0 nmol Pt/g tumor vs. 3.6 ± 0.4 nmol Pt/g tumor, P=0.2217).

Similarly there was no difference in Pt concentrations in kidney (19.0 ± 3.1 vs. 18.1 ± 3.8 nmol Pt/g tissue, P=0.6118), liver (8.4 ± 3.3 vs. 6.9 ± 1.2 nmol Pt/g tissue, P=0.2615), and brain (0.1 ± 0.06 vs. 0.09 ± 0.02 nmol Pt/g tissue, P=0.6666) between the two groups (cisplatin vs. cisplatin plus mesna).

Among the analyzed tissue, Pt concentrations were highest in the kidney, and lowest in the brain.

Cisplatin was incubated with mesna for 24 hours prior to injection into mice in group C (cisplatin mixed with mesna group, n = 8). Total Pt concentrations in tumors were compared between the cisplatin group and the cisplatin mixed with mesna group (fig. 4.2). There was a significant difference in tumor Pt concentrations between the two groups (4.1 ± 1.0 nmol Pt/g tumor vs. 1.7 ± 0.9 nmol Pt/g tumor, P=0.0002), which was a four-fold drop in tumor Pt concentrations in cisplatin mixed with mesna group as compared to cisplatin group.

Similarly there was significantly less Pt measured in plasma of cisplatin mixed with mesna group as compared to cisplatin group (0.121 ± 0.030 nmol

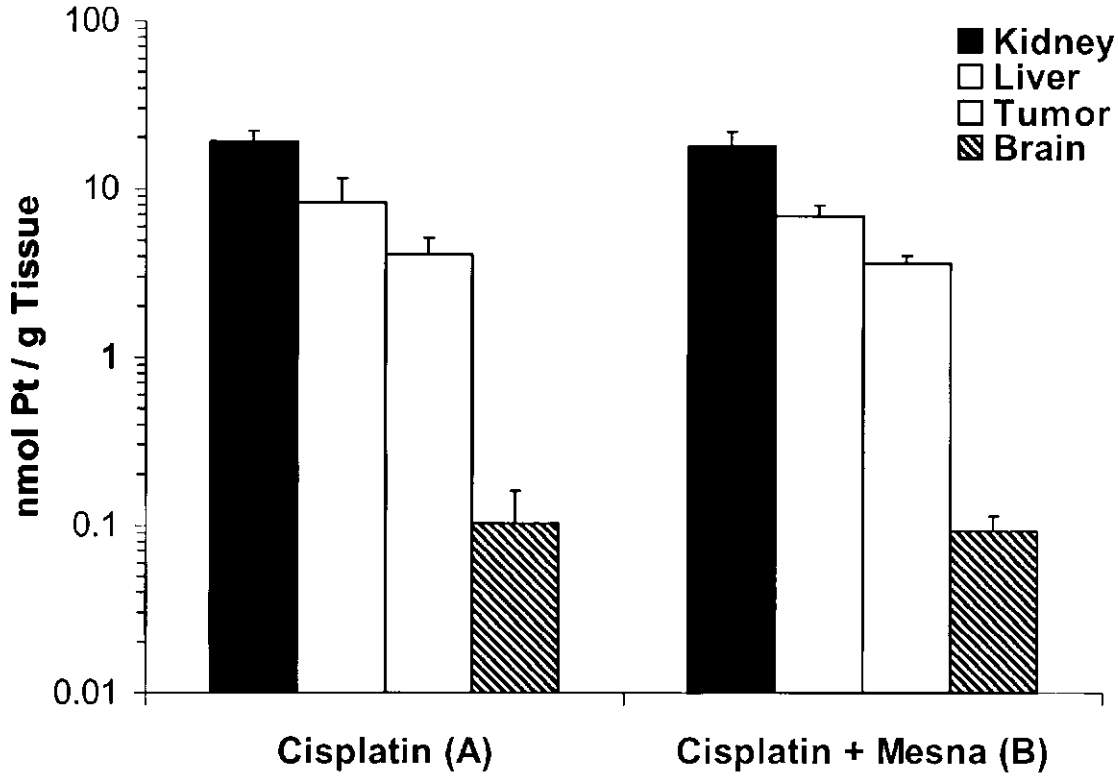


Figure 4.1. Comparison of various tissue and tumor Platinum concentrations in SCID/NOD mice treated with cisplatin alone (A) or cisplatin plus mesna (B). Pt levels were measured 24 hour after drug treatment. Cisplatin plus mesna group mice were given separate injections of each drug back to back, as quickly as possible (max. 30 second delay). There was no significant difference in Pt levels in any of the organs and tumors between the two groups. Mean values with SD represented, N = 8 mice per group.

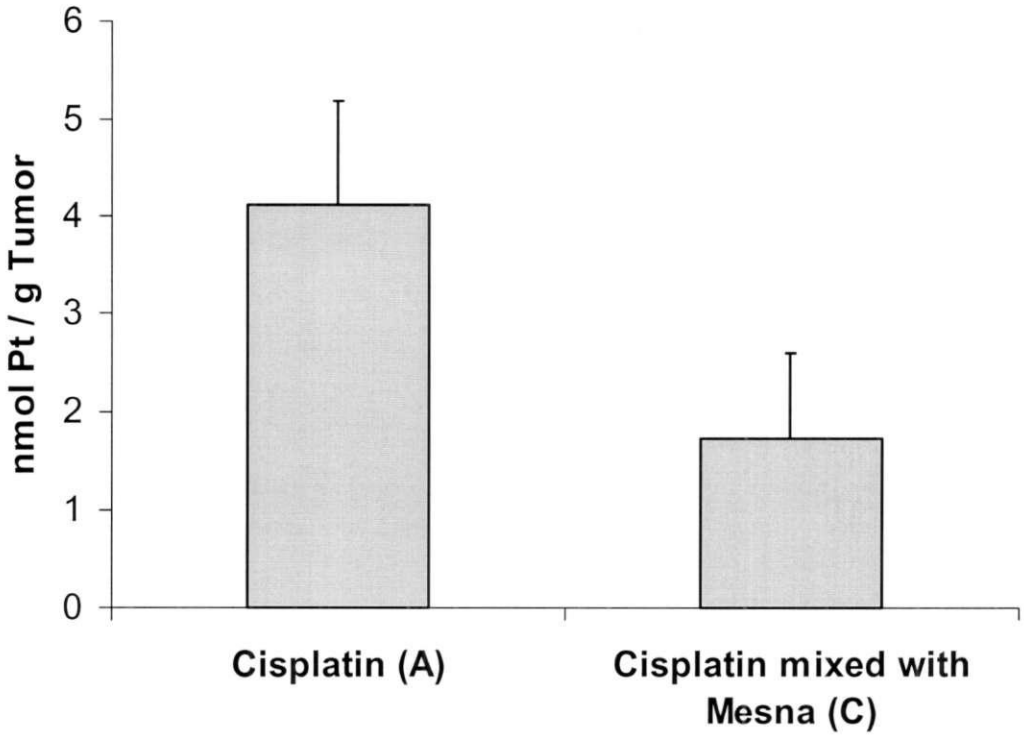


Figure 4.2. Platinum concentrations in tumors of mice injected with cisplatin alone (A) compared to mice given a single injection of cisplatin mixed with mesna (C). Pt levels were measured 24 hour after drug treatment. Cisplatin and mesna were incubated together in solution for 24 h prior to being injected into group C mice. There was a significant difference in tumor Pt levels between the two groups, P = 0.0002. Mean values with SD represented, N = 8 mice per group.

Pt/mL plasma vs. 1.42 ± 0.25 nmol Pt/mL plasma, P<0.0001), (fig 4.3). This meant approximately 90% of the Pt in group C mice was eliminated (excreted) by 24 hours, most likely because it was bound to mesna.

Influence of Mesna on tissue DNA and tumor DNA platinum concentrations in tumor bearing SCID/NOD mice

As for tissue Pt concentrations, there was no significant difference in Pt bound to DNA in kidney (203.3 ± 131.5 vs. 147.7 ± 58.2 fmol Pt/µg DNA, P=0.3025), liver (115.0 ± 47.8 vs. 133.5 ± 35.1 fmol Pt/µg DNA, P=0.3925), and tumor DNA (16.9 ± 5.6 vs. 15.4 ± 8.2 fmol Pt/µg DNA, P=0.6757) of cisplatin group vs. cisplatin plus mesna group (fig. 4.4).

However there was a two-fold reduction in Pt bound to DNA of tumors in cisplatin mixed with mesna group as compared to cisplatin group, which was significant (8.1 ± 4.0 vs. 16.9 ± 5.6 fmol Pt/µg DNA, P=0.0033), (fig 4.5).

Again, as for tissue Pt levels, the highest Pt concentrations were found in kidney DNA among the analyzed DNA samples. There was no Pt detected on DNA from brain samples, most likely because they were below the detection limit of our method.

Influence of mesna on renal toxicity of cisplatin

The renal toxicity of cisplatin was evaluated as the amount of Pt bound to DNA isolated from kidney. If lower concentrations of Pt were detected in cisplatin with mesna groups as compared to cisplatin alone with a significant difference, this would have been considered as renal protection by mesna. However there were no statistically significant reductions in Pt-DNA binding in kidneys in any of the mesna group mice (group B, group C, & group D) as compared to cisplatin group A mice (fig. 4.6).

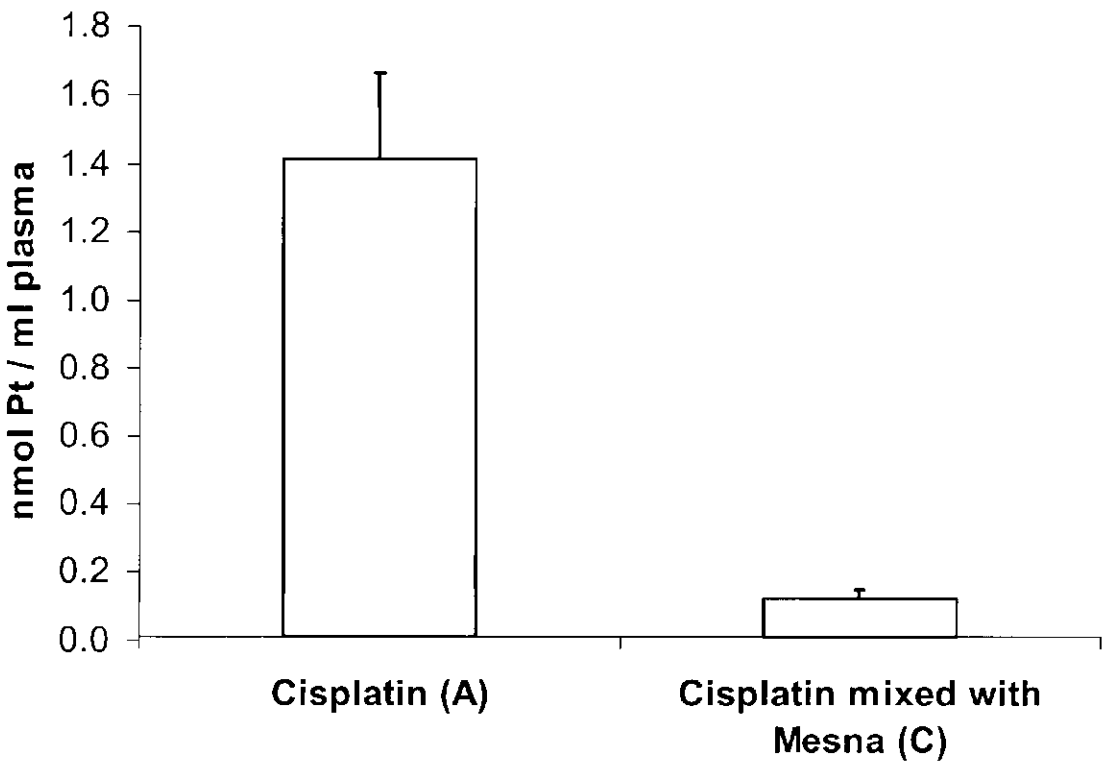


Figure 4.3. Comparison of plasma platinum concentrations in mice treated with cisplatin alone (A) or cisplatin mixed with mesna (C). Plasma Pt concentrations are 24 hour after drug treatment. There was a significant difference in plasma Pt levels between the two groups, $P < 0.0001$. Mean values with SD represented, N = 8 mice per group.

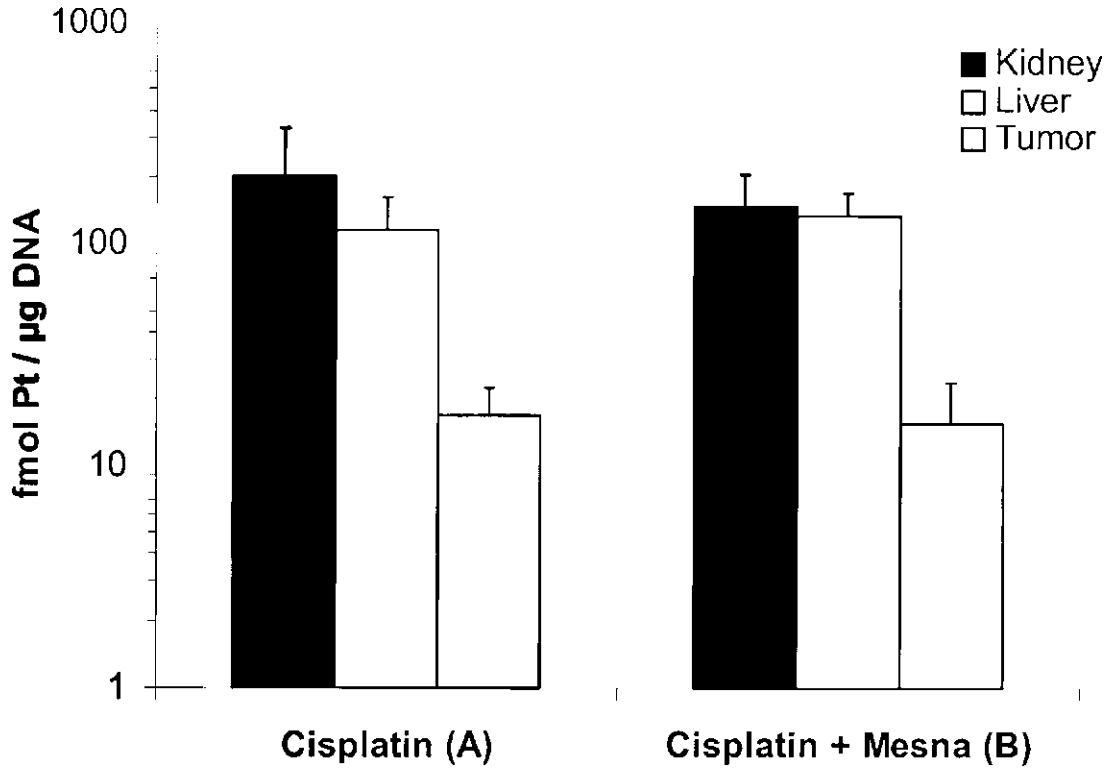


Figure 4.4. Comparison of Platinum bound to DNA isolated from tissue and tumors of mice treated with cisplatin (A) or cisplatin plus mesna (B). DNA Pt levels are 24 hour after drug treatment. As before, group B mice received back to back injections of each drug as quickly as possible. Mean values and SD represented, N = 8 mice per group.

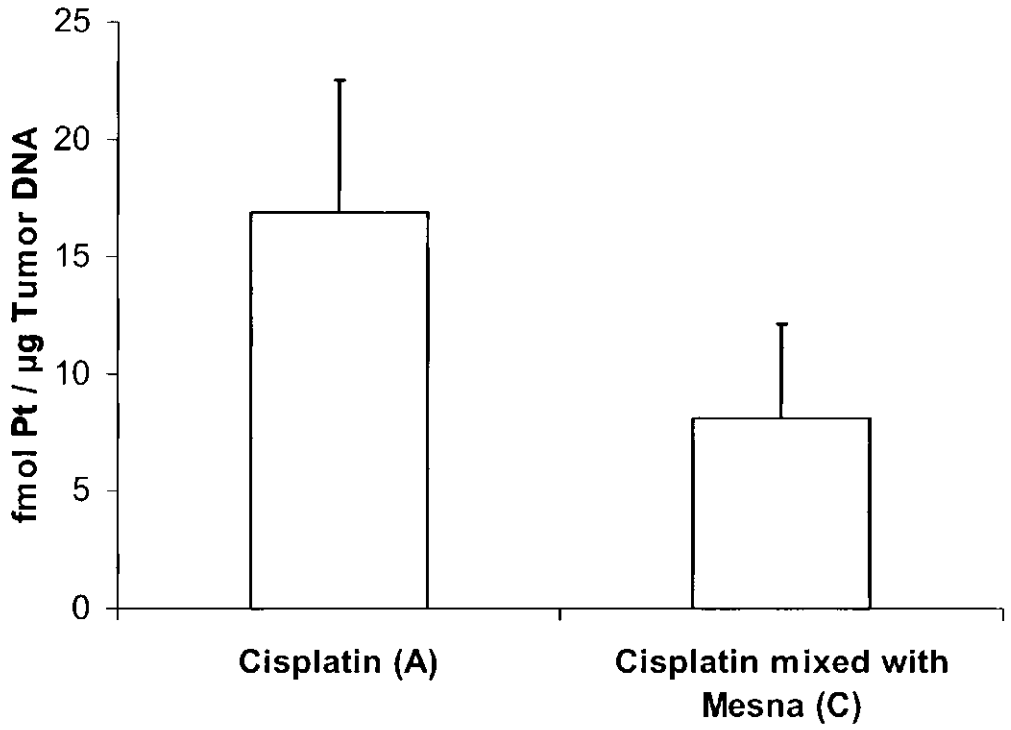


Figure 4.5. Platinum bound to tumor DNA in mice treated with cisplatin (A) or cisplatin mixed with mesna (C). Experimental conditions were as in figure 4.2. There was a significant difference in Pt DNA levels between the two groups, P = 0.0033. Mean values and SD represented, N = 8 mice per group.

Surprisingly even cisplatin mixed with mesna group (group C) was not significantly different from cisplatin group (186 ± 67 fmol Pt/μg DNA vs. 203 ± 131 fmol Pt/μg DNA, P=0.7506) in Pt levels bound to kidney DNA. This result was peculiar since as already mentioned, group C mice did have significantly lower Pt-DNA levels in tumor and also low plasma Pt levels as compared to cisplatin group mice (figures 4.5 and 4.3 respectively).

Overall it appeared that mesna did not offer any protection against renal toxicity of cisplatin as determined by this assay.

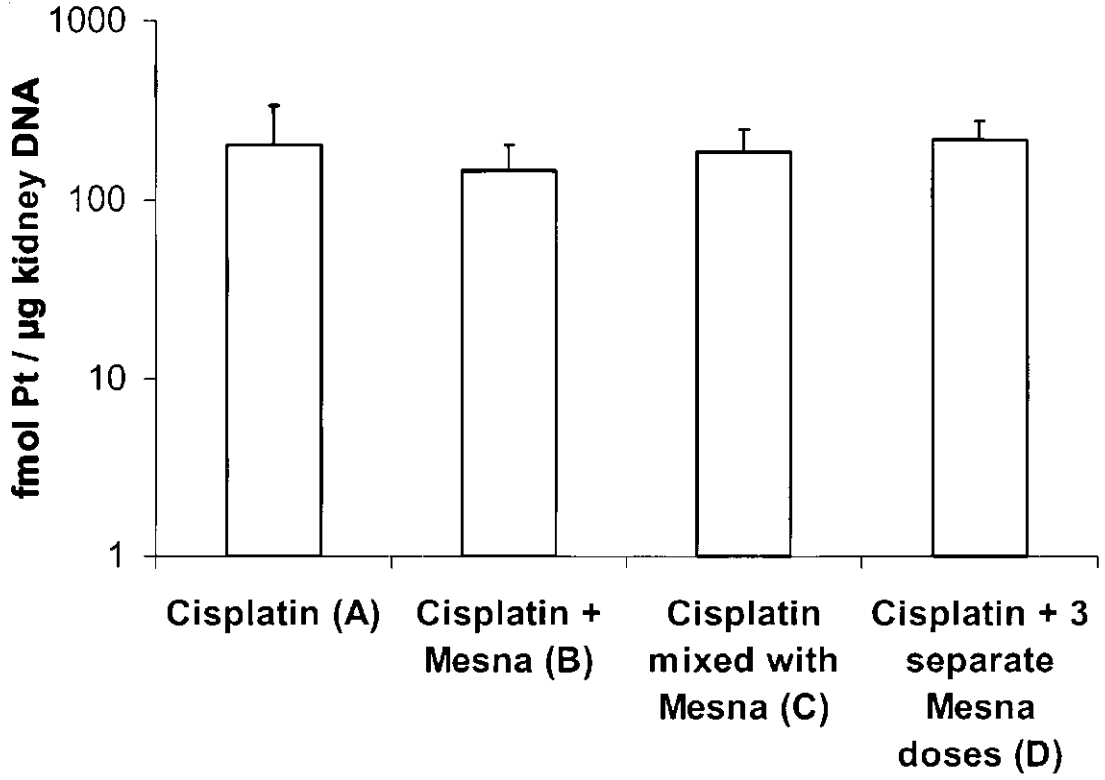


Figure 4.6. Effect of mesna on Platinum DNA binding of cisplatin in kidney. Group D mice received one mesna injection 5 minutes before the cisplatin injection, cisplatin injection at time zero, a second mesna injection 30 minutes after cisplatin, and a third mesna injection 6 hours after the cisplatin injection. Pt levels were measured 24 h after the cisplatin injection. Mean and SD represented with N = 8 mice per group for groups A-C and N = 6 mice for group D.

Discussion

Contrary to the hypothesis and different from *in vitro* results, when cisplatin and mesna were administered to tumor bearing SCID mice at the same time, tumor, tissue and Pt-DNA levels were not reduced as compared to cisplatin treated controls. Only when cisplatin and mesna were mixed prior to administration, a significant reduction in total tumor Pt and Pt-DNA levels were observed.

These results are reasonable based on pharmacokinetics of mesna and its reaction rate with cisplatin. After i.p. injection, 90% of mesna undergoes rapid oxidation in plasma and circulates as the chemically inert disulfide dimesna (Brock et al., 1982). Dimesna is extremely un-reactive toward cisplatin (Leeuwenkamp et al., 1991). Mesna and dimesna can not enter cells (Ormstad and Uehara, 1982) and therefore do not interfere with intracellular Pt-DNA binding of cisplatin. Additionally, mesna has a short half-life of 16.5 min in circulation (in rats) (Shaw et al., 1986), hence the remaining 10% mesna in circulation does not have enough time to react with cisplatin in a significant magnitude as supported by results presented here.

Similar *in vivo* results have been reported previously investigating antitumor efficacy of cisplatin in presence of mesna (Allan et al., 1986; Dorr and Lagel, 1989; Wagner et al., 1988). Antitumor efficacy of cisplatin in L1210 leukemia bearing mice was not affected by co-administration of mesna (Allan et al., 1986; Wagner et al., 1988). The experiments here led to a similar conclusion for the first time in a solid tumor model. Dorr and Lagel reported that when mesna was mixed directly with cisplatin, it reduced cisplatin's antitumor effect in mice bearing P-388 leukemia, but when mesna was administered five minutes after cisplatin it did not reduce cisplatin's antitumor efficacy (Dorr and Lagel, 1989). I found a reduction in total tumor Pt levels and cisplatin DNA binding in tumors when cisplatin and mesna were incubated together for 24 h and then injected into the animals. This experiment replicated a clinical scenario were

cisplatin and mesna are infused into patients through the same infusion line in 24 h infusion protocols. My results indicate that these patients are not getting the full benefit of their cisplatin treatment. Therefore such practice must be discontinued by modifying such protocols so that cisplatin and mesna do not come in contact together in solution and separate infusion lines should be used for each drug.

Nephrotoxicity is one of the main dose limiting factors in clinical cisplatin therapy. Since mesna does not alter the antitumor efficacy of cisplatin and since kidney cells are one of the only cell types that mesna is able to enter, it is possible that mesna might protect against nephrotoxicity of cisplatin. My results did not support this assumption as demonstrated by Pt-DNA binding of cisplatin in kidney (fig 4.6). Similar conclusions had been reached previously based on histological examinations of kidney after cisplatin/mesna treatment (DeWoskin and Riviere, 1992; Millar et al., 1985). Interestingly, unlike tumor DNA and plasma Pt, kidney Pt-DNA levels in cisplatin mixed with mesna group were not reduced (fig 4.6). This result could mean that cisplatin-mesna metabolites enter kidney cells and are able to bind DNA. Such a result supports the hypothesis of sulfhydryl activation of Pt-drugs in binding DNA (Natarajan et al., 1999).

One might argue that mesna did not reduce cisplatin's efficacy in this study because mesna was administered i.p. and that the outcome could have been different if mesna was given intravenously. Ormstad and colleagues found similar blood concentrations of mesna and dimesna after i.p. or i.v. administration of mesna (Ormstad et al., 1983). Mesna has a fast rate of absorption from the peritoneal cavity. Peak plasma concentrations were reached ten minutes after i.p. administration of mesna (Ormstad et al., 1983). Furthermore, the slope of disappearance curves for plasma mesna and dimesna after i.p. administration were similar to curves obtained after i.v. administration (Ormstad et al., 1983). Therefore, the route of administration of mesna is unlikely to change the outcome of the results reported here.

The highest Pt levels were detected in kidney and kidney DNA. This result was consistent with previous findings *in vivo* (Johnsson et al., 1995; Yoshida et

al., 1994) and is logical since renal excretion is the only route of cisplatin elimination. The lowest Pt levels were detected in brain, most likely due to cisplatin's difficulty in crossing the blood brain barrier.

Plasma Pt levels, similar to tumor Pt and tumor Pt-DNA were significantly reduced when cisplatin was mixed with mesna prior to injection (fig 4.3). Based on plasma Pt levels, 90% of the Pt was eliminated by 24 h. This finding is consistent with the elimination half-life of mesna but not for cisplatin alone. Most likely, cisplatin is bound to mesna so it can not enter cells. Therefore it is excreted from the body as fast as mesna's clearance. This means that Pt levels in plasma can be used as a nice model for predicting Pt tumor levels. One such model will be to compare pharmacokinetics of Pt-drugs in patients with or without concurrent mesna administration.

In summary my results demonstrated that mesna did not influence Pt accumulation and DNA binding of cisplatin in tumor and tissue of mice except when it was mixed directly with cisplatin prior to administration. These findings were contrary to the results from *in vitro* experiments in the same malignant glioma cells and can be explained based on pharmacokinetic properties of mesna *in vivo*.

A typical pediatric patient receiving cisplatin and mesna is given 20 mg/m^2 cisplatin and 4 X 500 mg/m^2 doses of mesna (based on oxazaphosphorine dose). Here we treated mice with a similar 1:100 cisplatin : mesna ratio and found that mesna does not reduce the activity of the Pt-drug. This outcome brings us one step closer to reassurance in safety of combining cisplatin with mesna in combination chemotherapy protocols, provided the two drugs are not mixed together in solution. Possibly it means that these patients are getting the full benefit of their Pt-drug treatment. However one can not extrapolate to humans based on the results from mice, therefore the next logical step was to examine pharmacokinetics of Pt-drugs in combination with mesna in patients.

Chapter Five: Influence of Mesna on Pharmacokinetics of Cisplatin and Carboplatin in Pediatric Patients

Methods

Patients

Twenty-nine pediatric patients receiving cisplatin or carboplatin, with or without mesna, at the Oncology Unit of the Southern Alberta Children's Hospital (Calgary, AB, Canada) were entered into this study. The patients received their chemotherapy as part of established protocols, hence clinical treatment decisions for the participating patients were not affected by this study. All patients and/or their parents gave informed consent prior to entering the study. The study protocol was approved by the Child Health Research Committee of the Southern Alberta Children's Hospital and by the Conjoint Health Research Ethics Board of the Faculty of Medicine, University of Calgary. Details of treatment, patient characteristics, and diagnosis for each patient are summarized in table 5.1.

Sampling

Blood samples were obtained from a central line prior to infusion and 1, 4, 8, 12, and 24 h after the start of the Pt-drug infusion. Blood (3.0 mL) was collected into non-heparinized tubes and serum prepared within 10 minutes of sample collection by centrifugation at 1000 x *g* for 10 minutes at 4 °C. Serum was then removed and 400 µL placed in an Amicon Centrifree device for preparation of protein free serum ultrafiltrates as described in chapter 3. Serum and serum ultrafiltrates were stored at –20 °C until analyzed (< 1 week) for total and free platinum concentration.

From two babies in the study, patients #20 and #24 only 1.0 mL of blood was collected at time points 0, 1, and 24 hour.

Table 5.1. Characteristics of Pediatric Patients Studied. Patient #6 was analyzed twice, once on day one of cisplatin treatment (6A), and again on day five (6B). Patient #10 was also analyzed twice, once on day one of carboplatin treatment (10A), and again on day two (10B). Serum creatinine and serum proteins were determined at the clinical laboratory before the Pt treatment. Abbreviations: F, female; M, male; BW, body weight; SCr, serum creatinine; C, cisplatin; CB, carboplatin; VP, etoposide; IF, ifosfamide; BL, bleomycin; VC, vincristine; LO, lomustine; MTX, methotrexate, 5-FU, fluorouracil; DOX, doxorubicin; AM, amifostine; TO, topotecan.

[a] Three 500 mg/m^2 doses of mesna over 15 minute infusions, 1, 5, and 8 hours after end of the carboplatin infusion.

[b] Three 500 mg/m^2 doses of mesna over 15 minute infusions, 2, 6, and 9 hours after end of the cisplatin infusion.

[c] Six 360 mg/m^2 doses of mesna: mesna from time –1 to 0 relative to start of carboplatin infusion; mesna 1 hr after end of the carboplatin infusion over 3 hrs; mesna 4 hrs and 20 minutes after end of carboplatin infusion over 15 minutes; mesna 7 hrs after end of carboplatin infusion over 15 minutes; mesna 10 hrs after end of carboplatin infusion over 15 minutes; and mesna 23 hrs after end of carboplatin infusion over 1 hr.

[d] Six 360 mg/m^2 doses of mesna: mesna 1 hr after end of the carboplatin infusion over 3 hrs; mesna 4 hrs after end of the carboplatin infusion over 15 minutes; mesna 7 hrs after end of carboplatin infusion over 15 minutes; mesna 10 hrs after end of carboplatin infusion over 15 minutes; mesna 23 hrs after end of carboplatin infusion over 1 hr; and mesna 23.5 hrs after the end of the carboplatin infusion over 3 hrs.

[e] Five 360 mg/m^2 doses of mesna: mesna 1 hr before start of the carboplatin infusion over 1 hr; mesna at the same time as carboplatin infusion over 3 hrs; mesna 4 hrs after end of the carboplatin infusion over 15 minutes; ; mesna 7 hrs after end of carboplatin infusion over 15 minutes; and mesna 10 hrs after end of carboplatin infusion over 15 minutes.

[f] Six 12 mg/kg doses of mesna: mesna at the same time as carboplatin infusion over 1 hr; mesna 1 hr after end of the carboplatin infusion over 3 hrs; mesna 5 hrs after end of the carboplatin infusion over 15 minutes; mesna 8 hrs after end of carboplatin infusion over 15 minutes; mesna 11 hrs after end of carboplatin infusion over 15 minutes; and mesna 24 hrs after end of carboplatin infusion over 1 hr.

[g] Two 500 mg/m^2 doses of mesna: mesna 6 hrs after end of the cisplatin infusion over 15 minutes; and mesna 10 hrs after end of the cisplatin infusion over 15 minutes.

[h] Five 360 mg/m^2 doses of mesna: mesna at the same time as carboplatin infusion over 3 hrs; mesna 4 hrs after end of the carboplatin infusion over 15 minutes; ; mesna 7 hrs after end of carboplatin infusion over 15 minutes; mesna 10 hrs after end of carboplatin infusion over 15 minutes; and mesna 24 hrs after end of the carboplatin infusion over 3 hrs.

[i] 300 mg/m^2 mesna 1 hr after end of the cisplatin infusion over 15 minutes.

Table 5.1. Characteristics of pediatric patients studied.

Patient No.	Pt drug, Dose (mg/m^2)	Mesna Dose (mg/m^2)	Pt drug Infusion length (h)	Age (years)	Sex	BW (kg)	Surface Area (m^2)	SCr (µmol/L)	Total Serum Protein (g/L)	Day in cycle	Concurrent Chemotherapy	Diagnosis
1	CB, 150	1500 [a]	1	13	M	29.3	1.04	39	61	1	VP, IF	astrocytoma
2	C, 20	0	1	16	F	65.6	1.81	78	70	1	VP, BL	ovarian germ cell
3	C, 75	0	6.4	16	F	59.9	1.68	68	68	1	VC, LO	choroid plexus
4	C,75	0	6	14	F	33.4	1.17	52	69	2	VC	medulloblastoma
5	C,100	0	6	17	F	33.6	1.18	50	63	2	MTX, 5-FU	nasopharyngeal carcinoma
6A	C, 20	1500 [b]	1	6	F	26.2	0.94	42	62	1	VP, IF	brain stem glioma
6B	C, 20	0	1	6	F	26.2	0.94	41	63	5	none	brain stem glioma
7	C, 20	0	1	13	F	47.0	1.47	53	63	1	VP	glioblastoma multiforme
8	CB, 500	0	5	15	M	62.8	1.78	70	61	1	VP	neuroblastoma stage IV
9	C,40	0	1	15	M	65.9	1.82	67	62	2	VP	neuroblastoma stage IV
10A	CB,100	2160 [c]	1	12	F	35.6	1.20	54	na	1	VP, IF	medulloblastoma
10B	CB,100	2160 [d]	1	12	F	35.6	1.20	54	na	2	VP, IF	medulloblastoma
11	CB, 400	0	1	16	F	52.9	1.58	55	65	1	VP	osteosarcoma
12	C, 40	0	1	6	F	25	0.95	43	61	4	VP	neuroblastoma
13	C, 40	0	23	13	M	65	1.73	38	67	1	VP	chest germ cell tumor
14	CB, 300	0	1	2	M	12.8	0.57	35	65	1	VC	not known
15	CB, 400	1800 [e]	1	15	M	54.3	1.63	52	63	1	VP, IF	osteosarcoma
16	C, 75	0	6	9	F	27.2	1.02	43	68	1	VC, LO	medulloblastoma
17	C, 120	0	4	13	F	39.0	1.34	51	54 (low)	1	DOX	osteosarcoma
18	CB, 560	0	1	11	F	27.3	1.01	44	68	1	AM	hepatoblastoma
19	C, 30	0	23.5	8	M	27.0	1.00	38	61	1	TO	Ewings stage IV
20	CB, (10mg/kg)	72 (mg/kg) [f]	1	0.42	F	6.0	0.32	na	na	1	VP, IF	not known
21	C, 20	1000 [g]	1	13	M	66.4	1.76	46	na	1	VP, IF	brain stem glioma
22	CB, 400	1800 [h]	1	11	M	39.6	1.30	44	81	1	VP, IF	Ewings sarcoma
23	C, 20	0	1	8	F	32.5	1.06	43	70	1	VP, TO	brain stem glioma
24	CB, (18mg/kg)	0	1	0.25	M	5.8	na	22	54	1	VP, DOX	neuroblastoma
25	C, 40	0	1	2	F	14.4	0.60	14 (low)	na	1	VP	neuroblastoma stage IV
26	C, 20	0	1	10	M	39.7	1.28	51	76	1	VP, TO	glioblastoma multiforme
27	C, 20	300 [i]	1	8	M	35.9	1.20	38	70	1	VP, IF	intracranial germcell tumor
28	C, 20	0	1	10	M	41.1	1.30	38	na	1	VP, TO	glioblastoma multiforme
29	CB, 560	0	1	3	F	15.7	0.66	27	67	1	VP	neuroblastoma stage IIB

In eleven children, urine samples were collected as 0-12 h and 12-24 h pools. Total volume for each urine pool was recorded, and a 10 mL aliquot from each pool was stored at –20 °C until analyzed for platinum content.

Platinum analysis

Platinum drugs were measured as elemental Pt by atomic absorption spectrometry (AAS) as described in chapter 3, with the following modifications. Total Pt serum concentrations were determined after a 1:10 dilution with 0.1% HNO_3. Ultrafiltrate Pt concentrations were measured without dilution. Urine samples were diluted 1:10 or 1:100 when necessary in 0.1% HNO_3.

A separate calibration curve was run for each matrix with the calibrators made in the appropriate matrix (i.e. Pt was spiked into 1:10 blank serum or blank urine and H_2O for ultrafiltrate samples). Total serum Pt samples and urine samples were hot-injected into the furnace at 80 °C at a single volume of 12 µL per injection and free serum Pt samples were hot-injected at the same temperature but at a single volume of 20 µL. A standard curve covering the range of 250 to 1000 nmol/L Pt, and 50 to 500 nmol/L Pt was analyzed with each batch of total Pt, urine samples and free Pt samples respectively. A quality control sample was run before and after an analysis batch with a tolerance of ±15% for acceptance of the results. No more than eight samples were analyzed with one calibration curve.

The limit of detection for total Pt in serum and urine samples was 10.0 ng/mL (50.0 nmol/L) elemental Pt and 1.0 ng/mL (5.0 nmol/L) for free Pt samples.

Pharmacokinetic analysis

Both non-compartmental (model independent) and compartmental (model dependent) methods of analysis were used to describe pharmacokinetics of total and free Pt for cisplatin and carboplatin.

Using non-compartmental analysis, the area under the serum concentration-time curve from time zero to 24 h (AUC_{0-24}), total serum clearance from 0 to 24 h (CL_{24}), apparent volume of distribution (V), cumulative percentage of free Pt excreted in urine from time zero to 24 h (Ae%), and renal clearance (CL_R) values were calculated from the following equations (Gibaldi and Perrier, 1982):

$$AUC_{0-24} = \sum\left[\frac{(C_{n-1} + C_n)(t_n - t_{n-1})}{2}\right]$$

$$CL_{24} = \frac{D}{AUC_{0-24}}$$

$$V = \frac{D}{C_{max}}$$

$$A_e\% = \frac{A_e}{D} \times 100$$

$$CL_R = \frac{A_e}{AUC_{free\,0-24}}$$

in which C is the concentration at time t for serum samples at n time points, C_{max} is the maximum serum Pt concentration at the end of the infusion, D is the dose of elemental Pt, Ae is the cumulative amount of Pt excreted in urine from time zero to 24 h, and AUC_{free} is the area under the curve for protein free Pt.

Pharmacokinetic parameters were also determined by fitting a two-compartment open model with exponential decay using the ADAPT program (release IV) kindly supplied by Drs D'Argenio and Schumitzky (Biomedical Simulations Resource, University of Southern California, Los Angeles, California,

U.S.A.). A two-compartment pharmacokinetic model with constant-rate intravenous infusion (fig 5.1) was fitted to each data set for both total and free Pt. The following parameters were estimated using the model: Volume of the central compartment (*Vc*), transfer rate constants between the central and peripheral compartments (K_{cp}) & (K_{pc}), elimination rate constant (K_{el}), negative slope of the log-linear serum concentration vs. time curve during the initial distribution phase (α), and elimination phase (β). Alternatively these parameters can be estimated by the method of residuals as described by Gibaldi (pp. 433-443), (Gibaldi and Perrier, 1982).

Additionally the following model dependent parameters were calculated from the following equations (Gibaldi and Perrier, 1982):

$$CLt = Kel \times Vc$$

$$AUC_{0-\infty} = \frac{D}{CLt}$$

$$Vp = Vc \times \left(\frac{Kcp}{Kpc}\right)$$

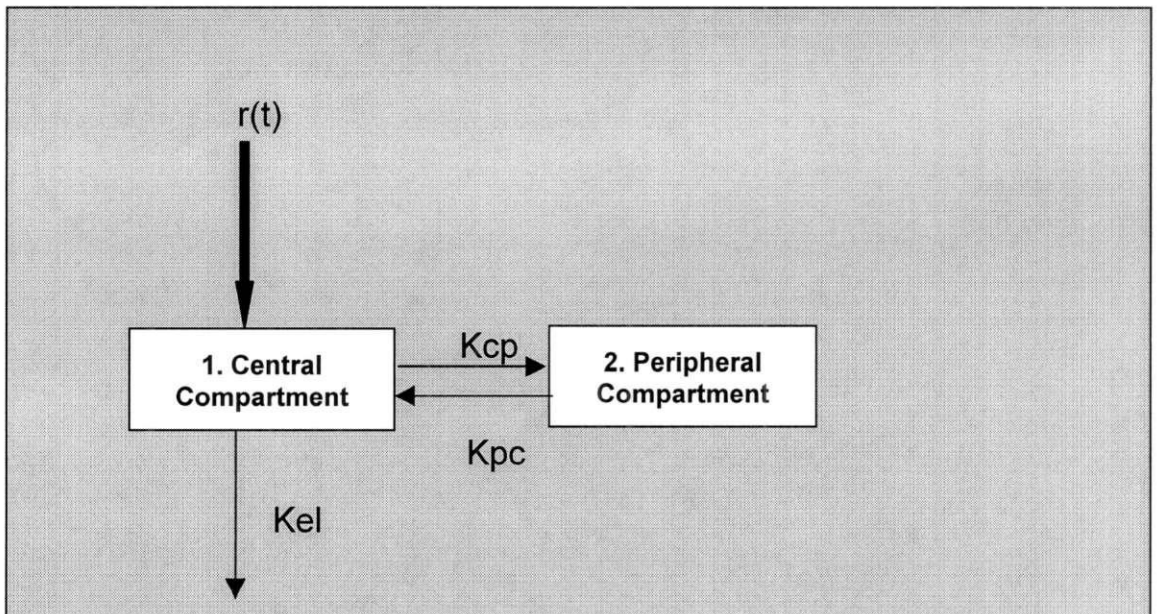
$$Vss = Vc + Vp$$

$$MRT = \frac{Vss}{CLt}$$

$$t_{1/2\alpha} = \frac{\ln 2}{\alpha}$$

$$t_{1/2\beta} = \frac{\ln 2}{\beta}$$

in which *CLt* is the total serum clearance, $AUC_{0-\infty}$ is the area under the serum concentration-time curve from time zero to infinity, *Vp* is the volume of distribution in the peripheral compartment, *Vss* is the apparent volume of distributions during steady-state, MRT is the mean residence time of Pt in serum, and $t_{1/2\alpha}$, $t_{1/2\beta}$ are the distribution and elimination half-lives for Pt respectively.



$$\frac{dx_1(t)}{dt} = -(K_{el} + K_{cp})x_1(t) + K_{pc}x_2(t) + r(t)$$

$$\frac{dx_2(t)}{dt} = K_{cp}x_1(t) - K_{pc}x_2(t)$$

Figure 5.1. Two compartment open pharmacokinetic model. The variables $x_1(t)$ & $x_2(t)$ represent the amount of drug in compartments one and two. Compartment one represents Pt in blood and compartment two represent Pt in tissue which includes Pt serum protein binding for free Pt species. The variable r(t) represents the rate of infusion of Pt into compartment one. The two differential equations describe the whole model but only the first equation is solved in the ADAPT program for x_1 .

Statistical analysis

The data were summarized as means ± standard deviations where appropriate. The data were checked for normal distribution by InStat software (Ver. 3, GraphPad Inc.), and the pharmacokinetic parameters were compared between the two groups by the un-paired Student's t-test (two-tailed, equal or un-equal variance based on the F-test). The level of significance was taken as $P < 0.05$.

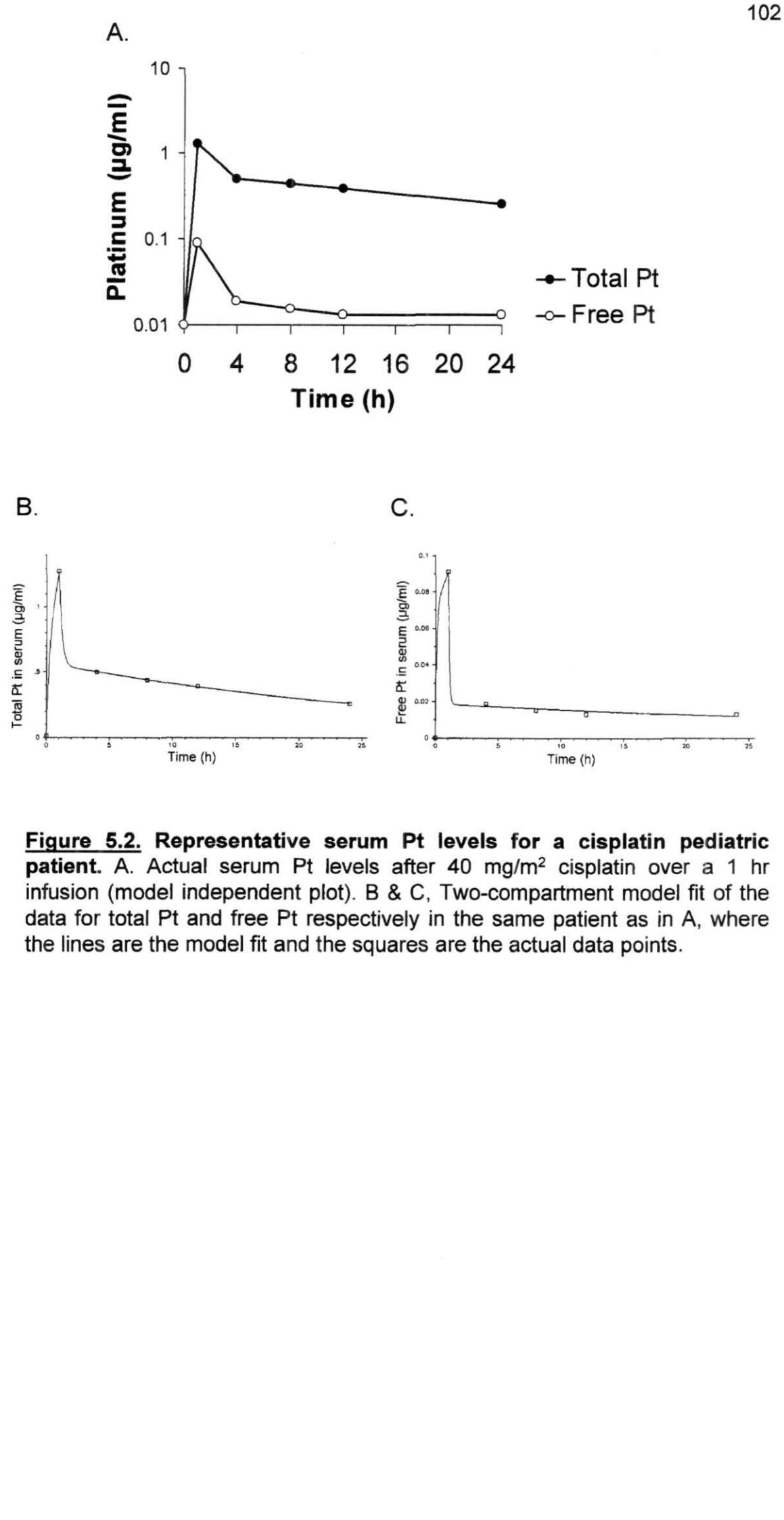
Results

Pharmacokinetics of cisplatin in children

Total Pt and free Pt concentrations were measured in the serum of 19 pediatric patients following the administration of cisplatin at doses of 20-120 mg/m^2 and various infusion schedules as indicated in table 5.1. Patient #6 was analyzed twice. Once on day one of the cisplatin cycle (which included mesna) and once on day five of the same cisplatin cycle (without mesna). Maximum serum Pt concentration and AUC for this patient on day five indicated accumulation of the cisplatin dose.

Figure 5.2a illustrates total and free Pt concentrations in the serum of a child during and after a 1-hour infusion of 40 mg/m^2 cisplatin. The pharmacokinetic parameters of the 19 patients determined by compartmental and non-compartmental analysis are listed in tables 5.2 and 5.3 for total and free Pt respectively.

Peak serum total and free Pt concentrations were observed at the end of the infusion and ranged from 0.69 to 4.14 µg/mL (3.5 to 21.2 µmol/L) and 0.025 to 0.205 µg/mL (0.13 to 1.05 µmol/L) respectively. Levels of both Pt species declined bi-exponentially, hence the two compartment model fit the data well in most cases (fig 5.2b-c). This provided for the calculation of half-life of distribution



<u>Figure 5.2.</u> Representative serum Pt levels for a cisplatin pediatric patient. A. Actual serum Pt levels after 40 mg/m^2 cisplatin over a 1 hr infusion (model independent plot). B & C, Two-compartment model fit of the data for total Pt and free Pt respectively in the same patient as in A, where the lines are the model fit and the squares are the actual data points.

Table 5.2. Pharmacokinetic summary of total Pt for cisplatin patients.

	Compartmental analysis									Non-compartmental analysis		
Patient	*Cmax* (μg/ml)	*Cmax* (μmol/L)	AUC* (mg.min/ml)	*CLt** (ml/min)	*Vc** (L)	*Vss** (L)	$t_{1/2\alpha}$* (min)	$t_{1/2\beta}$* (h)	MRT* (h)	AUC_{0-24} mg.min/ml	CL_{0-24} (ml/min)	*V* (L)
2	1.51	7.74	4.2	5.6	9.2	29.5	14	61	87	1.2	19.3	15.6
3	3.80	19.48	4.5E+03	1.8E-02	19.9	524.3	29	3E+05	5E+05	2.2	37.8	21.6
4	2.55	13.07	1.4	41.5	34.2	158.2	18	45	64	2.4	24.0	22.4
5	3.10	15.89	3.1	24.3	169.1	297.5	38	142	204	3.7	21.0	24.7
6A	1.36	6.97	1.7	7.0	8.5	20.3	53	35	48	0.9	13.3	9.0
6B	2.94	15.07	1.7E+08	7.3E-08	11.1	79.3	234	1E+10	2E+10	3.1	3.9	4.2
7	0.73	3.74	1.8	10.6	18.8	55.2	35	61	87	0.5	41.9	26.3
9	2.72	13.94	8.3	5.7	11.7	30.9	14	63	90	2.4	19.4	17.4
12	3.65	18.71	1.6E+07	1.6E-06	12.9	49.2	192	4E+08	5E+08	3.5	7.2	6.8
13	1.06	5.43	4.6E+04	9.8E-04	na	587.9	1	3E+07	1E+07	1.2	39.2	42.5
16	2.15	11.02	2.0	24.6	9.7	164.4	4	78	112	2.1	23.2	23.1
17	4.14	21.22	5.3E+02	0.2	56.2	177.8	57	1E+04	2E+04	3.7	27.9	25.2
19	0.69	3.54	na	na	na	na	na	na	na	0.6	30.7	28.2
21	0.93	4.77	2.7	8.5	21.4	44.7	52	61	87	0.7	32.4	24.6
23	0.95	4.87	1.7	8.2	13.0	32.7	39	47	66	0.7	19.6	14.5
25	1.28	6.56	1.0	14.9	4.2	26.4	9	21	30	0.6	24.2	12.2
26	1.30	6.66	5.8	2.9	7.0	26.7	13	107	154	1.1	15.7	12.8
27	1.14	5.84	2.7	5.7	17.4	40.8	89	85	119	0.9	17.1	13.7
28	0.96	4.92	2.1	8.1	13.9	47.4	56	70	97	0.5	33.3	17.6
Mean	1.95	9.97	3.0	12.9	26.0	75.0	34	67	96	1.7	23.7	19.1
SD	1.14	5.84	2.0	11.0	43.7	82.2	25	31	45	1.2	10.4	9.0
Max	4.14	21.22	8.3	41.5	169.1	297.5	89	142	204	3.7	41.9	42.5
Min	0.69	3.54	1.0	2.9	4.2	20.3	4	21	30	0.5	3.9	4.2

* Patients #3, 6B, 12, 13, 17,& 19 were excluded from statistical summary of compartmental-analysis since some PK parameters were exaggerated due to insufficient sampling time, and since some patients were analyzed on days other than first day of the Pt cycle.
na = not available

Table 5.3. **Pharmacokinetic summary of free Pt for cisplatin patients.**

	Compartmental analysis									Non-compartmental analysis				
Patient	*Cmax* (µg/ml)	*Cmax* (µmol/L)	AUC* mg.min/ml	*CLf** (ml/min)	*Vc** (L)	*Vss** (L)	$t_{1/2\alpha}$* (min)	$t_{1/2\beta}$* (h)	MRT* (h)	AUC_{0-24} mg.min/ml	CL_{0-24} (ml/min)	*V* (L)	Ae_{0-24} (%)	CL_R (ml/min)
2	0.045	0.23	0.14	170	107	1001	4	69	1	0.036	647	523	na	na
3	0.100	0.51	6.35E+04	1E-03	830	26070	34	2E+08	5E+06	0.052	1583	820	na	na
4	0.075	0.38	0.02	2318	1669	6528	55	36	1	0.056	1016	762	na	na
5	0.140	0.72	0.22	353	883	5844	35	194	7	0.112	684	547	na	na
6A	0.167	0.85	0.04	276	39	428	28	24	1	0.034	360	73	na	na
6B	0.055	0.28	5.49E+06	2E-06	116	422	10	2E+09	9E+07	0.056	220	221	na	na
7	0.032	0.16	0.03	655	213	1409	11	26	1	0.015	1265	607	na	na
9	0.090	0.46	3.33E+05	1E-04	648	3654	70	3E+08	5E+06	0.061	775	528	na	na
12	0.099	0.51	0.01	2995	7241	7244	1	3E+01	6E-04	0.100	248	249	na	na
13	0.045	0.23	na	na	na	na	na	na	na	0.032	1415	1000	47	668
16	0.062	0.32	0.07	704	59	6099	1	101	5	0.060	827	800	6	50
17	0.205	1.05	2.06E+03	5E-02	105	6415	6	1E+06	5E+04	0.135	773	508	45	346
19	0.025	0.13	na	na	na	na	na	na	na	0.030	656	920	37	243
21	0.057	0.29	0.14	162	350	999	62	73	1	0.035	660	401	na	na
23	0.073	0.37	0.13	104	145	824	43	95	3	0.033	412	190	32	130
25	0.091	0.47	0.07	240	18	759	4	39	4	0.030	525	171	na	na
26	0.055	0.28	0.09	181	261	752	54	50	1	0.032	523	303	42	220
27	0.045	0.23	0.11	140	202	919	18	77	2	0.028	554	347	22	121
28	0.054	0.28	0.05	324	244	852	57	33	1	0.024	702	312	43	299
Mean	0.080	0.41	0.09	469	349	2201	31	68	2	0.051	729	488	34	260
SD	0.047	0.24	0.06	614	474	2401	23	48	2	0.032	369	272	14	192
Max	0.205	1.05	0.22	2318	1669	6528	62	194	7	0.135	1583	1000	47	668
Min	0.025	0.13	0.02	104	18	428	1	24	1	0.015	220	73	6	50

* Patients #3, 6B, 12, 13, 17,& 19 were excluded from statistical summary of compartmental-analysis since some PK parameters were exaggerated due to insufficient sampling time, and since some patients were analyzed on days other than first day of the Pt cycle.
na = not available

and elimination for the Pt species. The mean distribution half-life was 34 ± 25 minutes and 31 ± 21 minutes for total Pt and free Pt respectively. The mean elimination half-life was 67 ± 31 hours and 68 ± 48 hours for total and free Pt respectively. By 24 hour (the end of our sampling time) there were still high levels of Pt present as indicated by the elimination half-life. This led to over-estimations of model-dependent parameters in patients 3, 6B, 12 and 17 for total Pt and patients 3, 6B, 9, 12, and 17 for free Pt. These patients most likely would have been described best by a three compartment model if further sampling times at 48 hour and 72 hour were available. Additionally patients 13, and 19 did not fit the two compartment model at all since these two patients received 23 hour infusions of cisplatin and we did not collect adequate post infusion samples from them (since this would have been a change to the ethically approved study protocol).

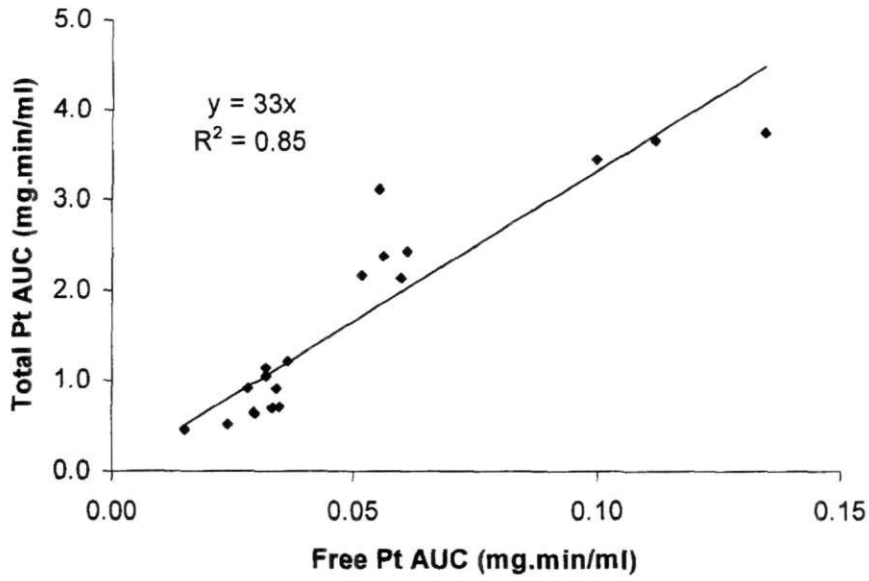
Cisplatin was highly protein bound, with an average 95% of the Pt being bound to serum proteins by 1 hour and 98% protein bound by 24 hour (fig 5.2a). There was a linear relationship between AUC total Pt and AUC free Pt (r^2 = 0.85), and this relationship was described by : AUC free = AUC total/33, (fig 5.3a).

Satisfactory urine collection were possible in 8 children and the mean urinary Pt elimination during 24 h was 34% ± 14 of the total Pt dose administered. This provided for calculation of renal clearance for free Pt which was 260 ± 192 (mL/min) which accounted for only 36% of free Pt clearance, indicating that cisplatin is subject to only limited direct urinary excretion and that there are other pathways of clearance besides renal elimination.

The relationship between non-compartmental total Pt AUC and infusion time was investigated (fig 5.3b), and it was concluded that the AUC for cisplatin is independent of the infusion time.

Also the relationships between a given surface-area based dose of cisplatin and total Pt non-compartmental parameters of *Cmax*, AUC, *CL*, and *V* were investigated (fig 5.4 a-d). Peak Pt concentrations and AUC increased

A.



B.

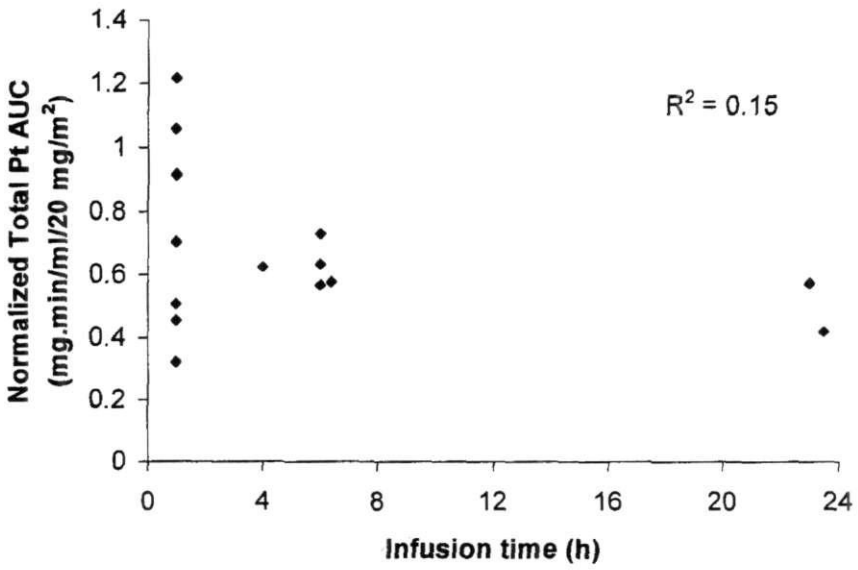


Figure 5.3. Relationship between total Pt AUC and free Pt AUC (A) and dependence of AUC on infusion time (B) in cisplatin patients. There was a high correlation between total Pt AUC and free Pt AUC, therefore free Pt behavior was highly dependent on total Pt (A). Total Pt AUC was independent of the infusion time (B). There was a high variability in AUC among the patients who received the same dose of the drug based on surface area. Only patients on first day of the Pt cycle were included in plot B.

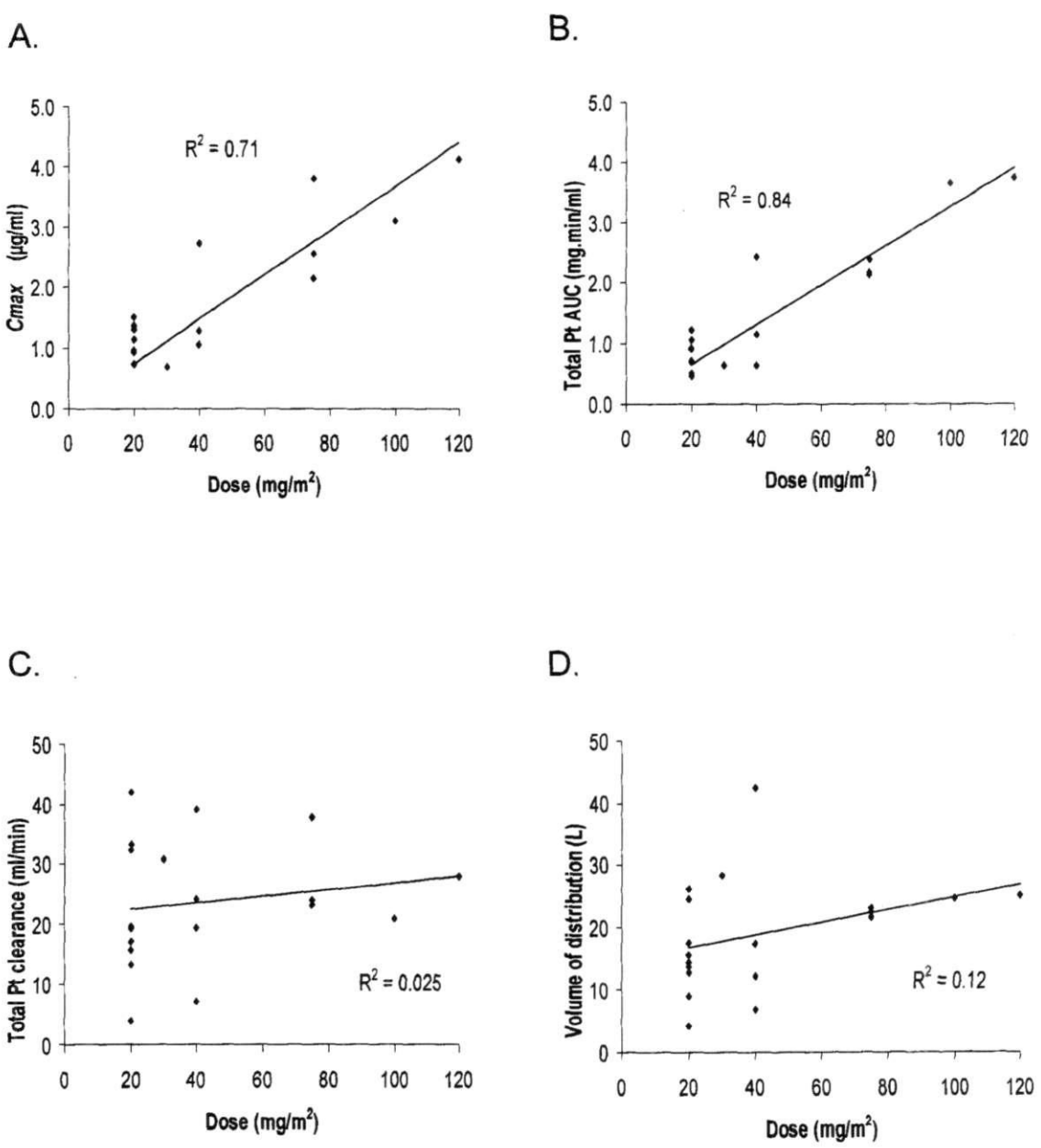


Figure 5.4. Relationship between surface-area based dose of cisplatin and total Pt non-compartmental pharmacokinetic parameters. Peak Pt concentration and AUC were dose dependent (A), and (B), but clearance and volume of distribution were not does dependent (C) & (D).

linearly with increasing dose, with linear correlation coefficients r^2 of: 0.71 and 0.84 respectively. This shows that these parameters are not appropriate for comparison between patients receiving different doses of cisplatin. Even among patients receiving the same dose there was approximately 2 fold variation in peak Pt levels (fig 5.4a), and 3-4 fold variation in AUC (fig 5.4b), implying that much of the variability in cisplatin pharmacokinetics is the result of factors other than body size as measured by surface area. Clearance and volume of distribution were not dose dependent (fig 5.4c-d), making these parameters a better choice for comparison of pharmacokinetics between patients receiving different doses of cisplatin.

Influence of mesna on pharmacokinetics of cisplatin in children

To investigate a possible influence of mesna on the pharmacokinetics of cisplatin, the mean values of compartmental and non-compartmental pharmacokinetic parameters of the two platinum species (total and free) were compared between the cisplatin group and cisplatin in combination with mesna group (table 5.4).

Only 3 patients out of 19 received mesna in combination with cisplatin in 2 and ½ years of our sample collection (table 5.1). These 3 patients all received 20 mg/m^2 of cisplatin over a 1-h infusion, therefore the best control group patients were the ones receiving the same cisplatin dose and infusion schedule on day one of the cisplatin cycle. No significant changes were observed for AUC, clearance, volume of distribution, MRT, $t_{1/2\alpha}$, and $t_{1/2\beta}$ when mesna was present in combination with cisplatin.

Pharmacokinetics of carboplatin in children

Total Pt and free Pt concentrations were measured in the serum of 12 pediatric patients following the administration of carboplatin at doses of 100-560

Table 5.4. Pharmacokinetic comparison of total Pt and free Pt in serum after treatment with cisplatin or in combination with mesna (CDDP + M).

Platinum species	Treatment	Dose Pt (mg)	*Cmax* (µg/ml)	AUC (mg.min/ml)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	MRT (h)	*CLt* (ml/min)	*CLt* (ml/min.kg)	*Vc* (L)	*Vc* (L/kg)	*Vss* (L)	*Vss* (L/kg)
		Compartmental analysis											
Total Pt	Cisplatin	18.0 ± 3.7	1.09 ± 0.31	3.1 ± 1.8	32 ± 18	69 ± 23	98 ± 33	7.1 ± 2.9	0.17 ± 0.08	12.4 ± 4.6	0.29 ± 0.12	38.3 ± 12.4	0.89 ± 0.32
Total Pt	CDDP + M	17.0 ± 5.4	1.14 ± 0.22	2.4 ± 0.6	65 ± 21	60 ± 25	85 ± 36	7.1 ± 1.4	0.19 ± 0.07	15.8 ± 6.6	0.38 ± 0.09	35.3 ± 13.1	0.86 ± 0.24
	P-value	0.741	0.805	0.54	0.057	0.619	0.607	0.999	0.763	0.414	0.341	0.755	0.897
Free Pt	Cisplatin		0.052 ± 0.015	0.09 ± 0.05	33 ± 25	54 ± 28	1.4 ± 1.1	287 ± 221	6.4 ± 4.7	194 ± 66	4.6 ± 1.9	968 ± 263	22.1 ± 5.7
Free Pt	CDDP + M		0.090 ± 0.067	0.10 ± 0.05	36 ± 23	58 ± 29	1.4 ± 0.8	193 ± 73	5.6 ± 4.3	197 ± 155	4.1 ± 2.3	782 ± 309	19.0 ± 5.8
	P-value		0.248	0.788	0.885	0.868	0.974	0.514	0.818	0.967	0.754	0.398	0.492
		Non-compartmental analysis											
Total Pt	Cisplatin			0.8 ± 0.3				26.0 ± 11.2	0.60 ± 0.26	17.3 ± 5.3	0.40 ± 0.12		
Total Pt	CDDP + M			0.8 ± 0.1				21.0 ± 10.1	0.49 ± 0.02	15.8 ± 8.0	0.37 ± 0.02		
	P-value			0.791				0.547	0.506	0.743	0.667		
Free Pt	Cisplatin			0.03 ± 0.01				710 ± 330	15.9 ± 6.7	387 ± 172	8.4 ± 2.7		
Free Pt	CDDP + M			0.03 ± 0.003				525 ± 152	13.0 ± 2.8	273 ± 176	6.2 ± 3.4		
	P-value			0.468				0.406	0.509	0.406	0.341		

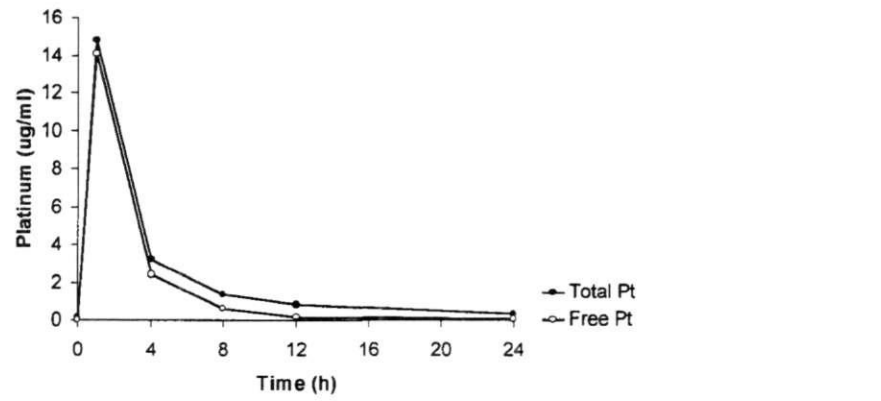
Mean ± SD shown, n = 5 patients for cisplatin group (cisplatin 20 mg/m^2 over 1 hr), and n = 3 patients for cisplatin plus mesna group (cisplatin 20 mg/m^2, mesna various schedules as in table 5.1)

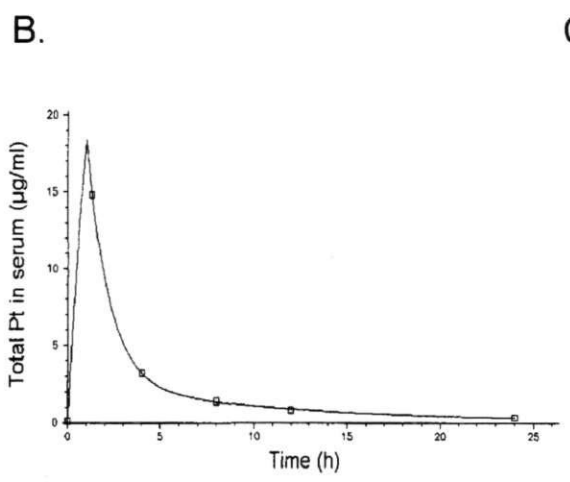
mg/m^2, with the exception of two babies; patients #20 and 24 that were dosed based on body weight and received 10 mg/kg and 18 mg/kg carboplatin respectively (table 5.1). Patient #10 was analyzed twice, once on day one, and again on day two of carboplatin treatment. Maximum serum Pt concentration and AUC for this patient on day two indicated that carboplatin dose accumulates less as compared to that of cisplatin.

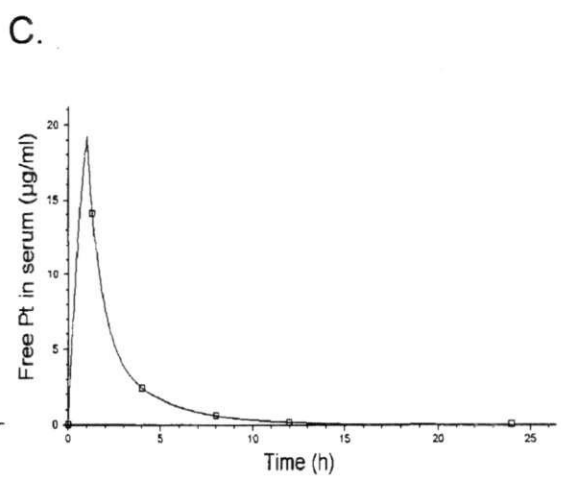
Figure 5.5a illustrates total and free Pt concentrations in the serum of a child during and after a 1-hour infusion of 300 mg/m^2 carboplatin. The pharmacokinetic parameters of the 12 patients determined by compartmental and non-compartmental analysis are listed in tables 5.5 and 5.6 for total and free Pt respectively. Both methods of pharmacokinetic analysis showed similar results.

Peak serum total and free Pt concentrations were observed at the end of the infusion and ranged from 6.54 to 27.70 µg/mL (33.5 to 142.0 µmol/L) and 2.23 to 33.43 µg/mL (11.4 to 171.4 µmol/L) respectively. Levels of both Pt species declined bi-exponentially, hence the two compartment model fit the data well in most cases (fig 5.5b). This provided for the calculation of half-life of distribution and elimination for the Pt species. The mean distribution half-life was 62 ± 10 minutes and 52 ± 18 minutes for total Pt and free Pt respectively. The mean elimination half-life was 30.5 ± 27.2 hours and 5.5 ± 3.6 hours for total and free Pt respectively. The non-compartmental $AUC_{0\text{-}24}$ for patients #20 and 24 could not be determined by the non-compartmental analysis alone because these patients had only 3 sampling time points. In these two patients the $AUC_{0\text{-}24}$ was determined by the $AUC_{0\text{-}\infty}$ of compartmental analysis using the formula:

$AUC_{0-24} = AUC_{0-\infty} - \frac{C_{24}}{\beta}$, where C_{24} is serum Pt concentration at 24 hours and β is the negative slope of the log-linear serum concentration vs. time curve during the elimination phase (Notari, 1987).







<u>Figure 5.5.</u> Representative serum Pt levels for a carboplatin pediatric patient. (A) Actual serum Pt levels after 300 mg/m^2 carboplatin over a 1 hr infusion (model independent plot). Unlike cisplatin, carboplatin is not highly protein bound and serum is almost free of Pt by 24 hours. (B) & (C), two-compartment model fit of the data for total Pt and free Pt respectively in the same patient as in (A), where the lines are the model fit and the squares are the actual data points.

Table 5.5. **Pharmacokinetic summary of total Pt for carboplatin patients.**

	Compartmental analysis									Non-compartmental analysis		
Patient	*Cmax* (µg/ml)	*Cmax* (µmol/L)	AUC (mg.min/ml)	*CLt* (ml/min)	*Vc* (L)	*Vss* (L)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	MRT (h)	AUC_{0-24} mg.min/ml	CL_{0-24} (ml/min)	*V* (L)
1	6.83	35.0	1.1	90	10.3	31.5	47	8	6	1.3	78	14.9
8	7.50	38.4	0.7	802	89.4	393.5	40	10	8	3.1	187	77.2
10A	6.54	33.5	4.2	19	9.6	99.9	82	78	89	1.7	46	11.9
10B	8.34	42.8	4.9	16	7.1	70.9	59	63	74	2.1	37	9.0
11	15.34	78.6	3.1	131	20.1	73.1	63	13	9	3.2	130	26.8
14	18.50	94.8	3.4	33	4.3	11.8	51	8	6	3.3	33	6.0
15	20.18	103.4	4.0	106	15.5	64.0	60	15	10	4.0	107	21.0
18	25.00	128.2	11.4	32	11.3	106.6	66	51	56	6.0	61	14.6
20	7.22	37.0	1.9	20	4.2	11.5	67	10	9	1.7	23	5.0
22	11.70	60.0	7.5E+07	4E-06	24.9	311.5	95	8E+08	1E+09	3.7	93	28.9
24	22.62	116.0	4.8	14	2.3	5.6	61	8	7	4.5	15	3.0
29	27.70	142.0	9.3	26	6.5	72.2	67	51	47	5.7	42	9.0
Mean*	15.83	81.1	4.8	49	9.1	54.7	62	30.5	31.3	3.3	57	12.1
SD*	8.13	41.7	3.2	43	5.5	37.1	10	27.2	32.2	1.7	37	7.4
Max*	27.70	142.0	11.4	131	20.1	106.6	82	78.5	89.1	6.0	130	26.8
Min*	6.54	33.5	1.1	14	2.3	5.6	47	7.6	5.8	1.3	15	3.0

* Patient #8 was left out of the statistical summary because of 5 hr infusion time and patient #22 was left out of the statistical summary because it did not fit the compartmental model due to insufficient sampling which created overestimation of some PK parameters.

Table 5.6. Pharmacokinetic summary of free Pt for carboplatin patients.

	Compartmental analysis									Non-compartmental analysis				
Patient	*Cmax* (µg/ml)	*Cmax* (µmol/L)	AUC (mg.min/ml)	*CLt* (ml/min)	*Vc* (L)	*Vss* (L)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	MRT (h)	AUC_{0-24} mg.min/ml	CL_{0-24} (ml/min)	*V* (L)	Ae_{0-24} (%)	CL_R (ml/min)
1	2.23	11.4	0.2	419	30.4	37.0	45	2.8	1.5	0.3	321	45.6	na	na
8	6.00	30.8	0.4	1437	86.1	183.1	24	2.5	2.1	2.1	279	96.5	na	na
10A	3.21	16.5	0.5	155	18.3	26.8	65	4.5	2.9	0.6	133	24.3	na	na
10B	4.14	21.2	0.6	126	13.8	27.7	60	7.3	3.7	0.7	108	18.8	na	na
11	8.36	42.9	1.1	368	35.1	67.4	57	8.3	3.1	1.3	308	49.1	na	na
14	19.50	100.0	2.4	47	3.5	5.7	30	2.2	2.0	2.5	45	5.7	na	na
15	20.40	104.6	2.4	175	14.0	18.7	46	2.7	1.8	3.1	137	20.7	66	90
18	29.45	151.0	4.2	88	9.3	13.4	67	7.3	2.5	4.9	75	12.4	66	49
20	8.30	42.5	0.5	73	1.6	1.6	14	0.4	0.4	0.5	73	4.7	na	na
22	18.50	94.8	2.9	115	14.3	25.3	75	10.2	3.7	2.5	136	29.5	75	101
24	33.43	171.4	6.4	11	1.6	2.2	59	3.1	3.4	6.4	11	2.0	na	na
29	8.55	43.8	1.1	209	19.9	47.4	56	11.6	3.8	1.3	177	28.0	na	na
Mean*	14.19	72.7	2.0	162	14.7	24.8	52	5.5	2.6	2.2	139	21.9	69	80
SD*	10.74	55.1	1.9	128	11.0	20.2	18	3.6	1.1	2.0	99	15.7	5	27
Max*	33.43	171.4	6.4	419	35.1	67.4	75	11.6	3.8	6.4	321	49.1	75	101
Min*	2.23	11.4	0.2	11	1.6	1.6	14	0.4	0.4	0.3	11	2.0	66	49

* Patient #8 was left out of the statistical summary because of 5 hr infusion time.

Carboplatin was much less protein bound as compared to cisplatin, with an average 5% of the Pt being bound to serum proteins by 1 hour and 5-15% protein bound by 24 hour (fig 5.5a). There was a linear relationship between AUC total Pt and AUC free Pt (r^2 = 0.49), and this relationship was described by : AUC free = [(AUC total – 2.1)/0.57], (fig 5.6).

Satisfactory urine collection was possible in 3 children and the mean urinary Pt elimination during 24 h was 69% ± 5 of the Pt dose administered. This provided for calculation of renal clearance for free Pt which was 80 ± 27 (mL/min) accounting for 58% of free Pt clearance, indicating that renal clearance and hence kidney function play an important role in elimination of carboplatin.

The relationships between a given surface-area based dose of carboplatin and total Pt non-compartmental parameters of *Cmax*, AUC, *CL*, and *V* were investigated (fig 5.7 a-d). Peak Pt concentrations and AUC increased linearly with increasing dose, with linear correlation coefficients r^2 of: 0.79 and 0.87 respectively. This shows that these parameters are not appropriate for comparison between patients receiving different doses of carboplatin. However, inter-patient variability of AUC was less than that observed for cisplatin (fig 5.7b). Clearance and volume of distribution were not dose dependent (fig 5.7 c-d), making these parameters a better choice for comparison of pharmacokinetics between patients receiving different doses of carboplatin.

Influence of mesna on pharmacokinetics of carboplatin in children

To investigate a possible influence of mesna on the pharmacokinetics of carboplatin, the mean values of compartmental and non-compartmental pharmacokinetic parameters of the two platinum species (total and free) were compared between the carboplatin group and the carboplatin in combination with mesna group (table 5.7). Both methods of pharmacokinetic analysis showed the same trends.

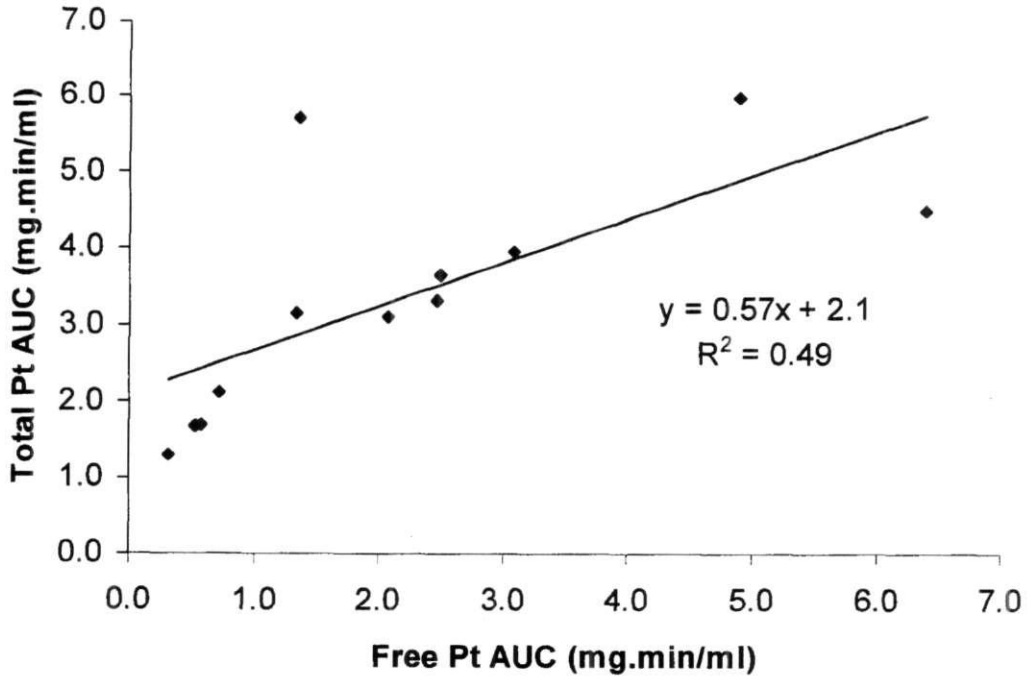


Figure 5.6. Relationship between total Pt AUC and free Pt AUC in carboplatin patients. There was a high correlation between total Pt AUC and free Pt AUC, therefore free Pt behavior was highly dependent on total Pt levels.

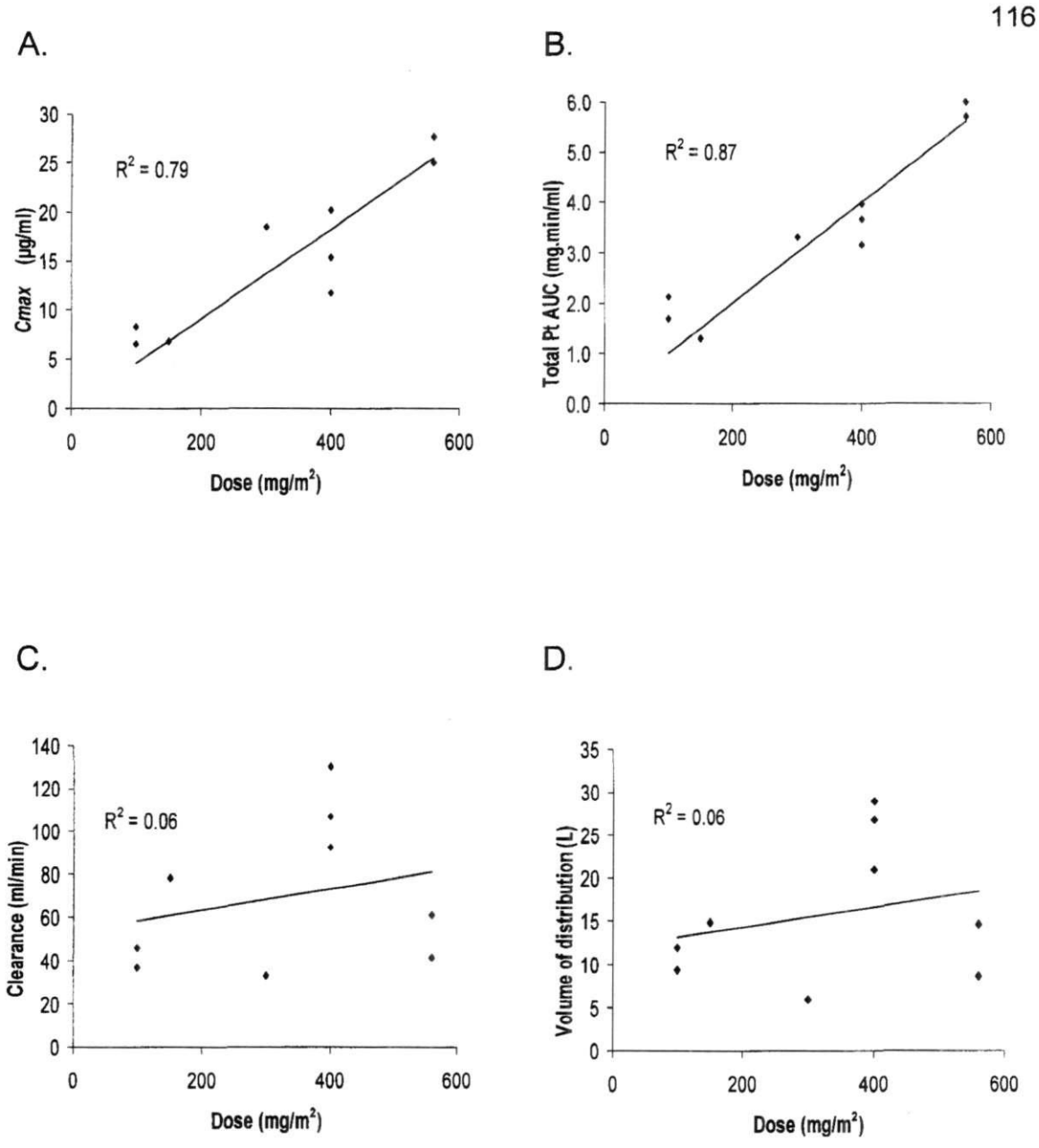


Figure 5.7. Relationship between surface-area based dose of carboplatin and total-Pt non-compartmental pharmacokinetic parameters. Peak Pt concentration and AUC were dose dependent (A), & (B), but clearance and volume of distribution were not dose dependent (C) & (D). Patients #20 & 24 were left-out of the plot because they were dosed based on body weight (kg). Patient #8 was left out of the plot because of 5 hr infusion time.

Table 5.7. Pharmacokinetic comparison of total Pt and free Pt in serum after treatment with carboplatin or in combination with mesna (carb + M).

Platinum species	Treatment	Dose Pt (mg)	Cmax* (µg/ml.400 mg/m²)	AUC* (mg.min/ml.400 mg/m²)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	MRT (h)	CLf (ml/min)	CLf (ml/min.kg)	Vc (L)	Vc (L/kg)	Vss (L)	Vss (L/kg)
					Compartmental analysis								
Total Pt	Carboplatin	239 ± 151	19.4 ± 3.9	5.6 ± 2.2	62 ± 7	26 ± 23	25 ± 24	47 ± 47	2.1 ± 0.6	8.9 ± 7.1	0.39 ± 0.03	53.9 ± 43.5	2.36 ± 1.76
Total Pt	Carb + M	176 ± 162	21.9 ± 8.2	10.8 ± 8.6	63 ± 13	35 ± 33	38 ± 41	50 ± 44	1.9 ± 1.4	9.3 ± 4.2	0.36 ± 0.20	55.6 ± 34.6	1.79 ± 0.70
	P-value	0.529	0.595	0.282	0.809	0.643	0.556	0.918	0.803	0.901	0.787	0.947	0.526
Free Pt	Carboplatin		15.4 ± 9.7	2.3 ± 1.3	54 ± 14	6.5 ± 3.9	3.0 ± 0.7	144 ± 146	5.8 ± 4.6	13.9 ± 13.9	0.56 ± 0.43	27.2 ± 28.8	1.1 ± 1.1
Free Pt	Carb + M		13.5 ± 5.4	2.5 ± 0.4	51 ± 22	4.6 ± 3.5	2.3 ± 1.3	177 ± 124	6.8 ± 5.1	15.4 ± 9.3	0.47 ± 0.29	22.9 ± 11.9	0.7 ± 0.4
	P-value		0.715	0.83	0.814	0.422	0.343	0.695	0.753	0.832	0.678	0.741	0.379
					Non-compartmental analysis								
Total Pt	Carboplatin			4.0 ± 0.6				56.3 ± 44.5	2.5 ± 0.2	11.8 ± 9.4	0.51 ± 0.03		
Total Pt	Carb + M			5.3 ± 2.2				64.0 ± 33.4	2.2 ± 1.0	15.2 ± 8.5	0.52 ± 0.25		
	P-value			0.307				0.751	0.516	0.54	0.961		
Free Pt	Carboplatin			2.3 ± 1.3				123 ± 121	5.0 ± 3.8	19.4 ± 19.3	0.79 ± 0.60		
Free Pt	Carb + M			2.3 ± 0.9				151 ± 87	6.0 ± 4.4	23.9 ± 13.5	0.78 ± 0.41		
	P-value			0.936				0.662	0.715	0.661	0.969		

Mean ± SD shown, n = 5 patients for carboplatin group (patient #8 was excluded from the mean calculations due to 5 hr infusion time. n = 6 patients for carboplatin plus mesna group.

* n = 4 patients for carboplatin group (patient #24 was excluded because of weight based dosing), n = 5 patients for carboplatin plus mesna group (patient #20 was excluded because of weight based dosing).

Six out of 12 patients received mesna in combination with carboplatin (table 5.1). These 6 patients all received various doses of carboplatin and mesna, which made comparison to the control group difficult. The best solution was to compare the non-dose dependent parameters normalized to body weight of each patient. No significant changes were observed for AUC, clearance, volume of distribution, MRT, $t_{1/2\alpha}$, and $t_{1/2\beta}$ when mesna was present in combination with carboplatin, probably indicating that, mesna does not influence pharmacokinetics of carboplatin in pediatric patients.

Discussion

For the first time pharmacokinetics of Pt-drugs in combination with mesna was investigated. The results indicated that treatment with mesna does not influence pharmacokinetics of cisplatin and/or carboplatin in pediatric patients. These results provide evidence against the hypothesis that mesna may reduce serum Pt levels of cisplatin, but support the hypothesis that pharmacokinetics of carboplatin may not be influenced in presence of mesna.

These answers are also reasonable based on the results obtained in SCID mice, chapter four, and what is known about pharmacokinetic behavior of mesna in patients.

After i.v. infusion mesna is oxidized in the blood to dimesna, an inactive disulfide (Brock et al., 1982). The mean half-life of mesna in patients has been reported to be 21.8 minutes (James et al., 1987), and in other studies reported to be 9 to 11 minutes (Goren et al., 1998). The short half-life of mesna, its metabolism to dimesna and its inability to enter cells most likely explain why mesna did not influence the pharmacokinetics of Pt-agents in patients. My results also verify the mathematical predictions of Leeuwenkamp et al. and Obrocea et al., that mesna will not influence pharmacokinetics of cisplatin (Leeuwenkamp et al., 1990) and carboplatin (Obrocea et al., 1998) in patients.

Nevertheless one should be cautious in reaching a firm conclusion from this study. The power of this study was weak with respect to sample size. Only 3 patients who received cisplatin plus mesna were enrolled into the study over two-and-a-half years of our sample collection. The best solution would be to use the results from this study for sample size calculation of a multi-center pharmacokinetic study. A larger sample population will also allow for selection of exact subject criteria with respect to dose and infusion schedule.

Even though no statistically significant difference was observed in the pharmacokinetic analysis, certain trends were observed that require further discussion and clarification. The half-life of distribution for total Pt in cisplatin plus

mesna group was doubled as compared to cisplatin group, but was not significant. This trend was not due to reduced Pt clearance and it did not have a significant impact on volume of distribution. However it can be explained by a decrease in protein binding of cisplatin by comparing the % ratio of free Pt AUC over total Pt AUC between the two groups. Mesna most likely competes with serum proteins in binding cisplatin, therefore a slight increase in % free Pt is observed as it is indicated by slower distribution/metabolism for total Pt. Decrease in protein binding of cisplatin in the presence of mesna was observed *in vitro* in chapter 3, (fig 3.4). Overall this did not make a significant contribution to pharmacokinetics of cisplatin in patients.

In carboplatin/mesna patients a trend in increasing total Pt AUC, elimination half-life and MRT was observed. This result was not due to reduced carboplatin clearance or an increase in distribution volume. Possibly this was due to a change in protein binding of carboplatin. It has been reported that the elimination half-life of total Pt is not dependent on kidney function but rather dependent on the turnover rate of proteins to which the Pt compound binds irreversibly (Korst et al., 1997; Korst et al., 1998; Vermorken et al., 1986). In vitro, mesna has no effect on protein binding of carboplatin (Obrocea et al., 1998). It is however possible that *in vivo* a mono-functional carboplatin-mesna metabolite might have a higher affinity for binding serum proteins, which might explain the trend in increasing AUC, elimination half-life and the MRT of total Pt for carboplatin. None of these pharmacokinetic parameters were statistically significant in this study, hence they did not influence the overall pharmacokinetic outcome for carboplatin.

In general, cisplatin was highly protein bound as compared to carboplatin, that was only slightly protein bound. This result was consistent with previous *in vitro* (van der Vijgh and Klein, 1986) and *in vivo* (Murry, 1997; O'Dwyer et al., 2000) reports. The difference in extent of protein binding was also evident in comparing the volume of distribution between the two Pt-drugs. Drugs that are highly protein bound have large volumes of distribution for the free drug moiety

(Evans et al., 1986). The *Vss* for free Pt in cisplatin patients was 100 times greater than that for carboplatin patients. Additionally since cisplatin was more protein bound to tissue proteins it also had longer elimination half-life and MRT for its total Pt moiety as compared to carboplatin.

Elimination of carboplatin was more dependent on renal function as compared to cisplatin. This result confirms previous pharmacokinetic reports (Murry, 1997; Peng et al., 1997; Tonda et al., 1996). Since carboplatin clearance is dependent on renal function, dosing formulas have been developed that individualize carboplatin dose for each patient based on glomerular filtration rate (GFR) (Calvert et al., 1989; Newell et al., 1993).

A notable feature of the pharmacokinetics of cisplatin in children was the variability in AUC achieved when the drug is administered on the basis of surface area (tables 5.2 & 5.3, figure 5.4b). To what extent this variability influences either the activity or toxicity of cisplatin in children is currently unknown; however, it prompts concern about the use of surface-area based dosing for cisplatin in children. A recent analysis has shown that cisplatin dose rate, but not total dose, is a significant determinant of nephrotoxicity in children (Skinner et al., 1998). Also in adults, peak free and total Pt plasma concentrations which are dependent on dose rate have been shown to relate to renal side-effects (Nagai et al., 1996; Reece et al., 1987). In this study I analyzed one cisplatin patient who experienced nephrotoxicity. Neither the peak free Pt, total Pt in serum or the AUC for this patient stood-out among other cisplatin patients analyzed as an indicator of nephrotoxicity. Interestingly though, this patient had the second fastest distribution half-life, and the second fastest clearance normalized per body weight, for total Pt among the cisplatin patients. Can these PK parameters be predictors for nephrotoxicity? Could it be that fast distribution of Pt leads to exceedingly high concentrations of Pt in the kidneys, leading to kidney failure? These questions certainly warrant a pharmacokinetic study of body-weight normalized total Pt clearance and distribution half-life correlation with nephrotoxicity.

Renal and hepatic function are two parameters that can influence inter-patient pharmacokinetic variation (Notari, 1987). Overall, the urinary excretion of Pt was 34% of the administered dose for cisplatin, a value consistent with pediatric data reported previously (Peng et al., 1997). Given that renal excretion accounts for less than half of the cisplatin dose administered (table 5.3), and that there is no relationship between GFR and the total plasma and renal clearance of free Pt (Peng et al., 1997), the variability in serum clearance and AUC observed (figs. 5.3, 5.4 b & c) must relate primarily to variations in the non-renal clearance of the drug. The non-renal clearance of cisplatin is thought to be largely due to tissue binding, as opposed to metabolism or biliary excretion (Peng et al., 1997). This observation prompted me to investigate the possibility of developing a pediatric dosing formula to tailor cisplatin dose on individual bases. The outcome is a preliminary pediatric dosing formula for cisplatin based on each patient's body weight and serum creatinine, findings of which will be published else where.

In summary, for the first time pharmacokinetics of Pt-drugs in combination with mesna was investigated. It was found that treatment with mesna does not influence pharmacokinetics of cisplatin and/or carboplatin in pediatric patients. This observation is important because it means that patients who are receiving concurrent Pt-drugs / Oxazaphosphorines / mesna are getting the full benefit of their Pt-drug treatment. Therefore, the clinical recommendation based on this study is that it is safe to combine mesna with Pt-drugs in combination chemotherapy, provided that mesna is not mixed in solution with Pt-drugs or infused through the same infusion line as Pt-drugs.

This study also established the start-up of a pharmacokinetics laboratory in Calgary, which made measurements of Pt-drugs possible in cancer patients for current and future therapeutic drug monitoring and pharmacokinetic studies.

Conclusions

Kinetics of Pt-drugs in reaction with mesna had been investigated previously. I extended these observations by providing the stoichiometry for these reactions. Mesna reacted with cisplatin in a molar ratio of 2 to 1. Taken together with the fast rate of this reaction it meant that cisplatin will be fully inactivated after coming into contact with mesna in solution. Surprisingly, mesna reacted with carboplatin in a molar ratio of 1 to 1. Considering the slow rate of this reaction, it meant that inactivation of carboplatin will not be biologically relevant.

Pt-DNA binding experiments in glioma cells showed that cisplatin was inactivated in the presence of mesna as evident from the reduced Pt-DNA binding. The reduced Pt-DNA binding followed from the reduced cellular accumulation of cisplatin. Taken together with the chemical results, this provided further proof for inactivation of cisplatin by mesna *in vitro*. Pt-DNA binding of carboplatin was not reduced in presence of mesna in the same experimental model. This strengthened the conclusion from the chemistry section that the reaction between mesna and carboplatin is too slow to have biological relevance.

In tumor-bearing SCID mice, mesna did not protect tumor cells or normal tissue form Pt-DNA binding of cisplatin, unless the two drugs were mixed together prior to injection to the animals. Also, as determined from pharmacokinetic analysis of pediatric patients, serum Pt levels for cisplatin and carboplatin were not influenced by the presence of mesna.

In conclusion, these findings show that mesna and the Pt-drugs are compatible *in vivo* if the two agents are not mixed in the same solution. However these findings also show that the direct admixture of cisplatin with mesna in solution results in a significant reduction of cisplatin's Pt-DNA binding. Therefore the clinical recommendation based on this thesis is that, mesna can be given at the same time as Pt-drugs, but it should be in its own separate infusion bag, and be infused from a different infusion line than that of the Pt-drugs.

Future Directions

Related to mechanisms of resistance to Pt-drugs, this thesis focused on two aspects of this problem: (1) Decreased cellular accumulation of Pt; and (2) inactivation by thiols, both of which were caused by mesna *in vitro*. Future investigations could examine synergistic effect of mesna on cells already resistant to Pt-drugs due to elevated GSH levels. These systems should be further investigated *in vivo*, since it was shown that mesna is compatible with Pt-drugs *in vivo*. It would be important to see if this conclusion still holds true in cells with elevated GSH levels.

Most cisplatin research has focused on its ability to modify genomic DNA (gDNA) in the nucleus. Recently an alternative cellular DNA target has been identified which could potentially play a role in the Pt-drugs mechanism. Mitochondrial DNA (mtDNA) has been identified as an additional target for cisplatin (Giurgiovich et al., 1997). There was a 6-fold higher proportion of Pt-DNA adducts in mtDNA, compared to gDNA (Giurgiovich et al., 1997). The preference for mtDNA was later attributed both to higher initial binding and to lack of removal of the Pt-DNA adducts (Olivero et al., 1997). It would be beneficial to investigate if mesna and/or other cellular thiols such as GSH would reduce Pt-DNA binding at mtDNA. Depending on the outcome, more insight will be added to understanding mechanism of action and resistance of Pt-drugs. Additionally, one can imagine designing a new class of Pt-drugs that would preferentially target mitochondrial DNA over gDNA. These new Pt-drugs might be useful in tumors that have become resistant to cisplatin based on increased nucleotide excision repair, a major pathway for removing cisplatin damage in gDNA.

Interaction of Pt-drugs with sulfur-containing compounds have primarily been associated with negative phenomena, such as the development of resistance or reduced activity of Pt-drugs. I took the same approach in starting this thesis. However I would like to propose an alternative outlook. Maybe we can

use sulfur-containing compounds to our advantage in designing new anticancer Pt-agents. In this approach, one could design new Pt-agents that contain sulfur-donor ligands of optimal kinetics which would shield the Pt-drug from attack of cellular thiols until the sulfur ligand is exchanged by the N7 of deoxy-guanosine. Finally one could think of totally new Pt(IV) compounds that need to be activated via reduction, but now using cellular thiol compounds.

References

Alberts, D.S., Liu, P.Y., Hannigan, E.V., O'Toole, R., Williams, S.D., Young, J.A., Franklin, E.W., Clarke-Pearson, D.L., Malviya, V.K. and DuBeshter, B., Intraperitoneal cisplatin plus intravenous cyclophosphamide versus intravenous cisplatin plus intravenous cyclophosphamide for stage III ovarian cancer. *N Engl J Med*, **335,** 1950-5. (1996).

Allan, S.G., Smyth, J.F., Hay, F.G., Leonard, R.C. and Wolf, C.R., Protective effect of sodium-2-mercaptoethanesulfonate on the gastrointestinal toxicity and lethality of cis-diamminedichloroplatinum. *Cancer Research*, **46,** 3569-73 (1986).

Al-Sarraf, M., Fletcher, W., Oishi, N., Pugh, R., Hewlett, J.S., Balducci, L., McCracken, J. and Padilla, F., Cisplatin hydration with and without mannitol diuresis in refractory disseminated malignant melanoma: a southwest oncology group study. *Cancer Treat Rep*, **66,** 31-5. (1982).

Anderson, M.E., Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact*, **111-112,** 1-14. (1998).

Andersson, A., Fagerberg, J., Lewensohn, R. and Ehrsson, H., Pharmacokinetics of cisplatin and its monohydrated complex in humans. *J Pharm Sci*, **85,** 824-7. (1996).

Andrews, P.A., Mann, S.C., Huynh, H.H. and Albright, K.D., Role of the Na+, K(+)-adenosine triphosphatase in the accumulation of cis-diamminedichloroplatinum(II) in human ovarian carcinoma cells. *Cancer Res*, **51,** 3677-81. (1991).

Andrews, P.A., Velury, S., Mann, S.C. and Howell, S.B., cis-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res*, **48,** 68-73. (1988).

Araujo, C.E., Cervellino, J.C., Pirisi, C., Pannunzio, O. and Callegari, J., Chemotherapy with high dose ifosfamide/mesna plus cisplatin for the treatment of ovarian cancer: a study of the Grupo de Estudio y Tratamiento Latino-Americano del Cancer. *J Surg Oncol*, **46,** 198-202. (1991).

Barnham, K.J., Djuran, M.I., del Socorro Murdoch, P., Ranford, J.D. and Sadler, P.J., Ring-Opened Adducts of the Anticancer Drug Carboplatin with Sulfur Amino Acids. *Inorganic Chemistry*, **35,** 1065-1072 (1996).

Basolo, F. and Pearson, R.G., The trans effect in metal complexes. *Progress Inorganic Chem.*, 381 (1962).

Bernal, S.D., Speak, J.A., Boeheim, K., Dreyfuss, A.I., Wright, J.E., Teicher, B.A., Rosowsky, A., Tsao, S.W. and Wong, Y.C., Reduced membrane protein associated with resistance of human squamous carcinoma cells to methotrexate and cis-platinum. *Mol Cell Biochem*, **95,** 61-70. (1990).

Blatter, E.E., Vollano, J.F., Krishnan, B.S. and Dabrowiak, J.C., Interaction of the antitumor agents cis,cis,trans-PtIV(NH3)2Cl2(OH)2 and cis,cis,trans-PtIV[(CH3)2CHNH2]2Cl2(OH)2 and their reduction products with PM2 DNA. *Biochemistry*, **23,** 4817-20. (1984).

Blommaert, F.A., Michael, C., van Dijk-Knijnenburg, H.C., Schornagel, J.H., den Engelse, L. and Fichtinger-Schepman, A.M., The formation and persistence of carboplatin-DNA adducts in rats. *Cancer Chemother Pharmacol*, **38,** 273-80 (1996).

Blommaert, F.A., van Dijk-Knijnenburg, H.C., Dijt, F.J., den Engelse, L., Baan, R.A., Berends, F. and Fichtinger-Schepman, A.M., Formation of DNA adducts by the anticancer drug carboplatin: different nucleotide sequence preferences in vitro and in cells. *Biochemistry*, **34,** 8474-80. (1995).

Borst, P., Evers, R., Kool, M. and Wijnholds, J., A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst*, **92,** 1295-302. (2000).

Bose, R.N., Ghosh, S.K. and Moghaddas, S., Kinetic analysis of the cis-diamminedichloroplatinum(II)--cysteine reaction: implications to the extent of platinum--DNA binding. *J Inorg Biochem*, **65,** 199-205. (1997).

Boven, E., van der Vijgh, W.J., Nauta, M.M., Schluper, H.M. and Pinedo, H.M., Comparative activity and distribution studies of five platinum analogues in nude mice bearing human ovarian carcinoma xenografts. *Cancer Res*, **45,** 86-90. (1985).

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72,** 248-54. (1976).

Briz, O., Serrano, M.A., Macias, R.I. and Marin, J.J., Overcoming cisplatin resistance in vitro by a free and liposome- encapsulated bile acid derivative: BAMET-R2. *Int J Cancer*, **88,** 287-92. (2000).

Brock, N., Pohl, J., Stekar, J. and Scheef, W., Studies on the urotoxicity of oxazaphosphorine cytostatics and its prevention--III. Profile of action of sodium

2-mercaptoethane sulfonate (mesna). *Eur J Cancer Clin Oncol*, **18,** 1377-87. (1982).

Calvert, A.H., Harland, S.J., Newell, D.R., Siddik, Z.H., Jones, A.C., McElwain, T.J., Raju, S., Wiltshaw, E., Smith, I.E., Baker, J.M., Peckham, M.J. and Harrap, K.R., Early clinical studies with cis-diammine-1,1-cyclobutane dicarboxylate platinum II. *Cancer Chemother Pharmacol*, **9,** 140-7 (1982).

Calvert, A.H., Newell, D.R., Gumbrell, L.A., O'Reilly, S., Burnell, M., Boxall, F.E., Siddik, Z.H., Judson, I.R., Gore, M.E. and Wiltshaw, E., Carboplatin dosage: prospective evaluation of a simple formula based on renal function. *J Clin Oncol*, **7,** 1748-56. (1989).

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. and Mitchell, J.B., Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Research*, **47,** 936-42 (1987).

Cersosimo, R.J., Cisplatin neurotoxicity. *Cancer Treat Rev*, **16,** 195-211. (1989).

Cervellino, J.C., Araujo, C.E., Sanchez, O., Miles, H. and Nishihama, A., Cisplatin and ifosfamide in patients with advanced squamous cell carcinoma of the uterine cervix. A phase II trial. *Acta Oncol*, **34,** 257-9 (1995).

Chatelut, E., Canal, P., Brunner, V., Chevreau, C., Pujol, A., Boneu, A., Roche, H., Houin, G. and Bugat, R., Prediction of carboplatin clearance from standard morphological and biological patient characteristics. *J Natl Cancer Inst*, **87,** 573-80. (1995).

Culine, S., Theodore, C., Bekradda, M., Farhat, F., Terrier-Lacombe, M.J. and Droz, J.P., Experience with bleomycin, etoposide, cisplatin (BEP) and alternating cisplatin, cyclophosphamide, doxorubicin (CISCA(II))/vinblastine, bleomycin (VB(IV)) regimens of chemotherapy in poor-risk nonseminomatous germ cell tumors. *Am J Clin Oncol*, **20,** 184-8. (1997).

Daugaard, G. and Abildgaard, U., Cisplatin nephrotoxicity. A review. *Cancer Chemother Pharmacol*, **25,** 1-9 (1989).

Dawson-Saunders, B. and Trapp, R.G., *Basic and Clinical Biostatistics*, second edition ed., Appleton and Lange, Norwalk, Connecticut (1994).

De Andres, L., Brunet, J., Lopez-Pousa, A., Burgues, J., Vega, M., Tabernero, J.M., Mesia, R. and Lopez, J.J., Randomized trial of neoadjuvant cisplatin and fluorouracil versus carboplatin and fluorouracil in patients with stage IV-M0 head and neck cancer. *J Clin Oncol*, **13,** 1493-500. (1995).

de Boer, J.G. and Glickman, B.W., Mutations recovered in the Chinese hamster aprt gene after exposure to carboplatin: a comparison with cisplatin. *Carcinogenesis*, **13,** 15-7. (1992).

Dedon, P.C. and Borch, R.F., Characterization of the reactions of platinum antitumor agents with biologic and nonbiologic sulfur-containing nucleophiles. *Biochem Pharmacol*, **36,** 1955-64. (1987).

DeWoskin, R.S. and Riviere, J.E., Cisplatin-induced loss of kidney copper and nephrotoxicity is ameliorated by single dose diethyldithiocarbamate, but not mesna. *Toxicology & Applied Pharmacology*, **112,** 182-9 (1992).

Dominici, C., Petrucci, F., Caroli, S., Alimonti, A., Clerico, A. and Castello, M.A., A pharmacokinetic study of high-dose continuous infusion cisplatin in children with solid tumors. *J Clin Oncol*, **7,** 100-7. (1989).

Dornish, J.M. and Pettersen, E.O., Protection from cis-dichlorodiammineplatinum-induced cell inactivation by aldehydes involves cell membrane amino groups. *Cancer Lett*, **29,** 235-43. (1985).

Dorr, R.T., Chemoprotectants for cancer chemotherapy. *Seminars in Oncology*, **18,** 48-58 (1991).

Dorr, R.T. and Lagel, K., Interaction between cisplatin and mesna in mice. *Journal of Cancer Research & Clinical Oncology*, **115,** 604-5 (1989).

Doz, F., Berens, M.E., Dougherty, D.V. and Rosenblum, M.L., Comparison of the cytotoxic activities of cisplatin and carboplatin against glioma cell lines at pharmacologically relevant drug exposures. *J Neurooncol*, **11,** 27-35. (1991).

Eastman, A., Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells*, **2,** 275-80. (1990).

Eastman, A., Cross-linking of glutathione to DNA by cancer chemotherapeutic platinum coordination complexes. *Chem Biol Interact*, **61,** 241-8. (1987).

Eastman, A., The Mechanism of Action of Cisplatin: From Adduct to Apoptosis. *In:* B. Lippert (ed.), *Cisplatin: chemistry and biochemistry of a leading anticancer drug*, pp. 111-134, Wiley - VCH, Zurich (1999).

Eastman, A. and Barry, M.A., Interaction of trans-diamminedichloroplatinum(II) with DNA: formation of monofunctional adducts and their reaction with glutathione. *Biochemistry*, **26,** 3303-7. (1987).

Edmonson, J.H., Suman, V.J., Dalton, R.J., Bro, W.C., Gallenberg, M.M., Long, H.J., Levitt, R., Hatfield, A.K., Krook, J.E., Mailliard, J.A. and Gerstner, J.B., Comparison of conventional dose and double dose carboplatin in patients receiving cyclophosphamide plus carboplatin for advanced ovarian carcinoma: a North Central Cancer Treatment Group Study. *Cancer Invest*, **19,** 597-602 (2001).

Egorin, M.J., Van Echo, D.A., Olman, E.A., Whitacre, M.Y., Forrest, A. and Aisner, J., Prospective validation of a pharmacologically based dosing scheme for the cis-diamminedichloroplatinum(II) analogue diamminecyclobutanedicarboxylatoplatinum. *Cancer Res*, **45,** 6502-6. (1985).

Elferink, F., van der Vijgh, W.J., Klein, I. and Pinedo, H.M., Interaction of cisplatin and carboplatin with sodium thiosulfate: reaction rates and protein binding. *Clin Chem*, **32,** 641-5. (1986).

Endo, T., Kimura, O. and Sakata, M., Carrier-mediated uptake of cisplatin by the OK renal epithelial cell line. *Toxicology,* **146,** 187-95 (2000).

Evans, B.D., Raju, K.S., Calvert, A.H., Harland, S.J. and Wiltshaw, E., Phase II study of JM8, a new platinum analog, in advanced ovarian carcinoma. *Cancer Treat Rep*, **67,** 997-1000. (1983).

Evans, W.E., Schentag, J.J. and Jusko, W.J., *Applied pharmacokinetics : principles of therapeutic drug monitoring*, 2nd ed., p. xxiv, 1272, Applied Therapeutics, San Francisco (1986).

Fetscher, S., Brugger, W., Engelhardt, R., Kanz, L., Hasse, J., Frommhold, H., Lange, W. and Mertelsmann, R., Standard- and high-dose etoposide, ifosfamide, carboplatin, and epirubicin in 107 patients with non-small-cell lung cancer: a mature follow-up report. *Ann Oncol*, **10,** 605-7. (1999).

Fichtinger-Schepman, A.M., van der Veer, J.L., den Hartog, J.H., Lohman, P.H. and Reedijk, J., Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry*, **24,** 707-13. (1985).

Fichtinger-Schepman, A.M., van Oosterom, A.T., Lohman, P.H. and Berends, F., cis-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of cis-diamminedichloroplatinum(II). *Cancer Res*, **47,** 3000-4. (1987).

Fraval, H.N., Rawlings, C.J. and Roberts, J.J., Increased sensitivity of UV-repair-deficient human cells to DNA bound platinum products which unlike thymine

dimers are not recognized by an endonuclease extracted from Micrococcus luteus. *Mutat Res*, **51,** 121-32. (1978).

Gale, G.R., Morris, C.R., Atkins, L.M. and Smith, A.B., Binding of an antitumor platinum compound to cells as influenced by physical factors and pharmacologically active agents. *Cancer Res*, **33,** 813-8. (1973).

Gately, D.P. and Howell, S.B., Cellular accumulation of the anticancer agent cisplatin: a review. *Br J Cancer*, **67,** 1171-6. (1993).

Gerke, P., Filejski, W., Robins, H.I., Wiedemann, G.J. and Steinhoff, J., Nephrotoxicity of ifosfamide, carboplatin and etoposide (ICE) alone or combined with extracorporeal or radiant-heat-induced whole-body hyperthermia. *J Cancer Res Clin Oncol*, **126,** 173-7. (2000).

Giaccone, G., Splinter, T.A., Debruyne, C., Kho, G.S., Lianes, P., van Zandwijk, N., Pennucci, M.C., Scagliotti, G., van Meerbeeck, J., van Hoesel, Q., Curran, D., Sahmoud, T. and Postmus, P.E., Randomized study of paclitaxel-cisplatin versus cisplatin-teniposide in patients with advanced non-small-cell lung cancer. The European Organization for Research and Treatment of Cancer Lung Cancer Cooperative Group. *J Clin Oncol*, **16,** 2133-41. (1998).

Gibaldi, M. and Perrier, D., *Pharmacokinetics*, 2nd , rev. and expand -- ed., p. viii, 494, Dekker, New York (1982).

Giurgiovich, A.J., Diwan, B.A., Olivero, O.A., Anderson, L.M., Rice, J.M. and Poirier, M.C., Elevated mitochondrial cisplatin-DNA adduct levels in rat tissues after transplacental cisplatin exposure. *Carcinogenesis*, **18,** 93-6. (1997).

Go, R.S. and Adjei, A.A., Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *J Clin Oncol*, **17,** 409-22. (1999).

Godwin, A.K., Meister, A., O'Dwyer, P.J., Huang, C.S., Hamilton, T.C. and Anderson, M.E., High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci U S A*, **89,** 3070-4. (1992).

Goel, R., Cleary, S.M., Horton, C., Kirmani, S., Abramson, I., Kelly, C. and Howell, S.B., Effect of sodium thiosulfate on the pharmacokinetics and toxicity of cisplatin. *J Natl Cancer Inst*, **81,** 1552-60. (1989).

Gonzalez, V.M., Fuertes, M.A., Alonso, C. and Perez, J.M., Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol*, **59,** 657-63. (2001).

Gore, M.E., Fryatt, I., Wiltshaw, E., Dawson, T., Robinson, B.A. and Calvert, A.H., Cisplatin/carboplatin cross-resistance in ovarian cancer. *Br J Cancer*, **60,** 767-9. (1989).

Goren, M.P., Anthony, L.B., Hande, K.R., Johnson, D.H., Brade, W.P., Frazier, M.W., Bush, D.A. and Li, J.T., Pharmacokinetics of an intravenous-oral versus intravenous-mesna regimen in lung cancer patients receiving ifosfamide. *J Clin Oncol*, **16,** 616-21. (1998).

Goren, M.P., Lyman, B.A. and Li, J.T., The stability of mesna in beverages and syrup for oral administration. *Cancer Chemother Pharmacol*, **28,** 298-301 (1991).

Gosland, M., Lum, B., Schimmelpfennig, J., Baker, J. and Doukas, M., Insights into mechanisms of cisplatin resistance and potential for its clinical reversal. *Pharmacotherapy*, **16,** 16-39. (1996).

Graziano, S.L., Valone, F.H., Herndon, J.E., 2nd, Crawford, J., Richards, F., 2nd, Rege, V.B., Clamon, G. and Green, M.R., A randomized phase II study of ifosfamide/mesna/cisplatin plus G-CSF or etoposide/cisplatin plus G-CSF in advanced non-small cell lung cancer: a Cancer and Leukemia Group B study. *Lung Cancer*, **14,** 315-29. (1996).

Harland, S.J., Newell, D.R., Siddik, Z.H., Chadwick, R., Calvert, A.H. and Harrap, K.R., Pharmacokinetics of cis-diammine-1,1-cyclobutane dicarboxylate platinum(II) in patients with normal and impaired renal function. *Cancer Res*, **44,** 1693-7. (1984).

Harrap, K.R., Initiatives with platinum- and quinazoline-based antitumor molecules-- Fourteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res*, **55,** 2761-8. (1995).

Harrap, K.R., Preclinical studies identifying carboplatin as a viable cisplatin alternative. *Cancer Treat Rev*, **12 Suppl A,** 21-33. (1985).

Hongo, A., Seki, S., Akiyama, K. and Kudo, T., A comparison of in vitro platinum-DNA adduct formation between carboplatin and cisplatin. *Int J Biochem*, **26,** 1009-16. (1994).

Horwich, A., Sleijfer, D.T., Fossa, S.D., Kaye, S.B., Oliver, R.T., Cullen, M.H., Mead, G.M., de Wit, R., de Mulder, P.H., Dearnaley, D.P., Cook, P.A., Sylvester, R.J. and Stenning, S.P., Randomized trial of bleomycin, etoposide, and cisplatin compared with bleomycin, etoposide, and carboplatin in good-prognosis metastatic nonseminomatous germ cell cancer: a Multiinstitutional Medical Research Council/European Organization for Research and Treatment of Cancer Trial. *J Clin Oncol*, **15,** 1844-52. (1997).

Hoskins, P.J., O'Reilly, S.E., Swenerton, K.D., Spinelli, J.J., Fairey, R.N. and Benedet, J.L., Ten-year outcome of patients with advanced epithelial ovarian carcinoma treated with cisplatin-based multimodality therapy. *J Clin Oncol*, **10,** 1561-8. (1992).

Howe-Grant, M.E. and Lippard, S.J., Aqueous Platinum (II) Chemistry; Binding to biological molecules. *In:* S. Helmut (ed.), *Metal Ions in Biological Systems, Metal complexes as anticancer agents.* Vol. 11, pp. 63-125, Marcel Dekker Inc., New York (1980).

Howell, S.B., Pfeifle, C.E., Wung, W.E. and Olshen, R.A., Intraperitoneal cis-diamminedichloroplatinum with systemic thiosulfate protection. *Cancer Res*, **43,** 1426-31. (1983).

Ishikawa, T. and Ali-Osman, F., Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem*, **268,** 20116-25. (1993).

James, C.A., Mant, T.G. and Rogers, H.J., Pharmacokinetics of intravenous and oral sodium 2-mercaptoethane sulphonate (mesna) in normal subjects. *Br J Clin Pharmacol*, **23,** 561-8. (1987).

Jamieson, E.R., Jacobson, M.P., Barnes, C.M., Chow, C.S. and Lippard, S.J., Structural and kinetic studies of a cisplatin-modified DNA icosamer binding to HMG1 domain B. *J Biol Chem*, **274,** 12346-54. (1999).

Johnsson, A., Olsson, C., Nygren, O., Nilsson, M., Seiving, B. and Cavallin-Stahl, E., Pharmacokinetics and tissue distribution of cisplatin in nude mice: platinum levels and cisplatin-DNA adducts. *Cancer Chemotherapy & Pharmacology*, **37,** 23-31 (1995).

Kartalou, M. and Essigmann, J.M., Mechanisms of resistance to cisplatin. *Mutat Res*, **478,** 23-43. (2001).

Kartalou, M. and Essigmann, J.M., Recognition of cisplatin adducts by cellular proteins. *Mutat Res*, **478,** 1-21. (2001).

Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saijo, N., Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res*, **51,** 3237-42. (1991).

Kelland, L.R., Preclinical perspectives on platinum resistance. *Drugs*, **59,** 1-8; discussion 37-8. (2000).

Kerbusch, T., de Kraker, J., Keizer, H.J., van Putten, J.W., Groen, H.J., Jansen, R.L., Schellens, J.H. and Beijnen, J.H., Clinical pharmacokinetics and pharmacodynamics of ifosfamide and its metabolites. *Clin Pharmacokinet*, **40,** 41-62. (2001).

Knox, R.J., Friedlos, F., Lydall, D.A. and Roberts, J.J., Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1- cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res*, **46,** 1972-9. (1986).

Kool, M., de Haas, M., Scheffer, G.L., Scheper, R.J., van Eijk, M.J., Juijn, J.A., Baas, F. and Borst, P., Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res*, **57,** 3537-47. (1997).

Koropatnick, J. and Pearson, J., Altered cisplatin and cadmium resistance and cell survival in Chinese hamster ovary cells expressing mouse metallothionein. *Mol Pharmacol*, **44,** 44-50. (1993).

Korst, A.E., van der Sterre, M.L., Eeltink, C.M., Fichtinger-Schepman, A.M., Vermorken, J.B. and van der Vijgh, W.J., Pharmacokinetics of carboplatin with and without amifostine in patients with solid tumors. *Clin Cancer Res*, **3,** 697-703. (1997).

Korst, A.E., van der Sterre, M.L., Gall, H.E., Fichtinger-Schepman, A.M., Vermorken, J.B. and van der Vijgh, W.J., Influence of amifostine on the pharmacokinetics of cisplatin in cancer patients. *Clin Cancer Res*, **4,** 331-6. (1998).

Kreissman, S.G., Rackoff, W., Lee, M. and Breitfeld, P.P., High dose cyclophosphamide with carboplatin: a tolerable regimen suitable for dose intensification in children with solid tumors. *J Pediatr Hematol Oncol*, **19,** 309-12. (1997).

Lassen, U., Kristjansen, P.E., Osterlind, K., Bergman, B., Sigsgaard, T.C., Hirsch, F.R., Hansen, M., Dombernowsky, P. and Hansen, H.H., Superiority of cisplatin or carboplatin in combination with teniposide and vincristine in the induction chemotherapy of small-cell lung cancer. A randomized trial with 5 years follow up. *Ann Oncol*, **7,** 365-71. (1996).

Le Chevalier, T., Brisgand, D., Douillard, J.Y., Pujol, J.L., Alberola, V., Monnier, A., Riviere, A., Lianes, P., Chomy, P., Cigolari, S. and et al., Randomized study

of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: results of a European multicenter trial including 612 patients. *J Clin Oncol*, **12,** 360-7. (1994).

Lebwohl, D. and Canetta, R., Clinical development of platinum complexes in cancer therapy: an historical perspective and an update. *Eur J Cancer*, **34,** 1522-34. (1998).

Leeuwenkamp, O.R., Neijt, J.P., van der Vijgh, W.J. and Pinedo, H.M., Reaction kinetics of cisplatin and its monoaquated species with the modulating agents (di)mesna and thiosulphate. *European Journal of Cancer*, **27,** 1243-7 (1991).

Leeuwenkamp, O.R., van der Vijgh, W.J., Neijt, J.P. and Pinedo, H.M., Reaction kinetics of cisplatin and its monoaquated species with the (potential) renal protecting agents (di)mesna and thiosulfate. Estimation of the effect of protecting agents on the plasma and peritoneal AUCs of CDDP. *Cancer Chemotherapy & Pharmacology*, **27,** 111-4 (1990).

Leone, B., Vallejo, C., Perez, J., Cuevas, M.A., Machiavelli, M., Lacava, J., Focaccia, G., Ferreyra, R., Suttora, G., Romero, A., Castaldi, J., Arroyo, A. and Rabinovich, M., Ifosfamide and cisplatin as neoadjuvant chemotherapy for advanced cervical carcinoma. *Am J Clin Oncol*, **19,** 132-5. (1996).

Links, M. and Lewis, C., Chemoprotectants: a review of their clinical pharmacology and therapeutic efficacy. *Drugs*, **57,** 293-308 (1999).

Logothetis, C.J., Samuels, M.L., Ogden, S.L., Dexeus, F.H. and Chong, C.D., Cyclophosphamide and sequential cisplatin for advanced seminoma: long- term followup in 52 patients. *J Urol*, **138,** 789-94. (1987).

Margolin, B.K., Doroshow, J.H., Ahn, C., Hamasaki, V., Leong, L., Morgan, R., Raschko, J., Shibata, S., SomLo, G. and Tetef, M., Treatment of germ cell cancer with two cycles of high-dose ifosfamide, carboplatin, and etoposide with autologous stem-cell support. *J Clin Oncol*, **14,** 2631-7. (1996).

Marty, M., Pouillart, P., Scholl, S., Droz, J.P., Azab, M., Brion, N., Pujade-Lauraine, E., Paule, B., Paes, D. and Bons, J., Comparison of the 5-hydroxytryptamine3 (serotonin) antagonist ondansetron (GR 38032F) with high-dose metoclopramide in the control of cisplatin-induced emesis. *N Engl J Med*, **322,** 816-21. (1990).

McEvoy, G.K., Cisplatin, *American Hospital Formulary Service Drug Information*, pp. 816-818, American Society of Health-Systems Pharmacists, Inc., Bethesda, MD (1999).

Meyer, W.H., Pratt, C.B., Poquette, C.A., Rao, B.N., Parham, D.M., Marina, N.M., Pappo, A.S., Mahmoud, H.H., Jenkins, J.J., Harper, J., Neel, M. and Fletcher, B.D., Carboplatin/ifosfamide window therapy for osteosarcoma: results of the st jude children's research hospital os-91 trial. *J Clin Oncol*, **19,** 171-82. (2001).

Millar, B.C., Siddik, Z.H., Millar, J.L. and Jinks, S., Mesna does not reduce cisplatin induced nephrotoxicity in the rat. *Cancer Chemotherapy & Pharmacology*, **15,** 307-9 (1985).

Miller, S.A., Dykes, D.D. and Polesky, H.F., A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, **16,** 1215 (1988).

Miller, V.A., Rigas, J.R., Pisters, K.M., Grant, S.C., Pfister, D.G., Heelan, R.T. and Kris, M.G., Ifosfamide plus high-dose cisplatin in patients with non-small cell lung cancer previously treated with chemotherapy. *Am J Clin Oncol*, **18,** 303-6. (1995).

Moore, M.J., Clinical pharmacokinetics of cyclophosphamide. *Clin Pharmacokinet*, **20,** 194-208. (1991).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65,** 55-63 (1983).

Mu, D., Hsu, D.S. and Sancar, A., Reaction mechanism of human DNA repair excision nuclease. *J Biol Chem*, **271,** 8285-94. (1996).

Murry, D.J., Comparative clinical pharmacology of cisplatin and carboplatin. *Pharmacotherapy*, **17,** 140S-145S. (1997).

Nagai, N., Kinoshita, M., Ogata, H., Tsujino, D., Wada, Y., Someya, K., Ohno, T., Masuhara, K., Tanaka, Y., Kato, K., Nagai, H., Yokoyama, A. and Kurita, Y., Relationship between pharmacokinetics of unchanged cisplatin and nephrotoxicity after intravenous infusions of cisplatin to cancer patients. *Cancer Chemother Pharmacol*, **39,** 131-7 (1996).

Natarajan, G., Malathi, R. and Holler, E., Increased DNA-binding activity of cis-1,1- cyclobutanedicarboxylatodiammineplatinum(II) (carboplatin) in the presence of nucleophiles and human breast cancer MCF-7 cell cytoplasmic extracts: activation theory revisited. *Biochem Pharmacol*, **58,** 1625-9. (1999).

Newell, D.R., Pearson, A.D., Balmanno, K., Price, L., Wyllie, R.A., Keir, M., Calvert, A.H., Lewis, I.J., Pinkerton, C.R. and Stevens, M.C., Carboplatin pharmacokinetics in children: the development of a pediatric dosing formula. The

United Kingdom Children's Cancer Study Group. *J Clin Oncol*, **11**, 2314-23. (1993).

Notari, R.E., *Biopharmaceutics and clinical pharmacokinetics : an introduction*, 4th , rev. and expand ed., M. Dekker, New York (1987).

Obermair, A., Speiser, P., Thoma, M., Kaider, A., Salzer, H., Dittrich, C. and Sevelda, P., Prediction of toxicity but not of clinical course by determining carboplatin exposure in patients with epithelial ovarian cancer treated with a combination of carboplatin and cisplatin. *Int J Oncol*, **13,** 1023-30. (1998).

Obrocea, M.M., Nassim, M.A., Molepo, M.J., Shirazi, F.H., Gallant, G., Dulude, H., Vincent, M.D., Stewart, D.J. and Goel, R., In vitro carboplatin-mesna interaction in aqueous solution, human plasma and urine. *Oncol Rep*, **5,** 1493-8. (1998).

O'Dwyer, P.J., Stevenson, J.P. and Johnson, S.W., Clinical pharmacokinetics and administration of established platinum drugs. *Drugs*, **59,** 19-27. (2000).

Olivero, O.A., Chang, P.K., Lopez-Larraza, D.M., Semino-Mora, M.C. and Poirier, M.C., Preferential formation and decreased removal of cisplatin-DNA adducts in Chinese hamster ovary cell mitochondrial DNA as compared to nuclear DNA. *Mutat Res*, **391,** 79-86. (1997).

Omura, G., Blessing, J.A., Ehrlich, C.E., Miller, A., Yordan, E., Creasman, W.T. and Homesley, H.D., A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma. A Gynecologic Oncology Group Study. *Cancer*, **57,** 1725-30. (1986).

Oprea, A., Bazzazi, H., Kangarloo, B. and Wolff, J.E., The kinetics and mechanisms of the reaction of Mesna with cisplatin, oxiplatin and carboplatin. *Anticancer Res*, **21,** 1225-9. (2001).

Ormstad, K., Orrenius, S., Lastbom, T., Uehara, N., Pohl, J., Stekar, J. and Brock, N., Pharmacokinetics and metabolism of sodium 2-mercaptoethanesulfonate in the rat. *Cancer Res*, **43,** 333-8. (1983).

Ormstad, K. and Uehara, N., Renal transport and disposition of Na-2-mercaptoethane sulfonate disulfide (dimesna) in the rat. *FEBS Lett*, **150,** 354-8. (1982).

Pattanaik, A., Bachowski, G., Laib, J., Lemkuil, D., Shaw, C.F., 3rd, Petering, D.H., Hitchcock, A. and Saryan, L., Properties of the reaction of cis-dichlorodiammineplatinum(II) with metallothionein. *J Biol Chem*, **267,** 16121-8. (1992).

Pendyala, L. and Creaven, P.J., In vitro cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. *Cancer Research*, **53,** 5970-6 (1993).

Peng, B., English, M.W., Boddy, A.V., Price, L., Wyllie, R., Pearson, A.D., Tilby, M.J. and Newell, D.R., Cisplatin pharmacokinetics in children with cancer. *Eur J Cancer*, **33,** 1823-8. (1997).

Perez, R.P., Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer*, **34,** 1535-42. (1998).

Polyzos, A., Tsavaris, N., Kosmas, C., Petrikos, G., Giannikos, L., Kalahanis, N., Papadopoulos, O., Christodoulou, K., Giannakopoulos, K., Veslemes, M. and Katsilambros, N., Second-line chemotherapy with cisplatin-ifosfamide in patients with ovarian cancer previously treated with carboplatin-cyclophosphamide. *J Chemother*, **11,** 144-9. (1999).

Price, A., Shi, Q., Morris, D., Wilcox, M.E., Brasher, P.M., Rewcastle, N.B., Shalinsky, D., Zou, H., Appelt, K., Johnston, R.N., Yong, V.W., Edwards, D. and Forsyth, P., Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin Cancer Res*, **5,** 845-54. (1999).

Reece, P.A., Stafford, I., Abbott, R.L., Anderson, C., Denham, J., Freeman, S., Morris, R.G., Gill, P.G. and Olweny, C.L., Two- versus 24-hour infusion of cisplatin: pharmacokinetic considerations. *J Clin Oncol*, **7,** 270-5. (1989).

Reece, P.A., Stafford, I., Russell, J., Khan, M. and Gill, P.G., Creatinine clearance as a predictor of ultrafilterable platinum disposition in cancer patients treated with cisplatin: relationship between peak ultrafilterable platinum plasma levels and nephrotoxicity. *J Clin Oncol*, **5,** 304-9. (1987).

Reed, E. and Kohn, K.W., Platinum analogues. *In:* B.A. Chabner and J.M. Collins (eds.), *Cancer Chemotherapy: principles and practice*, pp. 465-490, J. B. Lippincott Co. (1990).

Reed, E., Ozols, R.F., Tarone, R., Yuspa, S.H. and Poirier, M.C., Platinum-DNA adducts in leukocyte DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. *Proc Natl Acad Sci U S A*, **84,** 5024-8. (1987).

Rixe, O., Ortuzar, W., Alvarez, M., Parker, R., Reed, E., Paull, K. and Fojo, T., Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-

resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. *Biochem Pharmacol*, **52,** 1855-65. (1996).

Rosenberg, B., Van Camp, L., GrimLey, E.B. and Thomson, A.J., The inhibition of growth or cell division in Escherichia coli by different ionic species of platinum(IV) complexes. *J Biol Chem*, **242,** 1347-52. (1967).

Rosenberg, B., Van Camp, L. and Krigas, T., Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. *Nature*, **205,** 698-699 (1965).

Rosenberg, B., VanCamp, L., Trosko, J.E. and Mansour, V.H., Platinum compounds: a new class of potent antitumour agents. *Nature*, **222,** 385-6. (1969).

Sauter, C., Cogoli, M. and Arrenbrecht, S., Interactions of cytotoxic and other drugs: rapid cell culture assay. *Oncology*, **43,** 46-9 (1986).

Shapiro, J.D., Rothenberg, M.L., Sarosy, G.A., Steinberg, S.M., Adamo, D.O., Reed, E., Ozols, R.F. and Kohn, E.C., Dose intensive combination platinum and cyclophosphamide in the treatment of patients with advanced untreated epithelial ovarian cancer. *Cancer*, **83,** 1980-8. (1998).

Shaw, I.C., Graham, M.I. and Jones, M.S., The fate of [14C]-mesna in the rat. *Arzneimittelforschung*, **36,** 487-9. (1986).

Skarlos, D.V., Samantas, E., Kosmidis, P., Fountzilas, G., Angelidou, M., Palamidas, P., Mylonakis, N., Provata, A., Papadakis, E., Klouvas, G. and et al., Randomized comparison of etoposide-cisplatin vs. etoposide-carboplatin and irradiation in small-cell lung cancer. A Hellenic Co-operative Oncology Group study. *Ann Oncol*, **5,** 601-7. (1994).

Skinner, R., Pearson, A.D., English, M.W., Price, L., Wyllie, R.A., Coulthard, M.G. and Craft, A.W., Cisplatin dose rate as a risk factor for nephrotoxicity in children. *Br J Cancer*, **77,** 1677-82. (1998).

Sorenson, C.M. and Eastman, A., Influence of cis-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. *Cancer Res*, **48,** 6703-7. (1988).

Stewart, D.J., Benjamin, R.S., Luna, M., Feun, L., Caprioli, R., Seifert, W. and Loo, T.L., Human tissue distribution of platinum after cis-diamminedichloroplatinum. *Cancer Chemother Pharmacol*, **10,** 51-4. (1982).

Sundquist, W.I., Lippard, S.J. and Stollar, B.D., Monoclonal antibodies to DNA modified with cis- or trans- diamminedichloroplatinum(II). *Proc Natl Acad Sci U S A*, **84,** 8225-9. (1987).

Swenerton, K., Jeffrey, J., Stuart, G., Roy, M., Krepart, G., Carmichael, J., Drouin, P., Stanimir, R., O'Connell, G., MacLean, G. and et al., Cisplatin-cyclophosphamide versus carboplatin-cyclophosphamide in advanced ovarian cancer: a randomized phase III study of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*, **10,** 718-26. (1992).

Tallen, G., Mock, C., Gangopadhyay, S.B., Kangarloo, B., Krebs, B. and Wolff, J.E., Overcoming cisplatin resistance: design of novel hydrophobic platinum compounds. *Anticancer Research*, **20,** 445-9 (2000).

Taylor, S.G.t., Murthy, A.K., Vannetzel, J.M., Colin, P., Dray, M., Caldarelli, D.D., Shott, S., Vokes, E., Showel, J.L., Hutchinson, J.C. and et al., Randomized comparison of neoadjuvant cisplatin and fluorouracil infusion followed by radiation versus concomitant treatment in advanced head and neck cancer. *J Clin Oncol*, **12,** 385-95. (1994).

Thomson, A.J., Williams, R.J.P. and Reslova, S., The chemistry of complexes related *cis*-$Pt(NH_3)_2Cl_2$. An anti-tumor drug. *Struct. Bond.*, **11,** 1 (1972).

Tonda, M.E., Heideman, R.L., Petros, W.P., Friedman, H.S., Murry, D.J. and Rodman, J.H., Carboplatin pharmacokinetics in young children with brain tumors. *Cancer Chemother Pharmacol*, **38,** 395-400 (1996).

Trimmer, E.E. and Essigmann, J.M., Cisplatin. *Essays Biochem*, **34,** 191-211 (1999).

Turchi, J.J. and Henkels, K., Human Ku autoantigen binds cisplatin-damaged DNA but fails to stimulate human DNA-activated protein kinase. *J Biol Chem*, **271,** 13861-7. (1996).

Ueoka, H., Kiura, K., Tabata, M., Kamei, H., Gemba, K., Sakae, K., Hiraki, Y., Hiraki, S., Segawa, Y. and Harada, M., A randomized trial of hybrid administration of cyclophosphamide, doxorubicin, and vincristine (CAV)/cisplatin and etoposide (PVP) versus sequential administration of CAV-PVP for the treatment of patients with small cell lung carcinoma: results of long term follow-up. *Cancer*, **83,** 283-90. (1998).

Urban, T., Chastang, C., Lebas, F.X., Duhamel, J.P., Adam, G., Darse, J., Brechot, J.M. and Lebeau, B., The addition of cisplatin to cyclophosphamide-doxorubicin-etoposide combination chemotherapy in the treatment of patients

with small cell lung carcinoma: A randomized study of 457 patients. "Petites Cellules" Group. *Cancer*, **86,** 2238-45. (1999).

van der Vijgh, W.J., Clinical pharmacokinetics of carboplatin. *Clin Pharmacokinet*, **21,** 242-61. (1991).

van der Vijgh, W.J. and Klein, I., Protein binding of five platinum compounds. Comparison of two ultrafiltration systems. *Cancer Chemother Pharmacol*, **18,** 129-32 (1986).

Van Echo, D.A., Egorin, M.J., Whitacre, M.Y., Olman, E.A. and Aisner, J., Phase I clinical and pharmacologic trial of carboplatin daily for 5 days. *Cancer Treat Rep*, **68,** 1103-14. (1984).

van Warmerdam, L.J., Rodenhuis, S., van der Wall, E., Maes, R.A. and Beijnen, J.H., Pharmacokinetics and pharmacodynamics of carboplatin administered in a high-dose combination regimen with thiotepa, cyclophosphamide and peripheral stem cell support. *Br J Cancer*, **73,** 979-84. (1996).

Vermorken, J.B., van der Vijgh, W.J., Klein, I., Gall, H.E., van Groeningen, C.J., Hart, G.A. and Pinedo, H.M., Pharmacokinetics of free and total platinum species after rapid and prolonged infusions of cisplatin. *Clin Pharmacol Ther*, **39,** 136-44. (1986).

Vollmer, C.M., Ribas, A., Butterfield, L.H., Dissette, V.B., Andrews, K.J., Eilber, F.C., Montejo, L.D., Chen, A.Y., Hu, B., Glaspy, J.A., McBride, W.H. and Economou, J.S., p53 selective and nonselective replication of an E1B-deleted adenovirus in hepatocellular carcinoma. *Cancer Res*, **59,** 4369-74. (1999).

Wagner, T., Kreft, B., Bohlmann, G. and Schwieder, G., Effects of fosfomycin, mesna, and sodium thiosulfate on the toxicity and antitumor activity of cisplatin. *Journal of Cancer Research & Clinical Oncology*, **114,** 497-501 (1988).

Williams, D.A. and Lokich, J., A review of the stability and compatibility of antineoplastic drugs for multiple-drug infusions. *Cancer Chemother Pharmacol*, **31,** 171-81 (1992).

Wils, J., van Geuns, H., Stoot, J., Bergmans, M., Boschma, F., Bron, H., Degen, J., Erdkamp, F., van Erp, J., Haest, J., Iding, R., Lalisang, F., de Pree, N., de Rooy, C., Snijders, M., Schepers, J., Vreeswijk, J., Wals, J., Werter, M., Wetzels, L., Smeets, J. and Schouten, L., Cyclophosphamide, epirubicin and cisplatin (CEP) versus epirubicin plus cisplatin (EP) in stage Ic-IV ovarian cancer: a randomized phase III trial of the Gynecologic Oncology Group of the Comprehensive Cancer Center Limburg. *Anticancer Drugs*, **10,** 257-61. (1999).

Wolff, J.E., Egeler, R.M., Anderson, R., Ujack, E., Iceton, S. and Coppes, M.J., Mesna inactivates platinum agents in vitro. *Anticancer Research*, **18,** 4077-81 (1998).

Yamada, M., O'Regan, E., Brown, R. and Karran, P., Selective recognition of a cisplatin-DNA adduct by human mismatch repair proteins. *Nucleic Acids Res*, **25,** 491-6. (1997).

Yoshida, M., Khokhar, A.R., Zhang, Y.P., Thai, G. and Siddik, Z.H., Kinetics of tissue disposition of cis-ammine/cyclohexylamine- dichloroplatinum(II) and cisplatin in mice bearing FSaIIC tumors. *Cancer Chemother Pharmacol*, **35,** 38-44 (1994).

Zamble, D.B., Mu, D., Reardon, J.T., Sancar, A. and Lippard, S.J., Repair of cisplatin--DNA adducts by the mammalian excision nuclease. *Biochemistry*, **35,** 10004-13. (1996).

Zwelling, L.A., Cisplatin and new platinum analogs. *Cancer Chemother Biol Response Modif*, **10,** 64-72 (1988).