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Chemical, Pharmacokinetic and Biological Aspects of Platinum-Based Drugs

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The use of cisplatin for the treatment of metastatic testicular cancer is a medical historical landmark given that, for the first time, patients could be cured with drug therapy. A third generation platinum-based drug, oxaliplatin, has been registered for use in the treatment of colorectal cancer. Cisplatin and oxaliplatin have limited cross-resistance and display markedly different toxicity profiles. Platinum-based drugs (PBD) are chemically transformed to produce a variety of compounds that are of importance with respect to both wanted and unwanted toxicities. DNA-dependent protein kinase (DNA-PK), best known for its role in repair of DNA double-strand breaks, is involved in signalling pathways leading to cell death. It is unclear whether or not DNA-PK is a determinant of the cytotoxicity of PBD. The monohydrated complex of cisplatin (MHC) is thought to be the main intracellular form of the drug. Based upon a HPLC technique developed at the Karolinska Hospital Pharmacy, MHC was isolated and quantified. MHC was found to be more cytotoxic than cisplatin. Low extracellular chloride concentration by itself had relatively little affect upon drug stability. In media with low chloride concentration and pH MHC was rapidly transformed. This could have practical implications given that solid tumours often have microenvironments that are extremely acidic. We describe a method for synthesizing the dihydrated oxaliplatin complex (DOC). DOC was formed by hydrolyzing oxaliplatin at 70^{o}C in sodium hydroxide. The cytotoxicity of DOC was compared to oxaliplatin in a lung cancer cell line. DOC was found to be significantly more cytotoxic than oxaliplatin. The pharmacokinetic profile of intact oxaliplatin was determined in patients receiving treatment for metastatic colon cancer. The *in vivo* clearance was compared with the rate of oxaliplatin degradation *in vitro* in whole blood samples taken from the patients immediately prior to the start of drug infusion. A good correlation between clearance of oxaliplatin *in vivo* and degradation *in vitro* was noted. We observed a short elimination half-life which is in sharp contrast to previous reports based upon analysis of platinum content. Trifluoperazine (TFP) has been shown to enhance the cytotoxicity of cisplatin. We tested a panel of phenothiazines for their inhibitory properties on DNA-PK. TFP was found to be an inhibitor of purified DNA-PK and DNA-PK in cell lysate. In a lung cancer cell line treated with TFP, DNA-PK activity was decreased with associated cleavage of DNA-PKcs and Ku86 after 30 minutes of incubation. Using a cell line defective in Ku86, we observed that the cytotoxicity of PBDs is enhanced especially with respect to cisplatin. While both drugs were able to induce apoptosis, no caspase-3 activation was noted after treatment with oxaliplatin. Cells lacking Ku86 had constitutional expression of activated p38. Oxaliplatin activated p38 more rapidly than cisplatin. We noted perinuclear translocation of p38 irregardless of Ku86 status in cells exposed to oxaliplatin. In cells exposed to cisplatin, p38 activation was localized to the nucleus in Ku86 deficient cells only. Our data supports a role for Ku86 in influencing the cytotoxicity of PBD. This influence differs depending upon the platinum carrier ligand in question.

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"cure sometimes, relieve often, comfort always"

Dr. Edward Trudeau

To my family (on both sides of the Atlantic)

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Manuscript

Abbreviations

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
AUC	Area under the curve
BRCP	Breast cancer resistance protein
CLL	Chronic lymphocytic leukemia
C_{max}	Maximum serum concentration
DACH	Diaminocyclohexane
DAPI	4,6´diamidino-2phenylindole dihydrochloride
DNA-PK	DNA dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DOC	Dihydrated oxaliplatin complex
DSB	Double-strand breaks
FMCA	Fluorescent microculture cytotoxicity assay
GC-NER	Global-coupled NER
GSH	Glutathione
GST	Glutathione-S-transferase
HMGB	High mobility group binding proteins
HPLC	High pressure liquid chromatography
IC50	50% inhibitory concentration
JNK	c-Jun-NH2-terminal kinase
LRP	Lung resistance-related protein
MAPK	Mitogen activated protein kinase
MHC	Monohydroxy complex (monoaqua)
MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
MRP	Multidrug resistance protein
NCI	National cancer institute
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PARP	Poly(ADP-ribose) polymerase
PBD	Platinum-based drugs
PD	Pharmacodynamics
P-gp	P-glycoprotein
PK	Pharmacokinetics
PUF	Plasma ultrafiltrate
TC-NER	Transcribed-coupled NER
TEER	Transepithelial electrical resistance
TDM	Therapeutic drug monitoring
TRAIL	TNF related apoptosis-inducing ligand
XIAP	X-linked inhibitory apoptosis factor

Background

Historical Perspectives

The ability of platinum-based compounds to inhibit cell division was an accidental discovery. Approximately 40 years ago, Rosenberg and colleagues were investigating the effects of electric fields on the growth of *E. coli*. They observed the formation of long bacterial filaments that were the result of extensive bacterial growth (up to 300 times normal length) without cell division. They hypothesized that various group VIII transition metal ions were responsible[215]. Four years later, the same research group published a paper defining platinum compounds as a new class of antitumour agents[216]. Interestingly, cisplatin was first described over one hundred years before Rosenberg's discovery[203].

In 1977, Einhorn and Donahue described the use of platinum-based combination chemotherapy in patients with disseminated testicular cancer. In this study, 80% of patients achieved a complete remission and 20% a partial remission[59]. The following year, cisplatin was approved for clinical use by the American Food and Drug Administration.

Cisplatin is now a key component in the treatment of both testes and ovarian cancer. Many lung and head and neck cancer chemotherapy regimes are based on this drug. The excitement surrounding the introduction of cisplatin for cancer treatment spawned research resulting in the synthesis of many second and third generation platinum-based compounds. Unfortunately, to date, only two other platinum-based drugs (PBD), carboplatin and oxaliplatin are registered for clinical use.

Already 30 years ago, diaminocyclohexane (DACH)-platinum compounds were of interest because they demonstrated, pre-clinically, different patterns of activity and resistance compared to cisplatin[130]. Tetraplatin was the first DACH-based platinum drug to reach a phase I clinical trial. Unfortunately, patients experienced severe and disabling neurotoxicity that stopped further development of the drug and cast a shadow over DACH-based platinum drugs in general[46]. The synthesis of the DACH compound oxaliplatin was described in 1977. Approximately 12 years later, the drug was tested for the first time in a clinical setting. In 1996, oxaliplatin was registered in France for the treatment of colon cancer[46].

Biochemical Properties

Understanding the chemical properties of cisplatin and oxaliplatin has proven to be challenging and many controversies remain. Through non-enzymatic chemical changes, these drugs form a variety of biotransformation products[5,57,156,291]. It is generally considered that it is these "activated" compounds that account for the cytotoxicity and side-effects of these agents[212]. There is no evidence supporting enzymatic processes in the chemical transformation of PBD. Once formed, the biotransformation products are very reactive and bind to a vast array of cellular contents such as proteins, phospholipids, thiols, RNA and DNA[51,57,212,244,277,291].

The classic model of cisplatin biotransformation involves replacing the chloride ions with better leaving groups such as water. The low intracellular chloride concentration, relative to the extracellular environment, helps promote the formation of these electrophilic aquated species. The exact role of chloride in determining the cytotoxicity of cisplatin is disputed. A study in which the intracellular chloride ion concentration was substantially lowered could not demonstrate any changes in (1) the amount of platinum diffusing into the cell, (2) the amount of DNA platination or (3) the degree of cytotoxicity[115]. Nevertheless, it has been shown that the aquated species of cisplatin are at least 10 times more reactive than the parent compound[51] and that they inhibit ATPase one thousand times more than cisplatin[47].

The main intracellular form of cisplatin is thought to be the monohydrated complex (MHC). The formation of MHC is favored by a low chloride ion concentration and high pH[9,114]. At low pH, MHC is unstable[281]. The microenvironment of solid tumours is often characterized by hypoxia and acidosis. This might have practical implications for the stability of MHC in tumours and subsequent drug efficacy.

As many as 17 biotransformation products of oxaliplatin have been described[93]. As is the case for cisplatin, there is no evidence for metabolic processes in their formation. In the plasma ultrafiltrate (PUF) and urine of three patients receiving oxaliplatin, several biotransformation products were identified by mass spectrometry; most notably Pt-(DACH)Cl_2 and [Pt-(DACH)(OH_2)Cl]$^+$. Other compounds identified were suggestive of platinum conjugates with glutathione, methionine and other low molecular weight species[5]. The dihydroxy product (DOC) of oxaliplatin has been isolated and shown to have significantly greater cellular uptake and cytotoxic properties than its parent compound[156,269]. However, DOC is thought to represent a very small amount of the total plasma platinum pool and therefore might not be a principle determinant of the cytotoxicity of oxaliplatin[156].

Pharmacokinetics

Studying the pharmacokinetics (PK) of drugs enables clinicians to optimize dose and scheduling of treatment in accordance with the drugs` pharmacodynamics (PD). For drugs that are administered intravenously, pharmacokinetics is concerned with a given drugs` distribution, metabolism and elimination[210]. The relevance of a thorough understanding of pharmacokinetics in oncology is perhaps best exemplified by methotrexate. Therapeutic drug monitoring (TDM) using maximum serum concentration (C_{max}) and area under the the time concentration curve (AUC) have proven useful in minimizing unwanted toxicity and maximizing therapeutic utility[217]. Most pharmacokinetic data is collected in Phase I trials where the number of patients is small and efficacy data is not a major study end-point. The lack of pharmacokinetic data correlated to drug efficacy and side-effects accounts, in large part, for the limited use of TDM in cancer chemotherapy[217].

It was shown many years ago that increasing the dose-rate of cisplatin resulted in higher maximum plasma concentrations but lower drug exposure as measured by the AUC. The explanation for this is that cisplatin is principally eliminated through the kidney. There is a significant reabsorption of cisplatin in the renal tubules. Administering the drug at similar doses but at a higher dose-rate results in saturation of tubular reabsorption and consequently, greater renal clearance and lower AUC[74].

When platinum compounds are infused into the bloodstream, they rapidly form complexes with circulating plasma proteins. It is the free, ultrafiltrable, non-protein bound platinum compounds (PUF) that are important with respect to cytotoxicity[93]. Platinum that is bound has traditionally been looked upon as irreversible. This is probably not entirely true. At 24 hours after infusion of cisplatin, 95% of platinum is bound. In renal failure due to cisplatin, dialysis is capable of removing 60-80% of platinum from the blood[250]. In a study measuring platinum concentrations over 3 consecutive administrations, a constant 6% ratio of PUF to bound platinum was observed[82]. These studies support some degree of equilibrium between the bound and unbound fractions.

Using tumour xenograft models and a microdialysis technique, unbound platinum was found in the interstitial tissue of the tumour several minutes after an infusion of cisplatin. The amount of unbound platinum peaked at 30 minutes. The amount of platinum in tumour tissue was approximately 25% of that seen in the liver and kidney[287].

Following an infusion of oxaliplatin, approximately 5-30% is unbound, 10-30% protein-bound and 40% complexes with haemoglobulin and small molecular weight compounds in erythrocytes[5,151,166]. In a study of oxaliplatin in humans, one hour after infusion, approximately 40% of protein-bound oxaliplatin was accounted for by γ-globulins and 40% by albumin. Only 12% of the total unbound platinum pool was circulating as oxaliplatin. Of the urinary platinum pool, 50% was in the form of the parent compound. By three hours, no oxaliplatin was detectable in PUF and only 10% in urine. Oxaliplatin, which binds to red blood cells, was complexed to haemoglobin (60%) and low-molecular weight species (40%)[5].

The volume of distribution of a drug is influenced by its lipophilicity. Oxaliplatin is more lipophilic than cisplatin. In a review article on the pharmacokinetics of oxaliplatin, it was stated that oxaliplatin has a vastly greater volume of distribution than cisplatin[93]. We studied the pharmacokinetics of oxaliplatin based on the parent substance and not by measurement of platinum. Our study shows no difference in the volume of distribution between cisplatin and oxaliplatin[58]. Whether or not differences in lipophilicity result in cytotoxic advantages and/or toxic disadvantages is unclear. It has been proposed as a possible explanation for the greater neurotoxicity seen with DACH-platinum compounds[107]. Two studies examining platinum analogues found that intracellular drug uptake and cytotoxicity correlated with lipophilicity[152,172]. An interesting article was recently published in which the chloride ligands of cisplatin were replaced by an anionic surfactant. The cisplatin analogue was thereby dramatically more hydrophobic while still maintaining readily dissociable ligands. The authors were able to show as high as a five-fold increase in the intracellular concentration of the drug with a corresponding decrease in IC50[68].

Both cisplatin and oxaliplatin are eliminated principally through renal excretion. Kizu and co-workers found that at 24 hours post treatment with cisplatin and oxaliplatin in rabbits, 57% and 76% respectively of the total platinum pool was found in the urine[134]. Approximately 3 hours after an infusion of oxaliplatin one third of platinum is cleared by the kidney[50]. There is a strong correlation between the clearance of oxaliplatin and renal function. One observes higher AUC for free platinum in patients with impaired renal function but C_{max} is not affected[166]. Despite changes in the clearance of oxaliplatin in patients with impaired renal function, there is no evidence that this correlates with increased toxicity[251]. The half-life of cisplatin is almost double that of oxaliplatin reflecting the more rapid clearance of oxaliplatin from the blood (32 vs. 19 $L/h/m^2$)[58].

The pharmacokinetics of cisplatin and MHC have been reported in two studies[11,267]. Approximately 2% of commercially available cisplatin is in the form of MHC[11]. After a 1 hour infusion of cisplatin, 10% of the circulating drug concentration is in the form of MHC[267] and MHC represents 15% of the AUC[11].

Platinum retention many years after treatment has been reported[86]. The authors measured plasma platinum concentration in the blood of 61 patients treated with chemotherapy for testicular cancer. This was compared to a control group consisting of 20 patients cured by orchidectomy alone. The median follow-up time was 14 years with a range of up to 20 years. The mean platinum concentration in the treatment group was 64.9 pg/g plasma. All treatment patients had detectable platinum. In the control group, 18 patients had no detectable platinum and 2 patients were at the limit of detection (6 pg/g plasma)[86].

Intracellular Transport

There has been much debate about the transport of platinum into cells. Support for passive transport has been the inability to saturate the uptake of cisplatin and the lack of competitive inhibition using structural analogs[81,83]. In an electron microscopy study, the authors were able to demonstrate that after exposure to cisplatin, platinum was evident within 5 minutes at the cell membrane and nuclear envelope. There was no platinum evident in intracellular vesicles and no transmembrane channels were evident[23].

On the other hand, many pharmacological agents alter the transport of cisplatin suggesting a process more complex than simple diffusion[83]. More recent research in the area of cellular platinum transport has highlighted a coupling of cisplatin to copper. Deletion of the copper transport gene Ctr1, in both yeast and mammalian cells, decreased cisplatin uptake and resulted in diminished cytotoxicity[110]. It has also been shown in ovarian cancer cell lines that resistance to cisplatin is associated with changes in the influx and efflux of the drug and that this is accompanied by similar changes in copper transport acting through the copper transporter ATP7A[127]. In human hepatoma cells, resistance to copper was associated with resistance to cisplatin and vice versa[223]. A study comparing cisplatin, carboplatin and oxaliplatin in relation to the presence or absence of the copper transporters ATP7A and ATP7B has been published[225]. The presence of these copper transporters resulted in resistance to cisplatin and carboplatin but hypersensitivity to oxaliplatin. Increased cellular accumulation was noted for all three drugs in the presence of the transporters but less DNA platination for cisplatin and carboplatin and increased platination with respect to oxaliplatin. The authors suggest that ATP7A/ATP7B sequester platinum to sub-cellular vesicles thereby detoxifying the drugs. The copper transporters are not as effective in sequestering oxaliplatin resulting in its greater cytotoxicity[225]. Another publication by the same group showed that a 1.5 increase in ATP7A expression resulted in resistance for all three platinum drugs[226].

There is limited published data with respect to the cellular transport of oxaliplatin. There is support for passive diffusion. The intracellular uptake of oxaliplatin at 1 hour was linearly correlated to the drug concentration. At concentrations as high as 250 μM, there was no evidence of uptake saturation[171]. In comparing the intracellular uptake of cisplatin and oxaliplatin, both compounds showed a linear increase in intracellular platinum with increasing extracellular concentration. Oxaliplatin had a steeper drug concentration versus intracellular platinum concentration curve than cisplatin[85].

A study of cisplatin in breast cancer cell lines showed that intracellular uptake was evident after 1 hour and peaked at 5 hours. Platinum reached the nucleus after 3 hours. The amount of platinum in the nucleus in relation to the total intracellular platinum content was approximately 6%[150]. Two other studies suggest that once platinum reaches the interstitial tissue, its cellular uptake is rapid[23,173]. Within 5 minutes following exposure to cisplatin, platinum is present at both the cell membrane and nuclear envelope[23]. Using fluorescently tagged platinum analogues, platinum entered the cell rapidly but it took one hour to reach the nucleus. The platinum in the nucleus started to

decline after a few hours and was not detectable by 24 hours. In this model, almost no intracellular platinum was evident at 24 hours[173].

Ishikawa and co-workers discovered a mechanism for the export of intracellular cisplatin. A glutathione export pump was shown to actively remove cisplatin-GSH complexes. This pump was found to be functionally overexpressed in cisplatin resistant cells[111,112].

Toxicity

Besides its different spectrum of clinical efficacy, oxaliplatin differs from cisplatin with respect to toxicity. Cisplatin induced nausea and vomiting is both severe and prolonged. The drug frequently causes hearing loss and renal impairment. Following prolonged exposure to cisplatin, many patients experience peripheral neurotoxicity in the form of parasthesias. In comparison, oxaliplatin induced nausea is both milder and of shorter duration. Ototoxicity and nephrotoxicity are not problems with this drug. The dose-limiting toxicity of oxaliplatin involves the peripheral nervous system. Bone-marrow depression is relatively mild for both drugs, in contrast to carboplatin.

Neurotoxicity

If a cumulative threshold dose is achieved, all patients will develop disabling neurotoxicity from oxaliplatin. This is often reversible but resolution of symptoms can be delayed for more than a year. There are two distinct types of oxaliplatin induced neurotoxicity. A relatively acute cold-induced painful muscle spasm involving the area around the mouth and larynx can occur within the first day after receiving treatment. Patients are advised not to consume cold drinks or eat ice cream after having recently received oxaliplatin. This side-effect is not related to the cumulative dose of the drug and is completely reversible. Peripheral sensory and fine motor neuropathy is the other form of neurotoxicity associated with oxaliplatin. It is common, dependent upon the cumulative dose and the ultimate dose-limiting toxicity.

With standard doses of cisplatin, nephrotoxicity overshadows neurotoxicity. Prolonged use of cisplatin can result in peripheral sensory neuropathy usually of a relatively minor character. When higher doses are used, one observes disabling peripheral neurotoxicity within three courses of treatment[191].

There is some evidence to suggest that the biotransformation products of oxaliplatin are more neurotoxic than the parent compound. For example, Pt-(DACH)Cl_2 is more toxic to the dorsal root ganglia in rats[157]. Perhaps unexpectantly, the relative peripheral neurotoxicities of a panel of platinum-based compounds did not correlate with their respective accumulation in rat nervous tissue[230]. In another animal study, while the initial drug accumulation did not correlate with the relative degree of neurotoxicity, following an 8 week recovery period, a greater degree of neurotoxicity from oxaliplatin compared to cisplatin did correlate with platinum tissue retention[107].

With regard to acute neurotoxicity, it has been suggested that the oxalate group might immobilize calcium ions thereby altering the amplitude of voltage-gated sodium channels. Giving patients calcium and magnesium supplements has been suggested to diminish the severity and frequency of this form of oxaliplatin induced neurotoxicity[96]. Interestingly, there is clinical data for both cisplatin and oxaliplatin suggesting that infusions of glutathione significantly reduce the frequency and severity of neurotoxicity[33,204,242]. There is also data suggesting that cytotoxicity is not compromised by glutathione infusions[30,33,242]. In a randomized double-blind clinical trial in patients receiving cisplatin for the treatment of ovarian cancer, a glutathione supplement was administered in the experimental arm. The authors observed statistically significant greater neurotoxicity in the control arm with a trend towards a better response outcome in the glutathione treated patients[242]. A similar study in patients with colon cancer treated with oxaliplatin and glutathione also showed reduced neurotoxicity and a trend towards greater cytotoxicity in the glutathione arm[33].

Nephrotoxicity

One of the principle toxicities of cisplatin involves the proximal tubules of the kidney with the potential for renal failure. It is not the parent drug itself but rather a reactive thiol biotransformation product that is nephrotoxic. The formation of this reactive thiol starts with a circulating glutathione-platinum conjugate. This compound is cleaved by γ-glutamyl transpeptidase to form a cysteinyl-glycine-platinum conjugate which is further cleaved by diaminopeptidase to form cysteine-platinum and ultimately the reactive nephrotoxic thiol[258].

A recently published study examined the hypothesis that changes in ion transport in renal epithelial cells contribute to cisplatin induced nephrotoxicity[155]. The authors measured the transepithelial electrical resistance (TEER) of renal epithelial cells, growing in monolayer, following exposure to cisplatin, carboplatin or oxaliplatin. Cisplatin treatment resulted in a complete breakdown in TEER. Carboplatin or oxaliplatin did not alter TEER when the cells were exposed from the apical side. Basolateral application of oxaliplatin resulted in a reduction in TEER but not to the same extent as cisplatin. Cimetidine, which acts as an organic cation transport inhibitor, inhibited the changes in TEER following platinum drug exposure. Caspase 3 activation was observed following changes in TEER and the degree of activation related to the extent of change in TEER[155].

Prevention of nephrotoxicity from cisplatin involves administration of intravenous physiologic saline and mannitol. It has been demonstrated using cell lines and a rodent xenograft model that in a hypotonic environment there is greater renal platinum uptake and subsequent apoptosis compared to normal or hyperosmolar environments. The authors of this paper suggest that one should aim at maintaining a normal or even high urine osmolarity in patients being treated with cisplatin[205].

Ototoxicity

In contrast to oxaliplatin, cisplatin gives rise to significant hearing loss. Symptomatic ototoxicity was observed in 15% of patients receiving high dose weekly cisplatin[52]. As expected, administration of the cisplatin biotransformation product MHC results in greater hearing loss compared to administration of the parent compound[60]. In a study examining the affects of administration of physiological, acidic or basic saline to the round window in animals receiving cisplatin, it was noted that ototoxicity was increased in an acidic environment and decreased in an alkaline environment[252]. In acidic saline, MHC will be predominantly in its monohdyrated rather than monohydroxy form. This implies that MHC will be more reactive in an acidic environment[9]. In animal models, N-acetylcysteine, vitamin E or sodium thiosulphate administration prior to cisplatin provided protection from cisplatin induced ototoxicity[54,125,144,177].

Cytotoxicity

It has generally been accepted that platinum-based drugs exert their cytotoxic effects through the formation of platinum-DNA adducts[12,212]. This occurs predominantly at the N7 position of guanosine[19,277]. Loss of chloride is the rate-limiting step in the formation of these monofunctional adducts[19]. The vast majority of cisplatin-DNA adducts are of the intrastrand type between adjacent guanines[69,234].

Oxaliplatin was compared to cisplatin with respect to sequence, region-specificity and number of DNA lesions in both naked and cellular DNA[277]. The sites and types of these lesions were nearly identical. Cisplatin adducts were two to six times more frequent than oxaliplatin adducts but this was not reflected in greater cytotoxicity[277]. In another study, oxaliplatin formed fewer adducts than cisplatin, less lethal interstrand cross-links and approximately half as many DNA-protein cross links. The magnitude of repair of DNA adducts appeared to be similar for both drugs and oxaliplatin inhibited DNA-chain elongation to a greater extent than cisplatin[278]. Using equimolar concentrations of oxaliplatin and cisplatin, it was noted that oxaliplatin produced less DNA-platination but as many early DNA strand breaks as cisplatin[67].

In general, the degree of cytotoxicity of cisplatin correlates with the amount of DNA-platination[27,101,121,122,135,169,198,283]. However, a study in testicular cancer germ cell lines found no association between cisplatin DNA-platination and drug sensitivity[174] and a similar observation was made in a breast cancer cell line[159]. The location of cisplatin DNA-adducts has been shown to be similar for sensitive and resistant cell lines[169]. Although cisplatin is not thought of as a cell cycle-dependent drug, DNA-platination increased from non-dividing interphase cells to dividing cells. The highest degree of platination was seen in cells undergoing apoptosis[169].

With respect to a correlation between the cytotoxicity of oxaliplatin and DNA-platination, the literature is limited. It has been shown that there is a correlation between platinum levels in PUF and platination of DNA in circulating leukocytes[151] and that

oxaliplatin DNA-platination is linearly related to the drug concentration[171]. However, no correlation could be found between DNA-platination and cytotoxicity[14,171]. In a study comparing cisplatin with oxaliplatin the authors found that there was greater DNA-platination for both drugs in the more sensitive cell lines[101].

A study examining the biotransformation products of oxaliplatin, in relation to cytotoxicity and cellular uptake, was performed in a human colon cancer cell line[156]. DOC was the most cytotoxic of the substances tested (3-4 times). It also resulted in twenty times greater cellular uptake than the other compounds. In a study with the lung cancer cell line A549, we also showed that DOC is significantly more cytotoxic than its parent compound[269].

MHC is generally accepted as the main biotransformation product of cisplatin[19,212,231] and is considerably more reactive than the parent compound[51]. We showed that isolated MHC is more cytotoxic than the parent compound[281].

Platinum based chemotherapy might have important targets apart from nuclear DNA. It has been demonstrated that cisplatin and oxaliplatin can induce apoptosis independent of the cell nucleus[91,163]. It is interesting to note that mitochondrial DNA has been shown to have a two to fifty times greater propensity to be platinated than nuclear DNA[87]. Cisplatin reacts with phospholipids[244], inhibits amino acid transport[228], protein synthesis,[102,136] ATPases[47], uncouples oxidative phosphorylation, causes calcium efflux from the mitochondria[4] and selectively alters the intracellular concentrations of calcium and potassium[207]. The importance of these other targets in relation to cytotoxicity is unknown. Similar targets might also be relevant for oxaliplatin but there is limited published data available on this subject.

Drug Resistance

Using an NCI panel of cell lines, the cytotoxicity of cisplatin and oxaliplatin were compared[213]. The general conclusion from this study was that the two drugs had a different spectrum of cytotoxic activity and did not share the same mechanisms of drug resistance[213]. The importance of this finding is put into context if one considers that carboplatin is fully cross-resistant with cisplatin.

There is no simple explanation accounting for *de novo* or acquired platinum drug resistance. The literature in this area is vast (with respect to cisplatin) and clearly reflects a variety of potential mechanisms that are present in some cancers but not in others. If we assume that it is DNA that is the cytotoxic target of platinum drugs, one can divide mechanisms of resistance into those that reduce DNA-platination and those that reduce the impact of DNA-platination. The former category involves factors influencing intracellular drug import and export, factors influencing biotransformation and factors affecting platinum binding to thiol-containing proteins and non-protein sulphhydryl groups. The latter category includes factors that impact upon DNA repair and perhaps more importantly, factors that influence the signaling pathways that are set in motion as a result of DNA-platinum adduct formation. It makes intuitive sense that platinum drug resistance is often a multifactorial process.

Drug Uptake and Efflux

There are several studies supporting changes in drug uptake and/or efflux as a mechanism for cisplatin resistance[38,101,135,152,172]. However, many published reports have not been able to show any relation between drug uptake or excretion and cisplatin cytotoxicity[71,85,160,198].

There are few published reports correlating drug uptake/efflux with the cytotoxicity of oxaliplatin[101,171]. Two studies suggest that drug uptake is a factor in the development of oxaliplatin resistance. Mishima and co-workers found that drug uptake was reduced by approximately 75% in oxaliplatin resistant cells. However, there was no general correlation between IC50 and drug uptake[171]. In the other study, the authors found a 45% decrease in accumulation of oxaliplatin in resistant cells[101].

Reduced platinum accumulation as result of increased drug efflux has not been consistently demonstrated. Cisplatin, and probably oxaliplatin, are generally not considered as substrates for the transporter protein P-glycoprotein (P-gp)[63,118,235,290]. Nevertheless, there is evidence to suggest that transporter proteins may have a role in determining the cytotoxicity of cisplatin. There is very little published data concerning the efflux of oxaliplatin.

In human hepatoblastoma cells, cisplatin exposure resulted in the up-regulation of MDR1, the gene encoding for P-gp. The concomitant use of cisplatin and the P-gp inhibitor PSC 833 resulted in increased cytotoxicity[273]. The multidrug resistance

associated proteins (MRP) represent a family of efflux transporters associated with glutathione conjugates[139]. Increased resistance to cisplatin in a melanoma cell line was associated with increased expression of MRP2 both at the level of mRNA and protein[149]. The authors also observed an association between overexpression of MRP2 and a reduction in DNA-platination[149]. In human colon adenocarcinoma and mouse hepatoma cells, cisplatin resistance was associated with the overexpression of MRP1 and MRP2 and reduced intracellular cisplatin[31]. Fractionated irradiation in a lung cancer cell line was shown to cause an increased expression of MRP1 and MRP2 and this was associated with decreased cytotoxicity for cisplatin[104]. Seventy-two biopsies from patients with non-small cell lung cancer were studied in relation to sensitivity to platinum-based chemotherapy and the expression of the transporter proteins P-gp, MRP1, MRP2, MRP3 and a further drug efflux membrane transporter, the breast cancer resistance protein (BCRP). Only BCRP expression correlated with response to chemotherapy. The response rate was 44% in patients whose tumours did not express BCRP and 24% in tumours that did express the transport protein[285]. In 56 patients with advanced testicular germ-cell cancer, no correlation with chemosensitivity or survival was seen with respect to P-gp, MRP1 or BCRP but there was an inverse correlation between expression of the lung resistance-related protein (LRP) and survival[290]. LRP is not thought to be involved in drug export but rather intracellular drug distribution[229].

DNA-Repair

Platinum-based drugs have many cellular targets however most of the research efforts to date have concentrated on the formation of DNA adducts as being central to the drugs` cytotoxic qualities. The consequences of how a given cell reacts to platination of its DNA accounts, at least in part, for differences in cisplatin and oxaliplatin resistance.

Using a series of ovarian cancer cell lines with acquired resistance to cisplatin, an increased removal of platinum-DNA adducts was observed[121,122]. There were fewer interstrand cross-links in the resistant cell lines[122]. Cells with acquired resistance repaired interstrand cross-links faster than sensitive cell lines[121]. There are other studies that have not found a significant relationship between repair of platinated DNA and cytotoxicity[27,123]. In a study comparing cisplatin and oxaliplatin, an association between cytotoxicity and both adduct formation and repair was observed in cisplatin treated cells only[14]. Another comparison study found that decreased DNA-platination was a resistance factor for both drugs but no difference in DNA-repair was observed between sensitive and resistant cell lines for either drug[101].

Mismatch Repair (MMR)

The MMR system functions to maintain fidelity of the genome. It is specifically involved in the recognition and repair of single base mismatches as well as insertion-deletion loops in replicating DNA. The first and most well known association between mutation in components of MMR and the development of cancer comes from cases of

hereditary non-polyposis colon cancer[41]. There are six well described proteins comprising MMR. MSH2 and MSH6 form a dimer called MutSα which is involved in recognition of both single base mismatches and insertion-deletion loops. MSH2 and MSH3 form another dimer, MutSβ, which recognizes only the insertion-deletion loops. MutSα and MutSβ recruit MLH1 and PMS2 to help execute a process of recognition, excision and repair[41,247].

MMR recognizes cisplatin-DNA adducts[55]. Aberrant MMR function is associated with cisplatin resistance. This has been demonstrated in cell lines and clinical material[2,71,72,205,263]. Cisplatin provides a strong selection pressure following exposure in MMR deficient cells. One hour of treatment with cisplatin at IC50 was sufficient to enrich a population of MMR deficient cells from 5% at the onset to 81% after only 5 days of incubation[72].

Two publications, in 1996, provided initial insight into the biological differences between cisplatin and oxaliplatin[70,213]. Using a large panel of NCI cell lines, Rixe and co-authors suggested that cisplatin and oxaliplatin are not cross-resistant[213]. Fink and his research associates provided evidence that oxaliplatin DNA-adducts go unrecognized by the MMR system and that while aberrant MMR was a factor in cisplatin resistance, this was not the case for oxaliplatin[70]. It was later shown that defects in MLH1 or MSH6 but not MSH3 altered sensitivity to cisplatin[263]. This corresponded to increased replicative bypass ability of cisplatin adducts. No differences were seen in replicative bypass of oxaliplatin adducts whether MMR was aberrant or not[263]. Several other papers have addressed this theme and they are consistent with the findings above[37,71,184,288].

The p53 structural and functional homologue, p73, is associated with cisplatin induced cytotoxicity[239]. The activation and subsequent induction of apoptosis by p73 requires interaction with c-Abl[3,88,286]. MMR is also involved in p73 pro-apoptotic signaling[34,88,236]. This has been shown to act via MLH1[34] and PMS2[236]. Indeed, PMS2 was shown to be a requirement for cisplatin induced apoptosis acting via p73[236]. Deficiency in either MLH1 or PMS2 is associated with cisplatin resistance[88,236].

Nucleotide Excision Repair (NER)

Platinum-DNA adducts are thought to be principally excised and repaired by the NER system. NER is more complex than MMR and comprises about 30 different proteins[41,76]. In general, NER functions to remove bulky adducts usually about 25 nucleotides in length. The opposite DNA strand serves as a template for repair. The autosomal recessive disorder, Xeroderma pigmentosa, is characterized by an increased sensitivity to ultraviolet light. These patients develop skin cancers in early childhood. The molecular biological basis to this disorder is defective NER through mutation in genes involved in damage recognition. Seven defects have been identified so far[76].

NERs` influence on platinum-based drug cytotoxicity has been highlighted by work on both cell lines and clinical material[14,29,80,108,176,193,232,280]. One can divide NER

into 2 main functioning groups, each with different components. The transcribed area of active genes is repaired by Transcribed-Coupled NER (TC-NER) while the untranscribed areas of active genes and the untranscribed genome are repaired by Global-Coupled NER (GC-NER)[80]. It has been shown that defects in TC-NER but not GC-NER give rise to increased sensitivity to cisplatin[80]. In two studies using antisense targeting the NER components XPA and ERCC1, the authors were able to demonstrate a decrease in NER, increased apoptosis and lower IC50 levels for cisplatin[232,280]. In a recent publication, colon cancer cell lines with acquired resistance to oxaliplatin were shown to have an increase in ERCC1 mRNA compared to parental cell lines[29].

A study in seventy-three patients with metastatic colorectal cancer, treated with oxaliplatin, looked for associations between the presence of three polymorphisms of the NER gene XPD and overall survival. The authors found that in patients with the genotypes Lys/Lys, Lys/G1n, and G1n/G1n, the median survivals were 17.4, 12.8 and 3.3 months respectively[193].

There are only a few reports comparing NER in cisplatin and oxaliplatin treated cells. In an *in vitro* study, DNA-adducts, from both drugs, were repaired by NER with equal efficiency[211]. This result is contradicted in a study where twenty-four hours after drug exposure, 1-7% of cisplatin adducts remained compared to 6-24% for oxaliplatin[14]. In the same paper, ERCC1 and XPA expression correlated with the IC50 of oxaliplatin. There was a clear relationship between ERCC1 and cisplatin DNA-platination and IC50 but no relationship with XPA.

Patients with mutations in the BRCA1 gene are at increased risk of developing breast and/or ovarian cancer[182]. In men, prostate and breast cancer are associated with BRCA1 mutation[148]. BRCA1 is involved in many cellular processes such as DNA-repair and its deficiency results in chromosomal instability[266]. Error prone non-homologous end-joining (NHEJ) predominates over homologous end-joining in BRCA1 mutated cells[266]. Embryonic mouse stems cells deficient in BRCA1 are unable to carry out TC-NER[92]. It has been shown that following exposure to cisplatin, BRCA1 overexpression results in drug resistance while BRCA1 inhibition produces hypersensitivity[108]. This observation is corroborated in several other studies[26,208,253].

The high mobility group binding proteins (HMGB) like HMGB1 have been shown to interact with platinated DNA and thereby influence NER of these adducts[162]. There are a number of published studies with somewhat conflicting results but a general conclusion is that HMGB1 is a factor in determining resistance to cisplatin[100,181,195,274]. If the same is true for oxaliplatin remains to be seen. Interestingly, it has been shown that HMGB1 has a greater affinity for cisplatin adducts compared with oxaliplatin platinated DNA[37,264].

The ability of DNA polymerases to synthesize DNA past platinum adducts differs depending upon the platinum drug in question. Both polβ and polη appear to be more efficient in translesion DNA synthesis in oxaliplatin compared to cisplatin-platinated DNA[36].

Glutathione

Platinum-based drugs have a high affinity for thiol containing substances. The majority of the non-protein thiol pool is comprised of the tripeptide glutathione (GSH). GSH, at physiologically relevant concentrations, has been shown to bind platinum compounds and prevent either formation of DNA platination altogether or limit the binding to monofunctional adducts[56]. Blocking of cellular thiols by N-ethylmaleimide resulted in an eight fold increase in DNA-platination[222]. As might be expected, MHC has a greater affinity for GSH than cisplatin[188]. In a recently published study comparing GSH affinities in different platinum compounds, oxaliplatin and carboplatin exhibited highest and lowest affinities for GSH respectively. Oxaliplatin had a 1.4 fold greater reaction rate to GSH than cisplatin[98].

A variety of published studies have shown that increases in intracellular GSH can decrease sensitivity to cisplatin and vice versa[39,89,109,168,245,248,284,289]. Meijer and co-workers looked at the relationship between drug sensitivity and levels of GSH and glutathione-S-tranferase (GST) activity in seven platinum-based compounds (GST is an enzyme that promotes the conjugation of GSH to a wide variety of electrophilic compounds[259]). They found a good inverse correlation between GSH levels and drug sensitivity for all compounds but no relationship with respect to GST[168]. Depletion of GSH with DL-buthionine-S,R-sulfoximine (BSO) increased cytotoxicity for cisplatin but not for the DACH-platinum compound tetraplatin. However, no correlation could be found in a study comparing the cytotoxicity of cisplatin and oxaliplatin with levels of GSH or GST[15]. Gene amplification of GST-π in both head and neck cancer biopsy specimens and cell lines was found to be very frequent[45]. In this study, there appeared to be a relationship between GST-π amplification and resistance to cisplatin.

The relationship between GSH and platinum drug resistance is not based entirely upon DNA platination. Yang and coworkers looked at GSH and sensitivity to cisplatin in seven head and neck cancer cell lines[283]. Cytotoxicity was inversely correlated to GSH content. There was a correlation between cisplatin adduct formation and drug sensitivity but no correlation between GSH and adduct formation. GSH depletion with BSO increased the cytotoxicity of cisplatin without changing the degree of DNA-adduct formation.

A connection between increased GSH and cisplatin resistance, acting through Bcl-2, has been suggested[220]. Cisplatin resistance was observed in cells overexpressing Bcl-2. The overexpression of Bcl-2 was associated with a 3-fold increase in intracellular GSH but without any change in DNA-platination. Inhibiting the synthesis of GSH resulted in abrogating Bcl-2 mediated resistance without affecting Bcl-2 expression or the formation of DNA adducts[220]. There might also be a connection between c-Jun and GSH. In cells overexpressing and underexpressing c-Jun, GSH content was increased and decreased respectively with a corresponding change in sensitivity to cisplatin[192]. High levels of GSTπ are thought to influence the mitogen activated protein kinase (MAPK) pathways resulting in resistance to drugs like cisplatin[259].

There is limited data on the role of thiols in determining the cytotoxicity of oxaliplatin[15,61,246]. A suggestion that thiols can be of clinical importance for this drug come from a study on genetic polymorphisms of GST in patients with metastatic colorectal cancer receiving oxaliplatin. The authors found that patients with the (105)Val/(105)Val, (105)I1e/(105)Val and (105)I1e/(105)I1e genotypes survived 24.9, 13.3 and 7.9 months respectively[246].

Platinum-Based Drugs and Cell Death

Our understanding of programmed cell death is highlighted by the process of apoptosis. An updated review of cell death pathways has recently been published[189]. It is becoming increasingly evident that the effectiveness of cancer treatments require the induction of both apoptotic and non-apoptotic pathways of cell death[189].

Activation of the caspase cascade is at the center of inducing apoptosis. Triggering caspases is accomplished either from stimuli within the cell, for example DNA damage, or from external ligands binding to receptors on the cell surface. Fas ligand (FasL) and tumour necrosis factor related apoptosis-inducing ligand (TRAIL) are two examples of triggers of the extrinsic apoptotic pathway. It has been suggested that Fas-mediated apoptosis requires clustering of the Fas receptor into lipid bound "rafts" that float freely in the plasma membrane. Exposure to cisplatin has been shown to promote this clustering[141].

A key event in the intrinsic pathway is the permeabilization of the outer mitochondrial membrane (MOMP)[94]. MOMP results in the release of a variety of apoptosis promoting compounds such as cytochrome *c*. This represents, for the most part, a "point of no return" with subsequent caspase activation and apoptotic cell death. MOMP is a pivotal target for the regulation of apoptosis. Members of the Bcl-2 family promote or inhibit MOMP. For example, Bcl-2 is anti-apoptotic in that it blocks MOMP whereas Bax and Bak are pro-apoptotic and stimulate MOMP[94].

The release of cytochrome *c* into the cytosol results in the formation of a complex comprising cytochrome *c*, apoptotic protease activating factor-1 (Apaf-1) and caspase 9. This complex is called an apoptosome and is responsible for activating the executioner caspase, caspase-3[49]. It should be noted that there is data supporting the concept of caspase-independent apoptosis. In cisplatin treated human heptoma cells, pan-caspase inhibition does not completely block apoptosis[131]. The release of apoptosis-inducing factor (AIF) from the mitochondria following MOMP can induce apoptosis without caspase activation[124].

The classic view of two distinct apoptotic pathways, one intrinsic and the other extrinsic, is an oversimplification[218]. It has been shown that caspase-8 activation, following receptor ligand interaction, results in the subsequent cleavage of Bid. This, in turn, causes release of cytochrome c from the mitochondria[146,159]. Cytochrome *c*, acting via caspase-6, can activate caspase-8[44].

A key regulator of apoptosis is p53. It has been shown that p53 can up-regulate Fas and Trail receptors[21,180,279]. Following exposure to cisplatin, up-regulation of Fas and Trail receptors were noted in p53-wild type cells only[180,279]. p53 can also regulate apoptosis by directly interacting with Bcl-2 and Bax resulting in their inhibition or activation respectively[40].

Cisplatin and oxaliplatin activate both of the classical pathways of apoptosis. Cisplatin can induce Fas, FasL,[79,180] activate caspase-8, caspase-9, caspase-3, Bax, cause the release of cytochrome c, and cleavage of poly(ADP-ribose) polymerase (PARP)[16,103,105,161,180,272]. Oxaliplatin has also been shown to induce both Fas and FasL[165,209] and induce apoptotic cell death through caspase-8, caspase-9, caspase-3, Bax, Bak, cytochrome *c* release and subsequent PARP cleavage[90,91,95,165,209,254].

It has been suggested that necrotic cell death might be more important in determining the cytotoxicity of oxaliplatin than it is with cisplatin[15]. In a study on a panel of colon cancer cell lines, no caspase-9 activation was observed following exposure to oxaliplatin suggesting limited involvement of the intrinsic apoptotic pathway[209].

Components of the apoptotic pathways are among the multitude of factors determining platinum-based drug sensitivity. This implies that these factors are potential therapeutic targets. In cisplatin sensitive cells, Fas antibody inhibited cisplatin-induced apoptosis. In resistant cells, FLIP (an inhibitor of the caspase-8/Fas associated death domain) was up-regulated and the use of FLIP antisense oligonucleotides promoted apoptosis[126]. Cisplatin has been shown to inhibit the expression and phosphorylation of FLIP[243]. Overexpression of Bcl-2 and Bcl-X_L was observed in cisplatin resistant ovarian cancer cell lines[282]. An oxaliplatin resistant colon cancer cell line showed no caspase-3 activation but unchanged Bak, Bcl-2 and Bcl-X_L expression[90]. The cell line was found to harbour a Bax mutation. The same research group recently reported that the mitochondrial apoptotic response to oxaliplatin is mediated by induction of Bax/Bak and that a defect in Bax/Bak activation accounts for drug resistance[91]. An antisense oligonucleotide, targeting Bcl-X_L has been shown to enhance oxaliplatin induced apoptosis[99]. The degree of enhancement was greater in p53 and Bax competent cell lines[99].

X-linked inhibitory apoptosis factor (XIAP) is an intracellular protein involved in the inhibition of apoptosis down-stream of mitochondrial signaling. Using an antisense targeting XIAP, prostate cancer cells were shown to increase their sensitivity to cisplatin by approximately twenty fold[8]. Cells reacted to cisplatin within twenty-four hours when XIAP was inhibited compared to ninety-six hours when exposed to cisplatin alone[8]. XIAP is a substrate of Akt and the phosphorylation of XIAP by Akt reduces XIAP degradation. This is associated with decreased caspase-3 activation following exposure to cisplatin[48]. In cells with dominant negative Akt, cytotoxicity to cisplatin was increased. This is at least partially accounted for by the lack of Akt-induced phosphorylation of XIAP. When cells were treated with a p53 inhibitor (pifithrin-alpha-hydrobromide) or had mutated p53, dominant negative Akt was no longer able to increase the cytotoxicity of cisplatin[75].

The impact of p53 on the cytotoxicity of cisplatin and oxaliplatin is unclear. In some models, p53 appears to be an important determinant of drug sensitivity[29,138,187,201,214,221] while in other models it does not[42,91,137,138,202]. Using a panel of cell lines from the NCI, it was shown that cisplatin had a tendency to cause less growth inhibition in those cell lines that were p53 mutated[187]. In a study in patients with lung cancer, only three of twenty patients with aberrant p53 expression receiving neoadjuvant cisplatin demonstrated a pathological tumour response to treatment[221]. Gene transfer experiments with adenovirus vectors suggest that cisplatin cytotoxicity is enhanced by p53[214]. On the other hand, loss of p53 in ovarian cancer cell lines increased sensitivity to cisplatin[201]. In two studies using xenograft models, the presence or absence of p53 mutation did not have any impact upon the sensitivity to cisplatin[42,137].

Cisplatin and a DACH-platinum compound were tested in human cancer cell lines for their abilities to phosphorylate p53 and activate downstream targets[175]. Both compounds were able to phosphorylate serine-15. In contrast to cisplatin, the DACH compound provided either weak or no phosphorylation of p53 at serine-392. There was no difference in the downstream induction of p21 or Mdm2 for either compound[175]. Oxaliplatin was shown to be less sensitive in p53 -/- cell lines[29]. No PARP cleavage was present after treatment with oxaliplatin in p53 null cells[29]. The cytotoxicity of both oxaliplatin and cisplatin and their induction of apoptosis were independent of p53 status in a mouse salivary tumour model[202]. In a study comparing cisplatin and oxaliplatin in cervical cancer cells, p53 function was abrogated by either the human papillomavirus (HPV) E6 oncogene or by using p53 dominant negative cells. Lack of a p53 response resulted in increased cytotoxicity for cisplatin but decreased cytotoxicity for oxaliplatin[138]. In a recent study using colon cancer cell lines, sensitivity to oxaliplatin was shown to be only marginally dependent upon p53[91].

Mitogen-activated Protein Kinases

Following exposure to toxic agents, cells engage in a variety of signaling pathways that will lead either to detoxification and cell recovery or cell death. The MAPK family pathways are clearly implicated in the development of cancer however their role is complex and there is support in the literature for either promotion or suppression of tumour development depending upon the model used. This area has recently been reviewed in detail[62]. One of the MAPK pathways implicated in platinum cytotoxicity is c-Jun-NH2-terminal kinases (JNK). A central component of this pathway is c-Jun. Activation of c-Jun by JNK can result in either apoptosis or resistance to cell death depending on the cell type and the degree of aberrant cell cycle regulation/proliferation[265].

The complex involvement of MMR, JNK, c-Abl and p73 in PBD induced apoptosis seems to represent important differences in signaling pathways between cisplatin and oxaliplatin. Cisplatin can induce p73[88,255]. The tyrosine kinase c-Abl phosphorylates and activates p73[88,260,286]. It has recently been shown that c-Jun prolongs the half-life of p73 and potentiates p73-mediated cell death[255]. Cisplatin induction of p73 is absent in cells that are c-Jun defective and this appears to be independent of c-Abl activation of p73[255] even though it has been shown that cisplatin induces p73 via c-Abl[88]. The MMR protein PMS2 interacts with p73[236]. Indeed, PMS2 is a requirement for p73 induced apoptosis triggered by cisplatin[236]. JNK and c-Abl were shown to be differentially activated by cisplatin depending upon the proficiency of MMR[183]. Oxaliplatin appears to have limited ability or no ability at all, to activate c-Abl or JNK irregardless of the status of MMR[184, 209].

The p38 MAPK pathway has been shown to be an inducer of apoptosis following exposure to oxaliplatin[209]. p38 has four isoforms, α,β,γ and δ that are differentially activated depending upon the strength of the upstream signal[7]. p38α is thought to be pro-apoptotic and increases expression of Bax and the Fas/CD95 receptor[206]. In contrast, p38β doesn't potentiate Fas-mediated casapse-8 activation[256] and TNF-α induced apoptosis is inhibited by p38β[97]. Cisplatin has also been shown to activate p38[154].

DNA-dependent Protein Kinase

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase. Through NHEJ DNA-PK plays an important role in V(D)J recombination and the repair of DNA double strand breaks (DSB)[32]. It is also involved in the process of telomere shortening[65]. In mice deficient in DNA-PK, one observes increased shortening of telomeres, a shorter life span, increased incidence of infections, increased intestinal atrophy, increased skeletal change associated with aging and an increased incidence of T-cell lymphomas[66]. It is becoming increasingly clear that DNA-PK not only senses and repairs DNA damage but that it also acts in signal transduction triggering cell death. This is discussed below.

The DNA-PK protein complex consists of a large 465 kDa catalytic subunit (DNA-PKcs) and a heterodimeric regulatory binding complex consisting of two smaller proteins, Ku86 and Ku70 respectively. It is the Ku complex that binds to the open-ended DNA strand and the c-terminus of Ku86 recruits DNA-PKcs[84,240].

Aberrant DNA-PK has been demonstrated in mice, dogs and horses[133,200]. In humans, two examples of defective DNA-PK have been reported[6,249]. In 12 of 14 patients with multiple myeloma, truncated Ku86 was found[249]. The Ku86 variant exhibited less binding to DNA strand breaks and did not bind and activate DNA-PKcs[249]. Two cell lines were developed from a patient with glioblastoma multifrome. These cells lines, M059K and M059J, are DNA-PKcs wild-type and mutated respectively[6]. It has been shown in murine and human cell lines that expression of Bcr-Abl results in decreased post-transcriptional expression of DNA-PKcs[53].

The relevance of DNA-PK to the treatment of cancer is unclear. With respect to ionizing radiation, the in-vitro data is consistent. Deficiency in DNA-PK results in hypersensitivity to ionizing radiation[22,35,43,53,113,145,153,164,196,224,240,249,268,270]. M059J, DNA-PKcs defective, is more radiosensitive compared to M059K, DNA-PKcs wild-type[270]. An antisense construct targeting Ku86 resulted in increased radiation sensitivity in human fibroblasts[164]. This has been shown in M059 as well[22]. A small increase in radiation sensitivity was also observed with antisense targeting Ku70[190]. Using antisense targeting DNA-PKcs in human non-small cell lung cancer cell lines, the authors were able to demonstrate increased sensitivity to ionizing radiation and decreased DNA DSB repair[224]. A recent study in cell lines derived from two patients with late radionecrosis, following radiation treatment, showed decreased repair of DSB and lower DNA-PK activity (6 to 10 fold) compared to control cell lines[153].

With regards to clinical material, the impression is that higher DNA-PK activity/expression is advantageous to cancer treatment. In patients with tonsillar cancer, higher expression of Ku86 correlated with better loco-regional control following radiation treatment and higher DNA-PKcs correlated with improved survival[77]. This observation was corroborated in biopsy material from 67 esophagus cancer patients where high DNA-PKcs content was associated with improved clinical benefit from radiation treatment[185].

The role of DNA-PK in influencing the sensitivity to chemotherapeutic agents is less well established. In patients with chronic lymphocytic leukemia (CLL), DNA-PK activity correlated with resistance to chlorambucil[179]. This was observed in *in vitro* testing of lymphocytes from these patients and in the clinical response to treatment with chlorambucil[179]. A correlation was also noted for resistance to etoposide and DNA-PK activity in cells from patients with CLL[64]. In DNA-PKcs deficient fibroblasts from scid mice, exposure to bleomycin but not etoposide or cisplatin resulted in increased cytotoxicity[170]. Rodent cells, deficient in either DNA-PKcs or Ku86, had increased sensitivity to cisplatin[178]. This was also seen in the parental cells treated with the non-specific DNA-PKcs inhibitor wortmannin[178]. In both Ku70 and Ku86 null cells, increased cytotoxicity was noted for bleomycin, vincristine, taxol, adriamycin and etoposide. Tumour cells from patients with multiple myeloma with truncated Ku86 were hypersensitive to both mitomycin and bleomycin[249]. Ovarian cancer cell lines, 20 times more resistant to cisplatin than their parental lines, were found to have enhanced expression of DNA-PKcs at both the transcript and protein levels[257]. Using a Ku86 antisense construct in M059, the authors found enhanced cytotoxicity for bleomycin and etoposide but not for cisplatin or chlorambucil[22]. An antisense targeting Ku70 showed minor increases in drug sensitivity for bleomycin but not for cisplatin[190]. Using a novel DNA-PK inhibitor, the cytotoxicity from topoisomerase II drugs was potentiated in a leukemia cell line[275]. Increased expression of Ku86 and increased Ku86-DNA binding was found in cells that were cross-resistant to both ionizing radiation and cisplatin[78].

DNA-PK plays a role in apoptotic signaling. In a study using mouse embryo fibroblasts, DNA-PK was shown to be necessary for phosphorylating p53 at the human equivalent of serine 15 (serine 18). The absence of phosphorylated serine 18 abrogated radiation induced apoptosis[276]. Gemcitabine incorporation into DNA resulted in the activation of DNA-PK with subsequent complexing of DNA-PK with p53. Serine 15 of p53 was phosphorylated and the p53/DNA-PK complex co-localized to the nucleus with concomitant induction of apoptosis[1]. Whole-body irradiation in DNA-PK mutant mice resulted in normal induction of p21 but no induction of Bax[271]. This suggests that DNA-PK is involved in the upstream signaling of p53 with regards to the regulation of apoptosis but not cell-cycle arrest.

It has recently been proposed that DNA-PK can signal adjacent cells[116]. Following exposure to cisplatin, the degree of cytotoxicity was shown to be dependent upon cell density. DNA-PK deficient cells had unchanged cytotoxicity to cisplatin at low cell density but increased cytotoxicity at high cell density. The authors provide evidence suggesting that their observations were related to signaling from DNA-PK through gap junctions resulting in cell death in neighboring cells[116].

In vitro, c-Abl is phosphorylated and activated by DNA-PK[129]. Conversely, c-Abl phosphorylates DNA-PK which results in the dissociation of DNA-PKcs from the Ku heterodimer[119]. In irradiated cells, Ku70 has been shown to coprecipitate with c-Abl in the cytoplasm[140]. Nevertheless, activation of c-Abl can occur in DNA-PK defective cells[233]. DNA-PK can act via the MAPK cascade by regulating JNK[132,194]. As mentioned above, it has recently been shown that c-Jun expression increases the half-life of p73 and

that a defect in this pathway results in resistance to cisplatin[255]. C-Jun can phosphorylate c-Abl[20]. In other words, there exists the possibility of a signal transduction pathway incorporating DNA-PK, c-Jun, c-Abl and ultimately p73.

Bax, a key pro-apoptotic member of the Bcl-2 family, has been shown to be physically and functionally related to DNA-PK through the Ku70 subunit[227]. Ku70 binds Bax and prevents its translocation to the mitochondria. This results in suppression of cytochrome *c* release[227]. Interestingly, following irradiation, Ku70 was shown to translocate to the cytoplasm[140].

There is evidence to suggest that cisplatin influences the function of DNA-PK. It has been demonstrated in DNA damaged by cisplatin that even though Ku86 can bind to DNA, DNA-PK is poorly activated[261]. A model has been proposed whereby Ku86 binds near the DNA terminus and subsequently, in an ATP independent fashion, migrates proximally allowing more Ku and DNA-PKcs to bind at the terminus. The presence of cisplatin results in less migration of Ku86 and subsequently less binding of DNA-PKcs to damaged DNA strands[262].

Thesis

Aim

Platinum-based chemotherapy has been in clinical use for approximately 25 years. Its clinical application has increased consistently over the years especially since the introduction of oxaliplatin for the treatment of colon cancer in the mid 1990s. The amount of research on these drugs is extensive. A Pub Med search using cisplatin as the key word results in 31,683 articles dating back to 1972. A similar search for oxaliplatin yields 1082 articles dating back to 1980. This thesis examines cisplatin and oxaliplatin with attention to their chemistry, pharmacokinetics and molecular biology.

To the best of our knowledge, platinum-based drugs are chemically, not biologically, transformed to more reactive compounds. These biotransformation products are an essential component to our further understanding of wanted and unwanted toxicities of these agents. Two of the papers in this thesis address the purification and cytotoxicity of the biotransformation products MHC and DOC for cisplatin and oxaliplatin respectively.

Most of our understanding of the pharmacokinetics of platinum-based drugs has been based on sensitive methodology for measuring platinum content in plasma or plasma ultrafiltrate. Our paper on pharmacokinetics was one of the first reports where the aim of the study was to describe the pharmacokinetics of oxaliplatin in humans with measurement of the parent compound.

From the outset of our studies on these drugs, we were interested in how DNA-PK might impact upon their cytotoxicity. It had been shown previously that the phenothiazine drug Trifluoperazine enhanced the *in vitro* cytotoxicity of cisplatin[197]. Using a panel of phenothiazines we looked at how these agents influence DNA-PK and cell death.

The last article in this thesis asked two questions. Do the Ku86 or DNA-PKcs components of DNA-PK play a role in determining the cytotoxicity of platinum-based drugs? If they do, does the platinum carrier ligand affect this role?

Materials and Methods

Cell lines

Five human lung cancer cell lines were used in this thesis. U-1285, U-1810 and A-549 were derived from patients with small-cell lung cancer, non small-cell lung cancer (large cell) and bronchioalveolar cancer respectively[24,25,147]. M059K, J were derived from a patient with glioblastoma multiforme[6]. M059K is DNA-PKcs proficient whereas M059J is DNA-PKcs deficient. Four rodent cell lines were studied. K1, AA8 are wild-type chinese hamster ovary cell lines and Xrs-6, V3 are the complementary cell lines defective in Ku86 and DNA-PKcs respectively[28,73].

Cytotoxicity Assays

Three types of cytotoxicity assays were used in this thesis. The fluorescent microculture cytotoxicity assay (FMCA[142]) and a tetrazolium dye test (MTT[219]) were the short term assays used in papers I, II and V. Clonogenic assay was used for the cell lines M059K, J that is presented in this thesis as supplemental information.

Clonogenic assay has the advantage of minimizing changes in the cell cycle as a compounding factor in interpreting cytotoxicity data. On the other hand, the assay is more labour intensive and requires a significantly longer time frame. In using short term assays like FMCA and MTT it should be taken into account that one might be underestimating cytotoxicity because of mechanisms that have not come into full effect or overestimating cytotoxicity because of cell cycle influences not actually leading to cell death. Nevertheless, there is data supporting the reliability of short term assays when compared to clonogenic assays[128,199,237]. Kawada and co-workers demonstrated that there is an overall correlation between MTT and clonogenic assay of 0.67[128]. This correlation was different for different classes of drugs. The correlation was 0.94 for platinum-based drugs (they studied 5 – including cisplatin and carboplatin but not oxaliplatin)[128]. Another study comparing cisplatin cytotoxicity estimated from MTT and a clonogenic assay also showed a very good correlation between the two methodologies[199]. In a tumour xenograph model, investigators were able to assess the predictability rates of *in vitro* drug tests for *in vivo* chemosensitivity. The predictability rates were 88% and 90% for MTT and clonogenic assays respectively[237].

Short incubation assays like MTT are clearly not as sensitive as clonogenic assays with respect to ionizing radiation[18]. MTT can be used in estimating the cytotoxicity from radiation however its use is limited by accuracy within relatively narrow dose-ranges. At high doses many cell types do not regain exponential growth in MTT assays[241].

Preparation of Biotransformation Products

MHC and DOC were isolated using an HPLC system with a porous graphitic filter. After overnight incubation of cisplatin in distilled water, the hydrolysis mixture was injected into the system using an alkaline aqueous mobile phase (0.5mM NaOH). DOC was formed rapidly by hydrolyzing oxaliplatin in NaOH at 70^{o}C. The hydrolysis mixture was injected into the HPLC system using methanol and NaOH as the mobile phase. Post-column derivatization with *N,N*-diethyldithiocarbamate and microwave heating was used for quantification of cisplatin, oxaliplatin and MHC. A reference equilibrium mixture (55% MHC) was used to estimate the concentration of MHC. The detection limit cisplatin, oxaliplatin and MHC was approximately 0.05 μg/ml. DOC was quantified photometrically without post-column derivatization.

Patients

Patients from the Department of Oncology, Karolinska Hospital who were already scheduled to receive the combination of oxaliplatin, 5-fluouracil and leucovorin for metastatic colorectal cancer were asked to participate in a pharmacokinetic study examining serum levels of oxaliplatin. The study was approved by the hospital ethics committee.

Measurement of Apoptosis

Apoptotic cell death was quantified using three complementary assays. In paper IV the TUNEL assay and cell morphology was used to estimate apoptosis. The TUNEL assay is based upon the presence of DNA fragmentation producing an excess of 3´-OH ends in cells undergoing apoptosis. These fragments can be labeled with biotinylated UTP in the presence of terminal deoxyribonucleotidyl transferase and visualized using avidin conjugated peroxidase or fluorscein. Cell morphological evidence of apoptosis is based on the presence of nuclear fragmentation. This is easily estimated by staining the nuclei with DAPI solution. In paper V, in addition to cell morphology, apoptosis was assayed by measuring the degree of caspase-3 activation. This was accomplished using a fluorescent tagged antibody targeting activated caspase-3 and detecting the number of fluorescent cells in a flow cytometer.

DNA-PK Expression and Activity

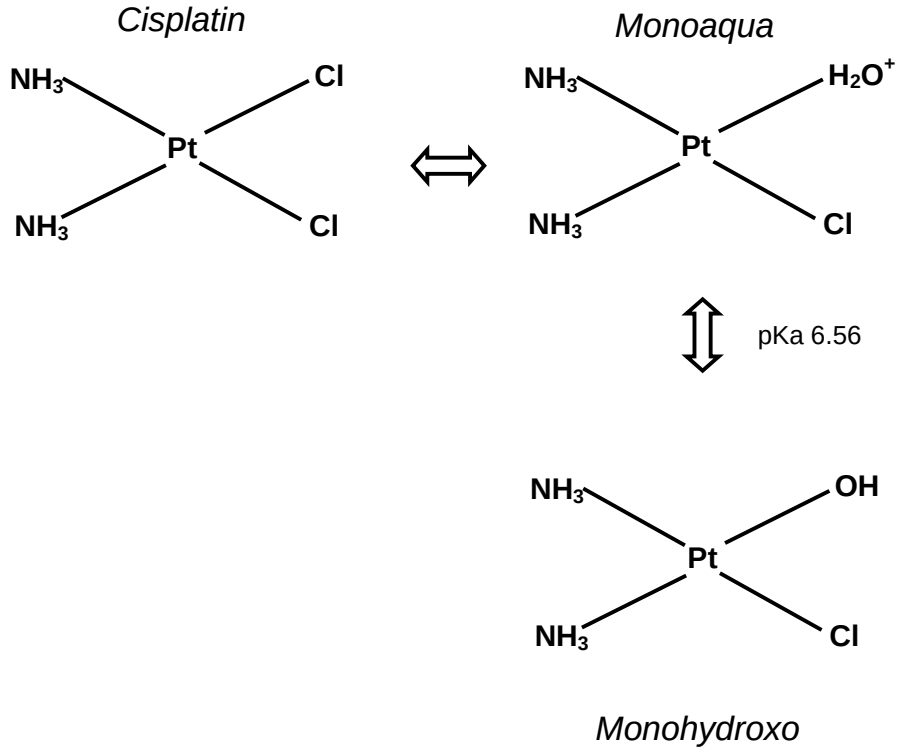
In paper IV DNA-PK expression was determined by Western blotting. This method allows for the separation of proteins by size as they move through a polyacrylamide gel under the influence of an electric current. The proteins from the gel are subsequently transferred to a nitrocellulose membrane and identified by an antibody targeting the protein in question. A secondary antibody is conjugated to horseradish-peroxidase. This enzyme enhances chemiluminescense which allows for identification of the protein on film.

The assay for DNA-PK activity is based on using a biotinylated p53 peptide as a DNA-PK substrate. Calf thymus DNA acts as an activator and [γ- ^{33}P] ATP is added to label DNA-PK. After the reaction is stopped, the biotinylated substrate is spotted onto a streptavidin impregnated membrane for subsequent quantification by liquid scintillation.

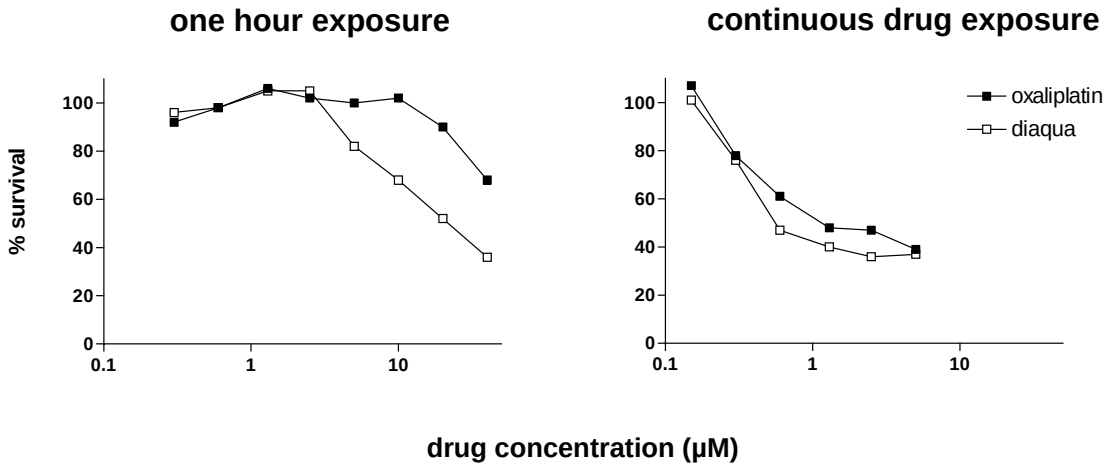
Results and Conclusions

In paper I, MHC was shown to be more cytotoxic than its parent compound cisplatin. The formation of MHC is favored by low chloride ion concentration and high pH[9,114]. We found that at low pH, MHC is unstable[281]. It has been shown that low extracellular pH increases the cytotoxicity of cisplatin[17,106,143] but not in cells that are subject to hypoxia[106]. Drug uptake was not affected by changes in pH[17]. There are conflicting results concerning whether or not the intracellular uptake of platinum is affected by the formation of MHC[114,238].

The acid-dissociation constant for MHC is 6.56[9]. Under normal physiologic conditions MHC would be expected to exist predominantly in the less reactive hydroxo form. Solid tumours often have an extracellular environment that is predominantly acidic. This implies that under these conditions, MHC would be more reactive but less stable.

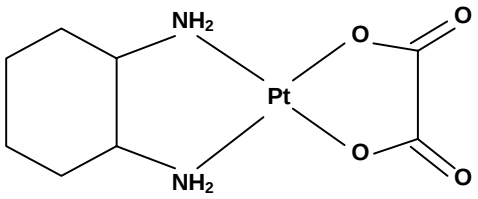


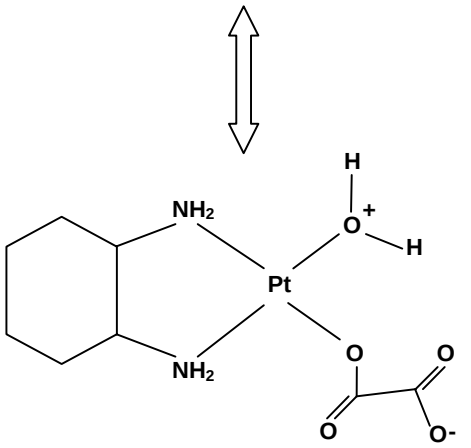
In paper II, a method is described for isolating DOC. DOC is clearly shown to be more cytotoxic than its parent compound. This result confirms the work of Luo and co-workers where DOC was also shown to have a greater cellular uptake than Pt(dach)(H_2O)Cl, Pt(dach)Cl_2 and oxaliplatin[156]. It is unknown if the formation of DOC is clinically relevant. In two reports, DOC was not identified either in PUF or urine[5,158]. Pt(dach)(H_2O)Cl but not DOC was measured in the PUF and urine from three patients treated with oxaliplatin[5]. One possible explanation is that DOC is so reactive that insignificant amounts of the compound are freely circulating. In the figure below the cytotoxicities of oxaliplatin and DOC were measured over time. For simplicity only one hour and continual exposure are depicted but measurements were made at 90, 120 and 150 minutes as well. After 3 days of continual exposure no significant difference in cytotoxicity between the two compounds is evident. A large separation of the cytotoxicity curves was seen after 60 minutes of exposure. This experiment is consistent with the hypothesis that DOC, at least in part, accounts for the cytotoxicity of oxaliplatin.



The diagram below is a proposed mechanism for the alkaline hydrolysis of oxaliplatin to form DOC[117]. The process involves the formation of an intermediate oxalato monodentate compound. The opening of the ring is reversible with a half-life of 16 minutes under the conditions tested. As is the case for MHC, the dissociation constant for the ring opening step is below physiologic pH. This implies that at physiologic pH, the reaction favours the deprotonation of the open-ring form and subsequent formation of DOC. In the acidic environment of solid tumours, closure of the ring is favoured and the rapid formation of oxaliplatin would be expected. Whether or not this has clinical implications is unknown.

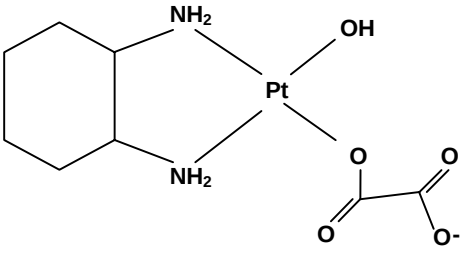
oxaliplatin



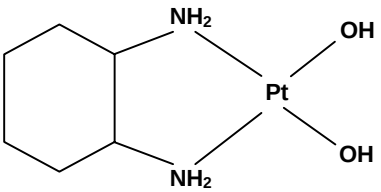


oxalato monodentate

pKa=7.16



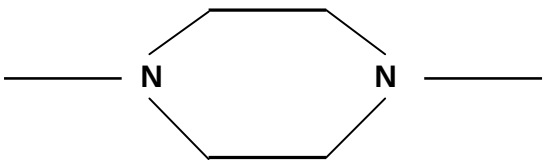
DOC



Paper III was a study in which the pharmacokinetics of oxaliplatin was assessed in a routine patient population receiving the combination of oxaliplatin and 5-fluorouracil for the treatment of metastatic colon cancer. Prior to this work, assessment of the pharmacokinetics of oxaliplatin was based almost entirely upon measurement of platinum content[93]. A blood sample was taken from each patient immediately prior to drug infusion to assess the *in vitro* degradation rate of oxaliplatin. On a theoretical basis there is no reason to suspect that 5-fluorouracil would impact upon the pharmacokinetics of oxaliplatin. There is somewhat conflicting evidence whether or not oxaliplatin affects the pharmacokinetics of 5-fluorouracil[120].

Our study established that the terminal half-life of oxaliplatin is approximately 14 minutes. This stands in sharp contrast to reports of up to 239 hours when platinum alone is assayed by inductively coupled plasma-mass spectrometry[93]. We also demonstrated that the *in vitro* degradation rate of oxaliplatin in whole blood significantly correlated with the clearance of oxaliplatin *in vivo*. The half-life of oxaliplatin is about 45 minutes *in vitro* compared to 14 minutes *in vivo*. Despite being more lipophilic than cisplatin, both drugs have similar volumes of distribution. Oxaliplatin has a clearance of 32 L/h/m^2 compared to 19 L/h/m^2 for cisplatin. This reflects the fact that oxaliplatin has a higher degradation rate than cisplatin in whole blood[10].

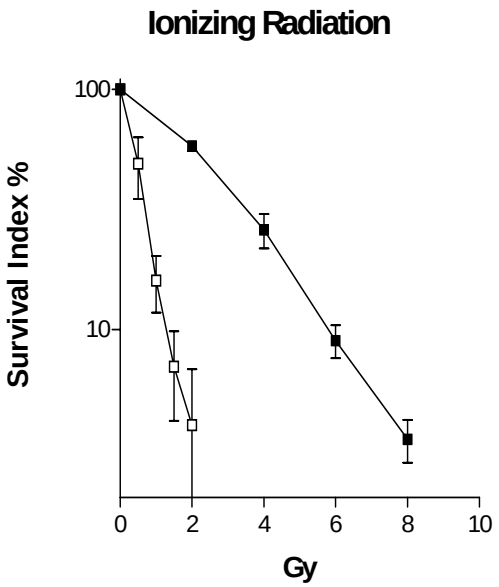
Trifluoperazine (TFP) potentiates the cytotoxicity of cisplatin[197]. Our research group was interested in DNA-PK and its impact upon the cytotoxicity of PBD. In paper IV we investigated phenothiazines as potential inhibitors of DNA-PK**.** Using a panel of phenothiazines and a thioxanthen, we found that compounds harbouring a piprasenyl group (TFP, fluphenazine, flupentixol) had the property of inhibiting DNA-PK in comparison to those compounds that did not have this group (chlorpromazine, promazine, trifluprom azine). TFP was found to be a specific inhibitor of both purified DNA-PK and DNA-PK from cell lysate. In U-1810 cells, DNA-PK activity was dramatically decreased after 30 minutes of incubation with TFP.



piprasenyl group

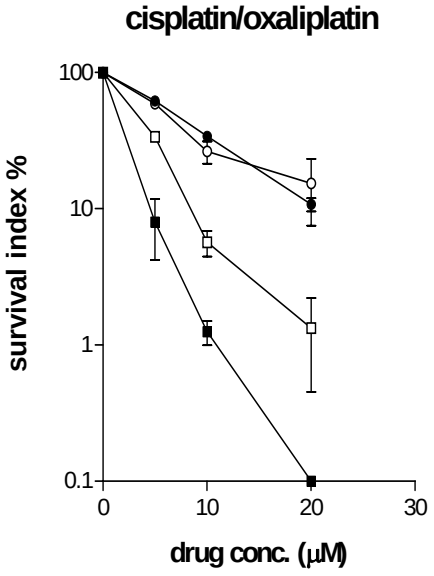
The doses of TFP used in this study are well above clinically relevant levels. At lower doses of TFP, we were not able to demonstrate any inhibition of DNA-PK or apoptotic cell death. TFP is a calmodulin inhibitor and it is possible that synergy between TFP and cisplatin is calmodulin based. In our study, calcium chelators or calmodulin inhibition had no influence on DNA-PK activity.

In paper V we asked whether DNA-PK influenced the cytotoxicity of PBDs and if this was dependent upon the carrier ligand in question. Using cell lines that are mutated at either DNA-PKcs or Ku86 we were able to show that cytotoxicity was enhanced for both drugs. Our data suggests that Ku86 might play a larger role in determining the cytotoxicity of cisplatin and that the catalytic subunit might be more important with respect to oxaliplatin. We also studied the influence of DNA-PKcs in the human glioblastoma cell line M059 following exposure to ionizing radiation as well as PBDs. This is additional data that was not included in paper V. The figures below represent cytotoxicity based upon clonogenic assay.

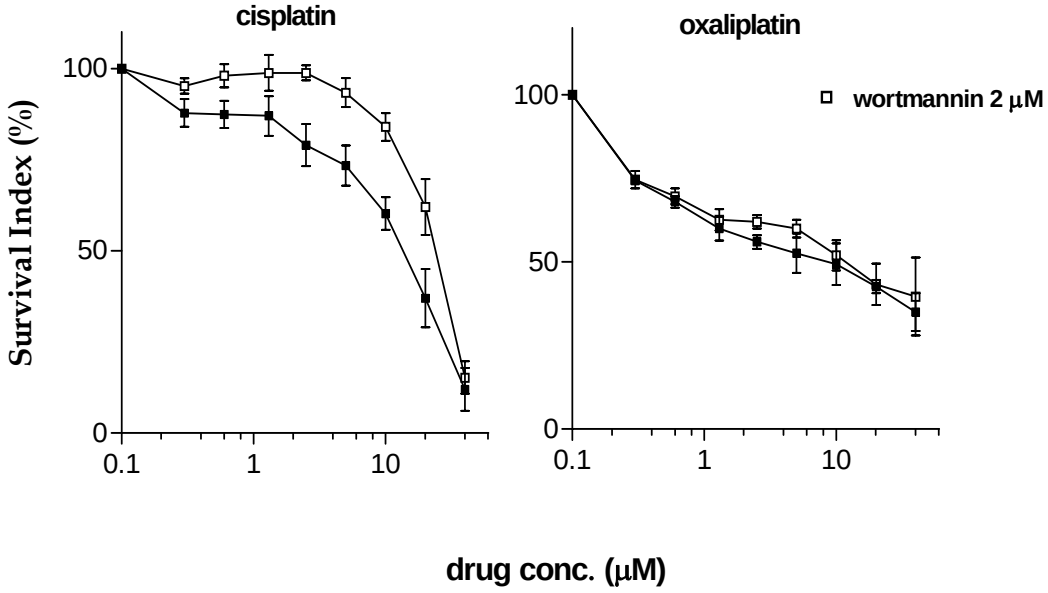


M059K is represented by filled squares and DNA-PKcs deficient, M059J, by the open squares. This figure demonstrates that cells deficient in DNA-PKcs are hypersensitive for ionizing radiation.

M059K and M059J are represented by filled and open shapes respectively. Cisplatin and oxaliplatin are represented by squares and circles respectively. In this cases the absence of DNA-PKcs results in resistance to cisplatin whereas oxaliplatin is unaffected by DNA-PK deficiency



Perhaps the most interesting observation from the figures above is that DNA-PKcs seems to protect M059 when exposed to ionizing radiation but enhances cytotoxicity with respect to cisplatin. A possible explanation for this is that we are observing DNA-PK`s role in repair of DSB in the case of ionizing radiation and DNA-PK´s role in apoptotic signaling with respect to cisplatin. The cytotoxicity of oxaliplatin doesn't seem to be affected by DNA-PKcs in M059. Using wortmannin as a non-specific inhibitor of DNA-PKcs, we compared the cytotoxicity of cisplatin and oxaliplatin in the cell line K1 with FMCA. This is depicted in the figure below. The data is consistent with our observations in M059.



We looked for differences in apoptotic signaling pathways depending upon the state of Ku86 and the platinum drug in question. Our study suggests that the kinetics and translocation of activated p38 differ between cisplatin and oxaliplatin and this is reflected in their relationship with Ku86. We did not observe caspase-3 activation in cells treated with oxaliplatin. This is in contrast to several reports demonstrating oxaliplatin induced caspase-3 activation[13,90,165,254].

Thoughts and Reflections

I would like to conclude this dissertation with general thoughts and reflections about the focus of our research questions. Over the last few years, funding of cancer research has become more concerned with applicability. This has its roots in a general disappointment over the lack of benefit to individual patients from cancer research despite extensive funding. A way to approach this problem is to shift resources to research projects that attempt to bridge the gap between pure science and clinical medicine – translational research. I believe that this approach is essentially correct but there are concerns. New knowledge for the sake of knowledge must be valued. Tomorrow's translational research will be based on today's discoveries. What isn't applicable now may turn out to be applicable later. Research will be heavily biased if we discount projects at the start because of uncertain applicability.

There is a point to re-visiting established drugs. It is interesting that we still debate the optimal scheduling of 5-fluorouracil some 40 years after its introduction to the clinic. Traditionally, phase I drug trials have sought to determine the maximum tolerated dose (MTD) of a drug and use this as a bench-mark for further clinical studies. The assumption is that it is beneficial to the patient to receive a dose that approximates the MTD without exceeding it. Another approach is metronomic chemotherapy. Metronomic chemotherapy makes use of our established drugs in a form of administration that allows for continuous exposure at low doses. The idea is to not allow the target cells a chance to recover while at the same time limiting unwanted side-effects. Using "old" drugs in new ways underlines the importance of a fresh look at the chemistry, pharmacokinetics and biology of these agents. This is especially important given that the new "fashionable" drugs along with their science are placing economic constraints that place the western world in a debate that is similar to AIDS treatment in underdeveloped countries. In other words, improved treatments are available but can we afford them?

I started my oncology training on a ward where high dose methotrexate was used to treat patients with osteosarcoma. PK analysis and TDM were well established in the treatment protocol. What struck me as odd was that this type of analysis wasn't done for any other of our chemotherapeutic agents. Why hasn't PK a more dominant role in how we use our drugs? The degree of exposure to cytotoxic agents is related to treatment outcome and side effects yet we don't use PK to help us define optimal exposure. Is it C_{max} or AUC that best defines drug exposure with respect to cytotoxicity? Perhaps the shape of the AUC curve is more important for certain drugs than AUC itself. There are several explanations for our lack of knowledge relating PK to treatment outcome. For many drugs there is little inter-individual PK variation thereby making a correlation of PK to treatment outcome or toxicity unlikely. There are few commercial drug assays available. This makes doing PK analysis both cumbersome and expensive. Phase I clinical trials usually have PK as a defined endpoint. These trials enter a small number of patients and are not designed to establish drug efficacy. The larger trials assessing drug efficacy often do not include PK as an endpoint. In short, there is a lack of PK analysis in large populations that would enable us to make conclusions about PK parameters and outcome. Population PK based on simple blood sampling protocols is a welcome development and should be included in larger clinical trials. At the same time it might be

of value if regulatory institutions placed a greater demand on trial protocols to do more extensive PK analysis prior to licensing.

Acknowledgements

Of the many pages that comprise a PhD thesis, my impression is that most people only read the List of Publications and the Acknowledgements. In this regard, I have taken my time to carefully reflect over the individuals who influenced me and my research during the nine years since I started this project.

I first met my adviser **Professor Rolf Lewensohn** over lunch at the former SSI conference room. At this meeting we discussed challenges facing the treatment of lung cancer. It was apparent to me then, as it is today, that Rolf is a visionary with a sincere goal to contribute to the improvement of treatment for patients with malignant disease. Rolf is an administrative magician. He has provided the conditions necessary for free and open discussion in our research group and the opportunity to pursue our own avenues of interest. My other adviser **Professor Hans Ehrsson** helped me to understand the complex world of platinum chemistry. Even though he didn't entrust me with the door code to the hospital pharmacy, I truly enjoyed dropping by and discussing platinum chemistry with him. I have often felt that the world would be a far nicer place to live in if there were more people like Hans Ehrsson.

I would like to thank Professor **Ulrik Ringborg** for accepting my somewhat unusual working schedule that made it possible for me do laboratory research while keeping active as a clinical oncologist.

Anders Nilsson is responsible for introducing DNA-PK to our research group (Should we kill him for this?). There are few people that I know in academics that have as broad a background as Anders. He became a mentor and more importantly a cherished friend. I am grateful to him for stimulating conversation concerning everything under the sun and for many moments of laughter and fun.

What I remember most from my first day at the old SSI lab is meeting two laboratory assistants who radiated energy and warmth. They made me feel welcome from the start. My sincere thanks to **Lillemor Laurén** and **Marianne Landegren** (by the way, I only tasted DMSO once).

I am truly grateful to my co-author **Ingrid Wallin**. Still not entrusting me with the hospital pharmacy door code, I was always welcome to visit, analyze and discuss our data. Ingrid has created an air of efficiency and professionalism in the lab and at the same time it feels warm, friendly and humble – thank-you.

Kristina Viktorsson was instrumental in helping me put the finishing touches on this thesis. She joined our research group a couple of years ago bringing new energy and enthusiasm. Kristina introduced me to the fascinating world of MAP kinases. She has

taken time off from her busy schedule to help me get through the “things to do” jungle of a PhD dissertation. Kristina- my true thanks.

The skillful assistance of my co-author **Birgitta Mörk** has enabled me to be clinically active through the latter part of my research. I feel a great deal of gratitude for her continual optimism in the face of cell lines that died if you looked crossed-eyed at them.

Åsa Holgerson sits across from me in our little cubby hole at the end of the lab. I must admit that she is much nicer to look at compared with the previous inhabitant (sorry Anders). Åsa is a light burning bright. We have had fruitful discussions about research, philosophy and music. Thank-you for always being a positive force in the lab and for allowing me to indoctrinate you into the wonderful world of jazz music.

My colleagues from Eskilstuna - **Jan-Erik Westlin, Ulf Bandmann, Gunilla Ljung, Peter Gorzov, Charlotta Mörth and Annika Karlsson**. You help make the practice of medicine fulfilling. I intended to stay 6 months and it became 5 years. My deepest thank-you to all of you.

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Thank-you to **Margareta Edgren and Ingegerd Hedlöf** for taking an interest and helping with our research questions on MO59.

Elin Jerremalm and Pernilla Videhult (how come they got trusted with the code to the pharmacy?) Thank-you for being a part of my roller-coaster ride through science.

A special thanks to **Staffan Eksborg** who bailed me out at least once when it came to computer problems and for his expert advice concerning the statistics in paper II.

Finally, a few words about the Swedish Yachnins. I am a very proud father with three terrific sons **Oscar, Kevin** and **William** and a wonderful wife **Thérese**. I hold no grudges that if it wasn’t for hockey, I would have finished this thesis several years earlier.

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