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BIOTRANSFORMATION OF THE ANTINEOPLASTIC DRUG OXALIPLATIN: IMPORTANCE FOR EFFECTS AND SIDE EFFECTS

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ABSTRACT

Oxaliplatin is a platinum-based cytostatic drug. It is used in combination with 5-fluorouracil and leucovorin for palliative and adjuvant treatment of metastatic colorectal cancer. The biotransformation of oxaliplatin is non-enzymatic and complexes with water, chloride, glutathione and methionine have been reported to be present in plasma ultrafiltrate from patients.

Oxaliplatin gives rise to DNA adducts and, consequently, DNA damage. This is the predominant explanation for its antineoplastic effect. It is not known to what extent the parent drug and/or its biotransformation products contribute to the cytotoxic effect. Common side effects of oxaliplatin treatment are nausea, vomiting and neurotoxicity.

To create a more solid ground for the understanding of the effects of oxaliplatin, we have elucidated the chemistry of the compound. Oxaliplatin contains a diaminocyclohexane and an oxalato ligand. In study I, hydrolysis of oxaliplatin was found to occur in two consecutive steps. In the first step, the oxalato ring was opened and in the second step it was lost, replaced by two water molecules. The ring-opening was reversible and the closing step was much faster than the opening step. In study II, the acid dissociation constant of the oxalato monodentate intermediate was determined to 7.23. At physiological pH less than 1 % of oxaliplatin will be present in the shape of the ring-opened intermediate.

The reaction of oxaliplatin with chloride was investigated in study III. The oxalato ring was opened, replaced by one chloride and then it was lost, replaced by another chloride. The initial reaction was fast with a half-life of 5-10 minutes. The cytotoxicity of the ring-opened species, $[Pt(dach)oxCl]^-$, was studied *in vitro* and was found not to exceed that of oxaliplatin. In study IV, the degradation of oxaliplatin in plasma ultrafiltrate was in good agreement with the degradation of oxaliplatin in the presence of a mixture of chloride, cysteine, methionine and glutathione at physiological concentrations.

It has been proposed that the acute neurotoxic side effects of oxaliplatin treatment involve voltage-gated ion channels. Since charged molecules can affect ion channel function, we hypothesized in study V that $[Pt(dach)oxCl]^-$ could be involved. We studied the effects of oxaliplatin and $[Pt(dach)oxCl]^-$ on voltage-gated potassium channels, but concluded that we did not see any effects in our *Xenopus* oocyte experimental system.

Apoptosis is commonly induced by DNA-damaging drugs. Apoptotic pathways induced by oxaliplatin and cisplatin were compared in study VI. While cisplatin-induced apoptosis was dependent on calcium and superoxide, oxaliplatin-induced apoptosis was not.

LIST OF PUBLICATIONS

I. Jerremalm E, Videhult P, Alvelius G, Griffiths WJ, Bergman T, Eksborg S, Ehrsson H (2002) Alkaline hydrolysis of oxaliplatin − isolation and identification of the oxalato monodentate intermediate. *J Pharm Sci* **91**: 2116-2121.

II. Jerremalm E, Eksborg S, Ehrsson H (2003) Hydrolysis of oxaliplatin − evaluation of the acid dissociation constant for the oxalato monodentate complex. *J Pharm Sci* **92**: 436-438.

III. Jerremalm E, Hedeland M, Wallin I, Bondesson U, Ehrsson H (2004) Oxaliplatin degradation in the presence of chloride: identification and cytotoxicity of the monochloro monooxalato complex. *Pharm Res* **21**: 891-894.

IV. Jerremalm E, Wallin I, Yachnin J, Ehrsson H (2006) Oxaliplatin degradation in the presence of important biological sulphur-containing compounds and plasma ultrafiltrate. *Eur J Pharm Sci*, **28**: 278-83.

V. Broomand A, Jerremalm E, Yachnin J, Ehrsson H, Elinder F (2008) Oxaliplatin neurotoxicity – no general ion channel surface-charge effect. *Manuscript submitted.*

VI. Hellberg V, Wallin I, Eriksson S, Hernlund E, Jerremalm E, Berndtsson M, Arnér ESJ, Shoshan M, Ehrsson H, Laurell G (2008) Toxicity of cisplatin and oxaliplatin – the importance of cochlear kinetics for ototoxicity. *Manuscript submitted.*

CONTENTS

LIST OF ABBREVIATIONS

BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid, tetraacetoxymethyl ester
cdk2	cyclin dependent kinase 2
dach	diaminocyclohexane
DOC	dihydrated oxaliplatin complex
EI	electrospray ionisation
FAAS	flameless atomic absorption spectrometry
5-FU	5-fluorouracil
HAI	hepatic artery infusion
ICP-MS	inductively coupled plasma mass spectrometry
LC	liquid chromatography
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$Pt(dach)Cl_2$	dichloro complex
$[Pt(dach)Cl(H_2O)]^+$	monochloro monoaqua complex
$[Pt(dach)(H_2O)_2]^{2+}$	diaquated oxaliplatin complex
$[Pt(dach)oxCl]^-$	monochloro monooxalato complex
PGC	porous graphitic carbon
PUF	plasma ultrafiltrate
ROS	reactive oxygen species
SOD	superoxide dismutase
tiron	4,5-dihydroxy-1,3-benzene disulphonic acid
TMB	3,3',5,5'-tetramethylbenzidine
TUNEL	terminal deoxynucleotidyl transferase–mediated dUTP nick end-labelling

1 INTRODUCTION

1.1 COLORECTAL CANCER

Colorectal cancer is a major cause of cancer-related death in the developed countries (Saunders and Iveson, 2006). In Sweden, the colorectal cancer incidence represents about 11 % of all cancers, which makes it the third most common cancer after prostate and breast cancer (Socialstyrelsen, 2007). Colorectal cancer is essentially equally distributed between men and women. About half of the patients develop metastatic disease, particularly to the liver (Saunders and Iveson, 2006). The 10-year relative survival in Sweden is about 50 % (Socialstyrelsen and Cancerfonden, 2005).

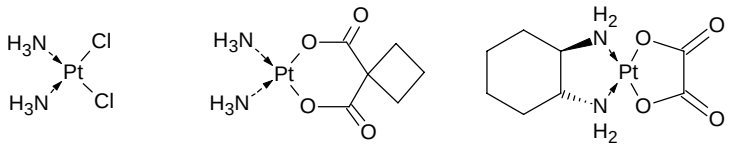
Colorectal cancer can manifest as change in bowel habits or blood in stool. Diffuse symptoms like fatigue, loss of appetite and weight loss are also common. The risk of developing colorectal cancer has a genetic component, but lifestyle factors such as diet and physical activity are also important. (Socialstyrelsen and Cancerfonden, 2005)

Surgery is the treatment of choice for primary disease (Bennouna and Douillard, 2002). Adjuvant chemotherapy is often used after surgery to avoid recurrence. Chemotherapy can also be used for palliative treatment of advanced disease. The standard chemotherapy has for the last 50 years been 5-fluorouracil (5-FU)-based. This cytostatic functions both as a thymidylate synthase inhibitor and as an inhibitor of the RNA synthesis. Unfortunately, the response rate of 5-FU monotherapy is only about 20 % in advanced disease and the overall survival is about one year. Combination therapy with the thymidylate synthase modulator leucovorin improves response rates slightly, but not the overall survival. New cytostatic agents against colorectal cancer include the oral 5-FU pro-drugs capecitabine and tegafur, the thymidylate synthase inhibitor raltitrexed, the topoisomerase I inhibitor irinotecan and oxaliplatin. Oxaliplatin and irinotecan work in synergy with 5-FU/leucovorin treatment and addition of either of these to 5-FU/leucovorin has almost doubled the overall survival in advanced disease (Schmoll, 2002). Furthermore, antibody-based therapy has evolved in recent years. Currently, one epidermal growth factor receptor antibody (cetuximab) and one vascular endothelial growth factor antibody (bevacizumab) are in use against colorectal cancer, in combination with chemotherapy (Patiyil and Alberts, 2006).

This thesis has been devoted to oxaliplatin, one of the newer cytostatic agents, commonly used against metastatic colorectal cancer. Oxaliplatin is an elaboration of the well-established platinum drug cisplatin (figure 1).

1.2 CISPLATIN

More than 40 years ago Rosenberg and co-workers accidentally found an inhibitory effect of a soluble platinum salt on cell division of *Escherichia coli* cells (Rosenberg et al., 1965). This was the discovery of one of our most important chemotherapeutics – cisplatin (figure 1).



M_w=300 cisplatin | M_w=371 carboplatin | M_w=397 oxaliplatin

Figure 1. Platinum compounds used in Sweden today for the treatment of cancer.

The first tumours reported to be susceptible to cisplatin therapy were testicular cancer and ovarian cancer. Remarkably, cisplatin therapy was a cure for the majority of patients with advanced testicular cancer, previously fatal to these patients. Today, cisplatin has a place also in the treatment of other malignancies, such as non-small cell lung cancer, bladder cancer, head and neck cancer, as well as some paediatric malignancies. (Desoize and Madoulet, 2002)

During the first years of cisplatin use, nephrotoxicity was a serious side effect. Nowadays, this is well-handled by pre- and posthydration (Desoize and Madoulet, 2002). Other side effects are nausea, vomiting, ototoxicity and cumulative neurotoxicity (Di Francesco et al., 2002).

1.2.1 Development of other platinum compounds

Since the discovery of the tumour inhibitory effects of cisplatin, about 3000 platinum compounds have been synthesised and tested *in vitro*. Only 30 of these have entered clinical trials and few of them are still under consideration. Four platinum compounds are now used in the clinic: cisplatin, carboplatin, oxaliplatin and in Japan nedaplatin (Desoize and Madoulet, 2002). Among those in late stage clinical trials are picoplatin and satraplatin (Kelland, 2007).

Carboplatin is effective against about the same tumour types as cisplatin and is sometimes preferred because it has less side effects, the dose-limiting one is myelosuppression (Desoize and Madoulet, 2002). However, cisplatin seems to be superior to carboplatin in terms of therapeutic effect (Lokich, 2001). Carboplatin is given to patients at a dose of approximately 1.1 mmol/m^2 every four weeks, for cisplatin the dose is 0.17-0.25 mmol/m^2 every three to four weeks and for oxaliplatin the dose is 0.21 mmol/m^2 every other week (FASS, 2008).

1.3 CHEMISTRY OF THE PLATINUM COMPOUNDS

Cisplatin and oxaliplatin are square planar platinum complexes. Nucleophilic substitution reactions of these compounds have been suggested to occur by an S_N2 mechanism, involving a transition state in the shape of a trigonal bipyramid (figure 2). In solution, the reaction may be aided by for example two solvent groups positioned above and below the plane (Banerjea et al., 1957). The trans effect makes ligands that are placed *trans* to a soft nucleophile more rapidly substituted than ligands in *cis* position. For example, the chloride ligands of cisplatin are more likely to be substituted because of the soft amine (Reedijk, 2003).

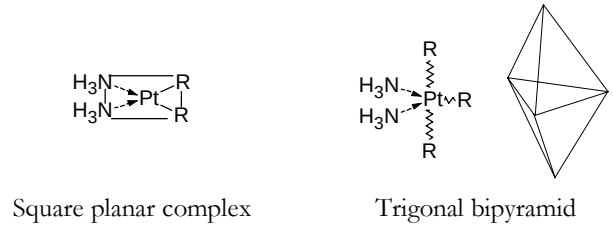


Figure 2. The planar structure of the platinum compounds and a proposed transition state.

1.3.1 Reaction kinetics

Substitution kinetics of square-planar Pt(II) complexes, where the nucleophile (Nu) is in large excess over the platinum complex, proceed by the general pseudo first order rate law $k_{obs} = k_1+k_2[Nu]$, where k_1 involves the rate constant for the reaction with the solvent and k_2 is the second order rate constant for the nucleophile. (Basolo et al., 1960; Dedon and Borch, 1987)

The reaction of cisplatin, carboplatin and Pt(dach)(malonate) with cysteine, methionine and glutathione has been studied and complexes could be formed via direct displacement, not requiring prior aquation (Dedon and Borch, 1987; Mauldin et al., 1988). The trans effect discussed in 1.3 is even more pronounced for the sulphur ligand (Barnham et al., 1995; El-Khateeb et al., 1999).

1.3.2 Hydrolysis

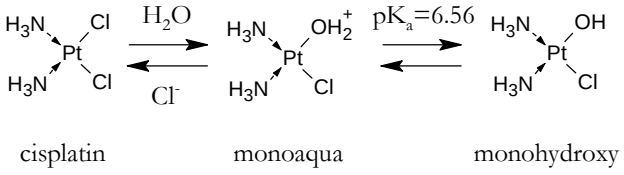


Figure 3. Hydrolysis of cisplatin.

The aquated species of cisplatin have been suggested to be of importance for the cytotoxic effect, since they are more reactive than the parent compound. Depending on the conditions (pH and chloride ion concentration), a monoaqua (figure 3) and a diaqua complex are subsequently formed (Miller and House, 1989). The acid dissociation constant for the monoaqua complex has been determined to 6.56 (Andersson et al., 1994). This means that at pH 7.4 the less reactive monohydroxy form (figure 3) will be

the dominating species. The active cisplatin species has been suggested to be the monoaqua species, which is more reactive than cisplatin itself (Knox et al., 1986). The monohydrated complex (monoaqua and monohydroxy, figure 3) was shown to be more cytotoxic than the parent compound in a cellular assay (Yachnin et al., 1998).

In a neutral water solution carboplatin is apparently stable. The acidic hydrolysis of carboplatin is a two step reaction, where the first step involves the formation of a ring-opened monodentate species. In the second step, the cyclobutanedicarboxylate ligand is lost (Canovese et al., 1988; Hay and Miller, 1998). The reaction with chloride has also been assumed to be a two-step reaction. The half-life of carboplatin in a 0.1 M chloride solution at pH 7 (37 °C) is 244 hours (Allsopp et al., 1991).

Oxaliplatin has been suggested to be hydrolysed *in vivo* by an intermediate step involving bicarbonate and/or phosphate (Mauldin et al., 1988; Graham et al., 2000; Di Francesco et al., 2002). However, the reaction of oxaliplatin with bicarbonate and phosphate is very slow under physiological conditions (Jerremalm and Videhult, unpublished observations). The dihydrated complex of oxaliplatin was shown to be more cytotoxic than the parent compound in an *in vitro* cytotoxicity assay (Videhult et al., 2002).

1.4 OXALIPLATIN

Platinum compounds containing a 1,2-diaminocyclohexane carrier ligand were studied in the 1970's and were demonstrated to be effective against for example cisplatin resistant mouse leukaemia cells (Burchenal et al., 1978). One of the isomers of a promising oxalato complex, oxaliplatin ([(1*R*,2*R*)-1,2-cyclohexanediamine-*N*,*N*´]oxalato(2-)-*O*,*O*´platinum), was first isolated and tested for antitumour activity by a Japanese group and was found to be more potent than the other isomers (*cis*-dach and *trans*-*S*,*S*-dach, figure 4) (Kidani et al., 1980). Oxaliplatin was also discovered to have synergistic effects with 5-FU (Mathé et al., 1989). The compound was registered in France in 1996, in the European Union in 1999 and in the United States in 2002 for the treatment of metastatic colorectal cancer (Graham et al., 2004).

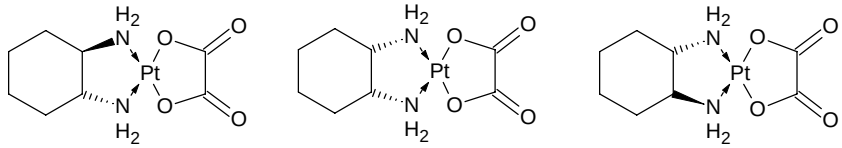


Figure 4. 1,2-Cyklohexanediamine isomers. Oxaliplatin=trans-R,R-dach.

1.4.1 Oxaliplatin therapy

In vitro experiments of oxaliplatin and 5-FU have suggested that the 5-FU sensitivity can be restored after oxaliplatin treatment, as seen by a reduced thymidylate synthase expression (Raymond et al., 2002). Commonly used oxaliplatin dosing regimens are 85 mg/m^2 given as an intravenous infusion every second week or 130 mg/m^2 every third week, of these the two week schedule appears to be preferable (Misset et al., 2000). In clinical studies of previously untreated patients with advanced colorectal cancer receiving oxaliplatin, 5-FU and leucovorin, the response rate ranged from 34 to 67 % and the overall survival was 15 to 20 months. It has also been shown that addition of oxaliplatin can overcome 5-FU resistance in patients with progressive disease. Oxaliplatin in combination therapy has been evaluated in clinical trials against for example recurrent ovarian cancer (Ferrandina et al., 2007), metastatic breast cancer (Airoldi et al., 2006), pancreatic cancer (Desai et al., 2007) and esophagogastric cancer (Cunningham et al., 2008).

Oxaliplatin can be used to convert unresectable liver metastases to resectability, the only way to possibly cure patients with inoperable advanced disease (Schmoll, 2002; Wicherts et al., 2007). Interestingly, hepatic artery infusion of oxaliplatin gives a possibility to reach liver metastases directly, decreasing the systemic toxicity. Guthoff et al. concluded that only about half of the oxaliplatin administered via hepatic artery infusion reaches the systemic circulation (Guthoff et al., 2003).

1.4.2 Side effects

In phase I trials of oxaliplatin, besides side effects like nausea and vomiting, the most common side effect was a reversible peripheral neuropathy, enhanced or triggered by cold and appearing within minutes or hours, characterised by paraesthesia and

dysaesthesia in hands, feet and in the oral region. The acute neurotoxic side effects were not immediately recognised as reversible, which can explain the protracted period of oxaliplatin's early development (Raymond et al., 1998). The mechanism of the acute neurotoxic side effects has been suggested to involve voltage-gated ion channels (see 1.5).

There is also a late onset neurotoxicity that occurs after several treatment cycles. A cumulative oxaliplatin dose of about 800 mg/m^2 causes problems with sensorimotor coordination, paraesthesias and dysaesthesias persisting between treatment cycles in about 10-15 % of the patients. This is the dose-limiting side effect of oxaliplatin treatment. Most patients recover within 4-6 months after treatment discontinuation (Carrato et al., 2002; Cassidy and Misset, 2002; Di Francesco et al., 2002; Gamelin et al., 2002). So far, no effective prevention strategy against the neurotoxic side effects of oxaliplatin has been presented (Albers et al., 2007).

1.4.3 Mechanism of action

The cellular uptake of oxaliplatin is likely to occur through passive diffusion and carrier-mediated transport systems (Zou et al., 1998; Ghezzi et al., 2004; Safaei and Howell, 2005). Platinum compounds with a diaminocyclohexane carrier are taken up about ten times faster than the corresponding diammine compounds (Zou et al., 1998). Oxaliplatin treatment (as well as cisplatin and carboplatin treatment) leads to platinum DNA adducts, mainly by binding to guanine residues of DNA and often as intrastrand crosslinks (Raymond et al., 1998; Chaney et al., 2005; Hah et al., 2007). This can lead to inhibition of the DNA replication, resulting in cell cycle arrest and apoptosis (Di Francesco et al., 2002). However, the mechanism of action may also involve other cellular systems. For both cisplatin and oxaliplatin it has been shown that an apoptotic response can occur also in enucleated cells (Mandic et al., 2003; Gourdier et al., 2004). Furthermore, the thioredoxin system, an important cellular redox system, is inhibited by cisplatin and oxaliplatin that bind to a selenocysteine-containing active site on thioredoxin reductase (Arnér et al., 2001; Witte et al., 2005). The cisplatin-thioredoxin reductase complexes can induce rapid cell death (Anestål and Arnér, 2003).

One intriguing fact is that the reaction of oxaliplatin *in vitro* with DNA is very slow (Butour et al., 1985; Hah et al., 2007). Considering oxaliplatin's short half-life *in vivo* (14 minutes (Ehrsson et al., 2002)), the DNA adducts may be formed by a biotransformation product of oxaliplatin. As an example (regarding cisplatin), an S ligand has been suggested to rearrange to an N ligand of a purin base due to more favourable thermodynamics (Reedijk, 2003). It is also evident, from *in vitro* studies, that at equimolar doses, oxaliplatin produces much fewer DNA adducts than cisplatin but is still equally potent (Woynarowski et al., 2000). This could, at least in part, be explained by a different conformation of cisplatin and oxaliplatin derived platinum-DNA adducts and differences in the DNA repair mechanism (Chaney et al., 2005).

1.4.4 Pharmacokinetics

After a two-hour infusion of oxaliplatin, 40 % of the platinum in blood is irreversibly bound to red blood cells. Of the remaining platinum about half is bound to plasma proteins and the rest is ultrafiltrable (Mani et al., 2002). Most pharmacokinetic studies

on oxaliplatin are based on measurements of total "free" platinum by flameless atomic absorption spectrometry (FAAS) or inductively coupled plasma mass spectrometry (ICP-MS). Terminal half-lives of about 200 hours have been described (using the more sensitive method ICP-MS), most likely reflecting the breakdown of platinum-containing proteins (Ehrsson et al., 2002). Erythrocyte platinum content declines even more slowly, with a half-life of up to 50 days, reflecting the turnover of red blood cells (Lévi et al., 2000). However, the pharmacokinetics of intact oxaliplatin has recently been evaluated by Ehrsson and co-workers, who concluded that the elimination half-life was only 14 minutes. The maximum concentration of oxaliplatin in blood ultrafiltrate after a 2-hour infusion of 85 mg/m^2 was about 4 μM and the volume of distribution was rather low, 0.26 l/kg (Ehrsson et al., 2002), similar to that of cisplatin (Andersson et al., 1996).

1.4.5 Biotransformation products

The biotransformation of oxaliplatin is non-enzymatic (Graham et al., 2000; Mani et al., 2002). In plasma ultrafiltrate (PUF) from patients treated with oxaliplatin, biotransformation products from reaction with water, chloride, glutathione and methionine have been reported (Allen et al., 1998; Allain et al., 2000). In one study of three patients receiving oxaliplatin, products in PUF one hour after the end of infusion were $Pt(dach)Cl_2$, $[Pt(dach)Cl(H_2O)]^+$ (figure 5), intact oxaliplatin, a methionine adduct and at least two unidentified products (Allain et al., 2000). The diaqua complex $[Pt(dach)(H_2O)_2]^{2+}$, (figure 5) was reported in urine from patients (Allen et al., 1998), but not found in the other study (Allain et al., 2000).

In an *in vitro* assay, the cytotoxicity of $Pt(dach)Cl_2$, $[Pt(dach)Cl(H_2O)]^+$ and $[Pt(dach)(H_2O)_2]^{2+}$ was higher than that of oxaliplatin. The cytotoxicity was proportional to the cellular uptake (Luo et al., 1998). However, the $Pt(dach)Cl_2$ complex was demonstrated to be present in very low concentrations *in vivo* (Shord et al., 2002).

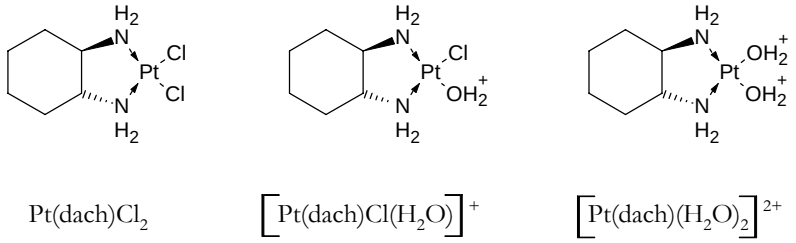


Figure 5. Chloride- and water-containing biotransformation products of oxaliplatin.

1.5 VOLTAGE-GATED ION CHANNELS

Excitable neurons and muscle cells act by generating an action potential, thereby communicating inter- and intracellularly. This is controlled by inward and outward currents through the cell membrane. The action potential is started by a small depolarisation, resulting in a rapid increase in sodium permeability that leads to influx of sodium ions. This is followed by a slower increase in potassium permeability causing potassium ions to flow out of the cell. The outward current of potassium ions and the closing of sodium (Na^+) channels terminate the action potential. (Rang et al., 2007)

Voltage-gated ion channels (conducting for example sodium, potassium and calcium) are membrane proteins that consist of four similar or identical protein domains (figure 6). Each domain contains six α-helices and the four domains are arranged around an aqueous pore composing the channel. The pore is built up by the short intramembrane segments between segment 5 and 6 in the four domains. Segment 4 is the positively charged voltage sensor that is responsible for the channel activation. Channel inactivation occurs when an intracellular part ("inactivation module", figure 6) of the protein blocks the channel. (Elinder and Århem, 2003; Yu et al., 2005)

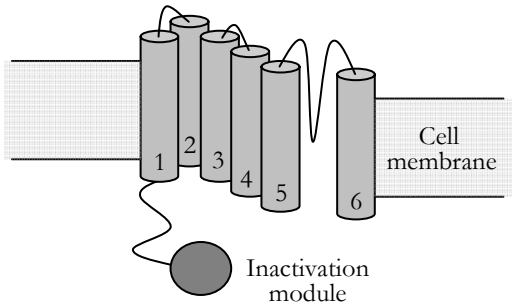


Figure 6. One of the four domains in a potassium ion channel. The pore is built up between segments 5 and 6 of the four domains.

The resting potential in neurons is negative to the outside by 60-80 mV. This is a result of open potassium (K^+) channels and outflow of potassium ions because of the higher intracellular than extracellular potassium concentration. Blocking K^+ channels or opening of Na^+ channels results in an increased electrical excitability. K^+ channels regulate the duration and frequency of the action potential discharges. (Rang et al., 2007)

A proposed mechanism of action of oxaliplatin neurotoxicity is the alteration of voltage-gated ion channel function. Oxaliplatin has been shown to shift the voltage dependence towards negative membrane potentials in Na^+ and K^+ channels (Adelsberger et al., 2000; Benoit et al., 2006). Intracellularly applied oxaliplatin was suggested to reduce the sodium current through chelation of calcium by oxalate (Grolleau et al., 2001). Furthermore, oxaliplatin induced multiple endplate potentials that were proposed to be caused by a slower inactivation of Na^+ channels (Webster et al., 2005).

1.6 APOPTOSIS

The aim of chemotherapy is to kill the tumour cells. Necrosis is one type of cell death, where the cell disrupts by swelling, causing inflammation in the surrounding tissue. Apoptosis, on the other hand, does not cause inflammation. It is characterised by nuclear and cytoplasmic condensation, blebbing of the plasma membrane and the formation of apoptotic bodies (small membrane-enclosed fractions of the cell), which are phagocytosed. (Fadeel and Orrenius, 2005)

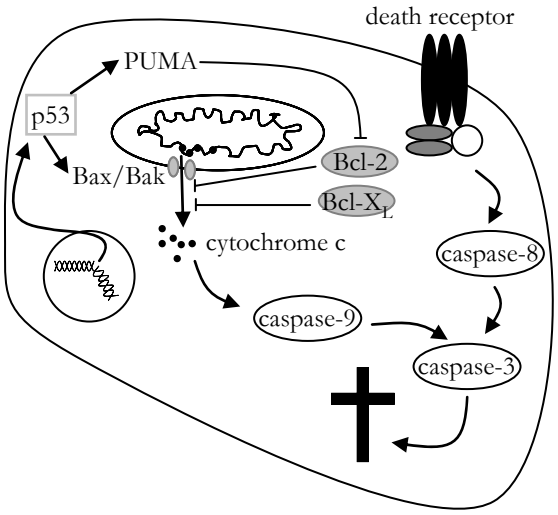


Figure 7. Apoptosis signalling. Intrinsic (release of cytochrome c from the mitochondria) and extrinsic (death receptor mediated) pathways.

There are two main pathways of apoptosis (a term first presented in the beginning of the 1970´s (Kerr et al., 1972)). The extrinsic pathway, which is important for the immune system, involves activation of death receptors, resulting in caspase-8 mediated activation of caspase-3 (figure 7). The intrinsic pathway is induced by for example DNA damage and involves cytochrome c release from mitochondria leading to activation of caspase-9 and caspase-3 (figure 7). The outcome of both pathways is cellular degradation and the formation of apoptotic bodies (Fadeel and Orrenius, 2005).

The tumour suppressor p53 is an important component of the apoptosis machinery. This protein can induce the expression of numerous genes, for example genes involved in growth arrest (such as p21), DNA repair and regulators of apoptosis (Weinberg, 2007). The p53 gene is often mutated in tumours, for example colon cancer (Vogelstein et al., 2000).

1.6.1 Oxaliplatin- *vs.* cisplatin-induced apoptosis

Because of the differences in effects and side effects of the platinum compounds, it has been proposed that their cellular modes of action may differ. For example, the expression of PUMA (a BH3-only protein involved in the induction of apoptosis through the intrinsic pathway, figure 7) was induced by oxaliplatin but not cisplatin in lovo and SW 116 colon cancer cell lines (Wang et al., 2006). Another study on HCT116 colon cancer cells indicated that the response to oxaliplatin unlike cisplatin was affected by p21 gene status (Hata et al., 2005).

1.6.2 Reactive oxygen species and calcium in apoptosis

It is known that a number of chemotherapeutic agents, including cisplatin, can increase cellular levels of reactive oxygen species (ROS) (Pelicano et al., 2004). One effect of ROS is p53 activation, but ROS can also be generated downstream of p53 activation (Martindale and Holbrook, 2002). ROS may affect the apoptotic machinery by catalysing the dimerisation of Bax, thereby facilitating cytochrome c release and subsequent caspase activation (figure 7). ROS could also act inside the mitochondria by oxidising cardolipin, which normally binds cytochrome c in the mitochondrial inner membrane, causing cytochrome c release (Neuzil et al., 2006).

Calcium is important in many aspects of apoptosis. For example, alterations in calcium homeostasis may cause endoplasmic reticulum (ER) stress. Prolonged ER stress can lead to caspase activation (Orrenius et al., 2003). Cisplatin has been shown to induce some type of ER stress (Mandic et al., 2003). Calcium can also trigger mitochondrial permeabilisation resulting in cytochrome c release (Orrenius et al., 2003).

2 AIM OF THE THESIS

The overall aim of this thesis was to elucidate the chemistry of oxaliplatin that can be important for the biological effects and for the pharmaceutical preparation.

3 MATERIALS AND METHODS

3.1 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a standard method for separating and quantifying different substances. Various separation techniques have been used regarding cisplatin, for example anion- and cation-exchangers and reversed-phase chromatography. It is important to consider the reactivity of the platinum compounds with the mobile and stationary phases. For example acetonitrile, often used in mobile phases, has been shown to react with platinum compounds (De Waal et al., 1990). Analysis of oxaliplatin has been done on reversed-phase LC systems with an aqueous mobile phase (Luo et al., 1999; Allain et al., 2000; Ficarra et al., 2002). Lou et al. used solvent-generated cation-exchange to separate oxaliplatin and its biotransformation products (Luo et al., 1999). The separation method used in the present studies consisted of a porous graphitic carbon (PGC) column, previously used for the separation of cisplatin and its hydrated complexes (Ehrsson et al., 1995) and a methanol-based mobile phase.

3.1.1 Porous graphitic carbon

The Hypercarb™ (used in the present work) is a PGC column composed of flat sheets of hexagonally arranged carbon, making it very hydrophobic. At the same time, the graphitic surface is highly polarisable. Carbon columns are physically and chemically stable and tolerate a wide pH range. One disadvantage of the PGC is that non-polar compounds can be difficult to desorb from the surface. (Hanai, 2003)

3.1.2 Post-column derivatisation

When analysing oxaliplatin in samples where there is interference of other compounds (for example in plasma ultrafiltrate) on-line post-column derivatisation with ultraviolet detection was used. The platinum compound (in this case oxaliplatin) is allowed to react with *N*,*N*-diethyldithiocarbamate under microwave heating after the separation process. This is an efficient method with a low quantitation limit. (Ehrsson and Wallin, 2003)

3.2 MASS SPECTROMETRY

Mass spectrometry (MS) has become an important tool in the identification of biologically related targets. The mass spectrometer is composed of an ion source, a mass analyser and a detector (figure 7) measuring the mass-to-charge ratio (*m/z*).

3.2.1 Ionisation and mass analyser

In this thesis, electrospray ionisation (EI) with a quadrupole mass analyser was used (papers I and III). An advantage with EI is that it can be coupled directly to LC. The sample is passed through a small capillary and nebulised by a strong electric field. This produces multiply charged droplets and eventually ions that can enter the mass analyser (figure 7). The nebulisation process can be facilitated by a flow of for example nitrogen gas. The separation in a quadrupole is done by a combination of radio frequency voltages and direct current voltages applied between four rods (figure 7), allowing only

one *m/z* to reach the detector. The voltages are then varied to allow detection of different *m/z* and thereby generating a mass spectrum. (Glish and Vachet, 2003)

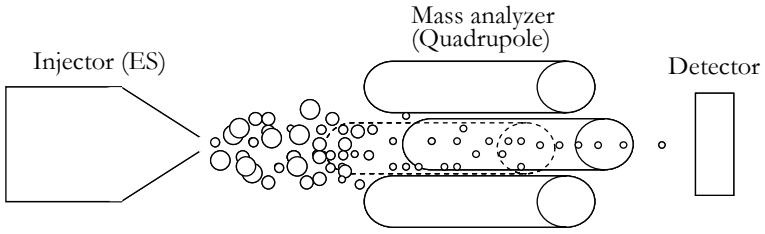


Figure 7. Schematic illustration of a mass spectrometer.

For a more unambiguous identification of a compound, tandem mass spectrometry was performed in paper III. The parent ion was dissociated to form daughter ions with a detectable *m/z*. Collision-induced dissociation of the parent ion by collision with argon gas was used. The instrument was a quadrupole-hexapole-quadrupole, where the quadrupoles function as MS and the middle hexapole functions as the collision cell.

3.2.2 Platinum and chlorine isotopes

Platinum has an isotopic distribution with the main isotopes ^{194}Pt (32.9 %), ^{195}Pt (33.8 %), ^{196}Pt (25.3 %) and ^{198}Pt (7.2 %). This gives a characteristic MS pattern. Chlorine, of importance for paper III, has two isotopes, ^{35}Cl (75.5 %) and ^{37}Cl (24.5 %). (Aylward and Findlay, 1994)

3.3 MEASUREMENT OF ION CHANNEL CURRENTS

In paper V, an experimental system with frog (*Xenopus laevis*) eggs (oocytes) that, after injection of mRNA, can express membrane-bound proteins, for example ion channels. Most electrophysiological measurements were done with the two-electrode voltage-clamp technique. Two electrodes are inserted into the oocyte. One is a membrane potential recording electrode and the other is a current-delivering electrode. This allows measurement of currents flowing through the ion channels. (Wagner et al., 2000)

3.4 CELLULAR ASSAYS

3.4.1 Cytotoxicity assays

In paper III, a classical method, the clonogenic assay was used. It is based on the fact that cells that are exposed to cytotoxic treatment lose the ability to divide and form colonies. The cells were seeded and exposed to the drug, washed and left for two weeks to form colonies. Then the colonies were stained and counted.

The clonogenic assay has a good correlation with *in vivo* chemosensitivity (Shimoyama et al., 1989). One disadvantage with the assay is that it is time consuming. There are cellular assays (for example sulphorhodamine B and the tetrazolium-based MTT) that are faster and appear to have a good correlation with the clonogenic assay, at least for platinum drugs (Perez et al., 1993; Kawada et al., 2002).

3.4.2 Apoptosis assays

Commonly used apoptosis assays are Annexin V staining (detecting early apoptotic events when phosphatidylserine, normally found intracellularly, is exposed extracellularly), the TUNEL assay (in apoptotic cells DNA is fragmented, resulting in many DNA ends that can be labelled) and a specific antibody for activated caspase-3. However, it is difficult to quantify the extent of apoptosis over time using these methods, since apoptotic cells disappear within a few hours. (Weinberg, 2007)

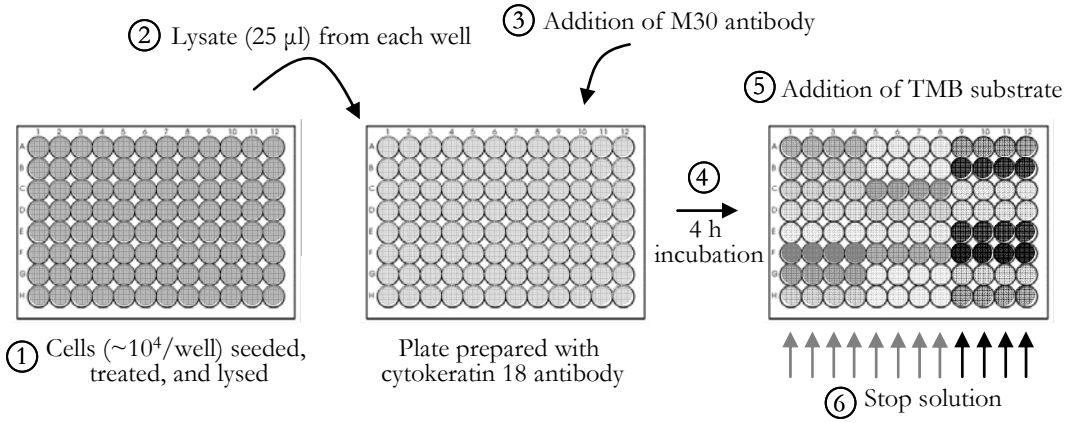


Figure 8. M30-Apoptosense® ELISA. 1. Cells are seeded in 96-well plates and treated on the following day. After for example a 24 hour incubation, cells are lysed directly in the medium. 2. An aliquot of each lysate is added to a well with a cytokeratin 18 antibody attached to the bottom of the well. 3. Next, the M30 monoclonal antibody, specific for the caspase-cleaved fragments of cytokeratin 18 is added and allowed to bind (4.). 5. Before reading the plate in a microplate reader, 3,3',5,5'-tetramethylbenzidine (TMB) substrate is added for colouring and the reaction is stopped by adding sulphuric acid (6.).

In paper VI, we used an apoptosis ELISA (enzyme-linked immunosorbent assay) for quantitation of apoptosis, M30-Apoptosense® (PEVIVA AB, Sweden). The assay (figure 8) is based on the measurement of caspase-cleaved fragments of the protein cytokeratin 18. The M30-Apoptosense® assay can be used with epithelial cells or other cells containing cytokeratin 18. One advantage of this type of assay is that it measures the accumulated levels of the stable cytokeratin 18 fragment instead of measuring for example caspase activity only at a certain time point. (Hägg et al., 2002)

4 RESULTS AND DISCUSSION

4.1 ALKALINE HYDROLYSIS OF OXALIPLATIN (PAPER I)

Earlier in our laboratory, the alkaline hydrolysis of oxaliplatin was explored in order to find a convenient way to synthesise the dihydrated oxaliplatin complex (DOC) and enable the study of the toxicity of this compound compared to oxaliplatin itself (Videhult et al., 2002). The dihydrated complex had previously been prepared by incubating $Pt(dach)Cl_2$ with silver nitrate, resulting in a mixture of $[Pt(dach)(H_2O)_2]^{2+}$ and $[Pt(dach)Cl(H_2O)]^{+}$ (Luo et al., 1998). Videhult et al. showed that DOC could be formed rapidly (within 30 minutes) at sodium hydroxide concentrations of 0.01-0.1 M at 70 °C and that the complex was more cytotoxic than oxaliplatin (Videhult et al., 2002).

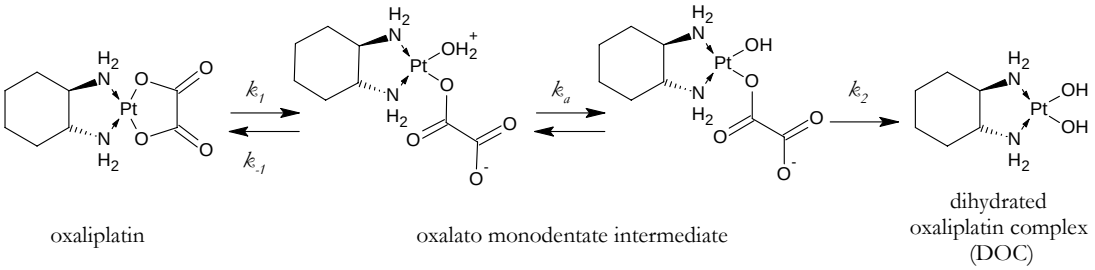


Figure 9. Hydrolysis of oxaliplatin.

In connection to these studies, the degradation of oxaliplatin in alkaline solution was studied. The reaction was fast with a half-life of 1.0 minute at 70 °C and 0.1 M sodium hydroxide when it was quenched on ice prior to the LC injection. As an alternative, quenching the reaction with succinic acid to pH 6 was tried. This resulted in a corresponding observed half-life of 3.6 minutes, suggesting that the reaction was partly reversed at this pH. We continued the study without the neutralization step. The reaction rate was almost independent of the concentration of the hydroxide ion, which is in agreement with previous findings for cisplatin (Miller and House, 1989). This led to the proposed mechanism that is shown in figure 9. A water molecule attacks the platinum atom, which leads to the opening of the oxalato ring. Depending on the pH, the oxalato ring will either close or the ring-opened intermediate will be deprotonated, enabling the formation of DOC. In a neutral water solution oxaliplatin will be "semi-stable", constantly opening and closing the oxalato ring. The formation of the monodentate intermediate and DOC was also followed by LC. The monodentate intermediate was identified by MS.

Allsopp et al. modelled a two-step alkaline hydrolysis for carboplatin. It was suggested that the hydroxide ion would attack platinum or, since hydroxide is a poor nucleophile for Pt, a carbonyl carbon (Allsopp et al., 1991). However, we found that the reaction rate for oxaliplatin was practically independent of the hydroxide ion concentration. We therefore believe that our model (figure 9) is more correct, at least for oxaliplatin.

4.2 DETERMINATION OF THE ACID DISSOCIATION CONSTANT (PAPER II)

Oxaliplatin is apparently stable in water solutions with a half-life of 157 hours at 37 °C, pH 7.4. To be able to calculate the acid dissociation constant (k_a) of the intermediate, we first wanted to know the rate constant (k_{-1}, figure 9) of the ring-closing reaction at 37 °C. When the pH is at least 2 pH units below pk_a, the observed rate constant of the ring-closing reaction (k_{obs}) equals k_{-1}.

$$k_{obs} = \frac{k_{-1}}{1 + \frac{k_a}{a_h}} \qquad \textit{Equation 1}$$

The intermediate was isolated by collecting fractions from the LC system and the closing reaction was studied photometrically at pH 4 and temperatures where the reaction was slow enough to be measured with sufficient precision (8-25 °C). The rate constant at 37 °C was obtained by extrapolation of k_{-1} to this temperature using the Arrhenius equation (equation 2), giving k_{-1}=14.6 min^{-1}.

$$k = A \times e^{-Ea/RT} \qquad \textit{Equation 2}$$

The observed rate constant for the ring-closing reaction in the pH range 8.3-8.8 was determined by investigating the formation of oxaliplatin by LC. The pH range was limited by either a very fast reaction (pH<8.3) or by the formation of DOC and the re-formation of the intermediate from oxaliplatin (pH>8.8). Using equation 1 with k_{-1} as a constant, k_a was determined to 5.9×10^{-8} (pk_a=7.23) by nonlinear regression analysis.

At pH 7.4 only about 0.7 % of the total platinum will appear as the ring-opened intermediate. The importance of the ring-opened complex in the *in vivo* situation is not known. However, we have noticed in an apoptotic assay of oxaliplatin in a colon cancer cell line that the apoptotic response increases with pH (unpublished observation). This may have a connection to the ring-opened intermediate or to an increased formation of DOC.

Some studies have implied that carbonate could serve as an activator of oxaliplatin (Luo et al., 1999; Ravera et al., 2007). However, our own (unpublished) experiments indicate that the crucial factor in these types of experiments is pH.

4.3 THE MONOCHLORO COMPLEX (PAPER III)

Previously, a biphasic degradation profile of oxaliplatin in the presence of chloride has been observed in our laboratory. Since the chloride concentration in blood is high (about 0.1 M), we wanted to examine the reaction more closely. The rate of the degradation of oxaliplatin at different chloride ion concentrations was studied. The reaction rate was dependent on the chloride ion concentration and the degradation was biphasic. At 0.1 M chloride, the half-life of oxaliplatin is about 17 h, while the first phase of the reaction has a half-life of only 5-10 minutes. This confirms the recommendation that oxaliplatin should not be mixed with chloride-containing solutions.

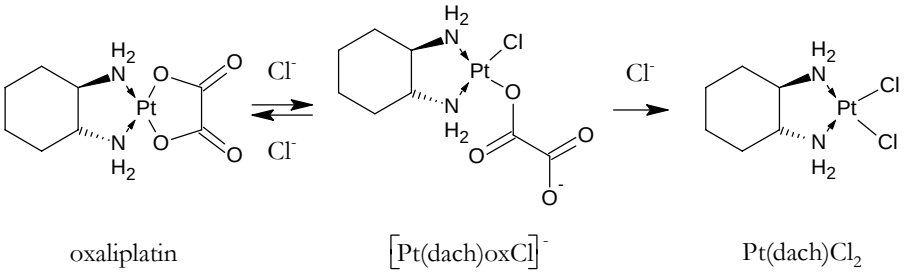


Figure 10. Oxaliplatin reacts with chloride.

The reaction of carboplatin with chloride has been suggested to occur in two steps (Allsopp et al., 1991). We proposed that a ring-opened intermediate, in conformity with the monohydrated complex, could explain the biphasic behaviour. By modifying the mobile phase, we separated two products that were identified by LC-MS(-MS) as the monochloro monooxalato complex ($[Pt(dach)oxCl]^-$) and the dichloro complex ($Pt(dach)Cl_2$), figure 10. The formation of $[Pt(dach)oxCl]^-$ was rapid and corresponded to the fast first phase of the degradation of oxaliplatin (figure 11).

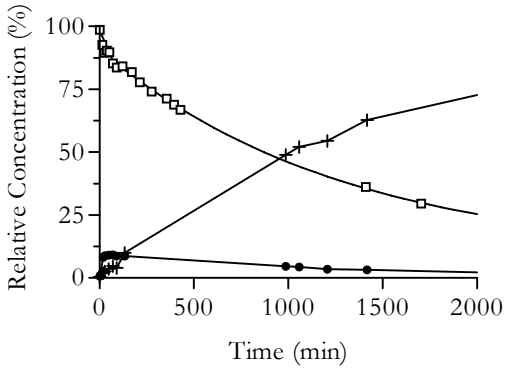


Figure 11. Degradation of oxaliplatin (□) and formation of $[Pt(dach)oxCl]^-$ (●) and $Pt(dach)Cl_2$ (+) in 0.1 M sodium chloride at 37 °C, pH 7.4.

The cytotoxicity of $[Pt(dach)oxCl]^-$ was evaluated by the clonogenic assay on a human colon adenocarcinoma cell line (HT-29) by comparing incubation in chloride-containing and chloride-free medium. In this setting, there was no difference in the cytotoxic effects. Nevertheless, it could be important to note that the chloride

concentration in the cytoplasm is lower than in the extracellular fluid (Jennerwein and Andrews, 1995), but in the nucleus the chloride concentration has been found to be considerably higher (Siebert, 1972). Perhaps the chloride content in the medium is of minor importance in this study.

Considering the high chloride content in plasma and the formation rate of the intermediate, it is reasonable to assume that $[Pt(dach)oxCl]^-$ is formed *in vivo*. In an *in vitro* study in blood plasma, negatively charged biotransformation products were suggested to be present, though they were not identified (Pendyala and Creaven, 1993). In contrast, due to the short *in vivo* half-life of oxaliplatin, the *in vivo* concentration of $Pt(dach)Cl_2$ should be very low. Indeed, Shord et al. showed that less than 3 % of oxaliplatin was biotransformed to $Pt(dach)Cl_2$ (Shord et al., 2002).

4.4 REACTION WITH SULPHUR-CONTAINING COMPOUNDS (PAPER IV)

It is well known that platinum compounds have a high affinity towards sulphur-containing compounds (Dedon and Borch, 1987). Complexes with methionine and glutathione have been observed in plasma from patients undergoing oxaliplatin chemotherapy (Allen et al., 1998; Allain et al., 2000). One objective of this paper was to compare the reactivity of oxaliplatin towards cysteine, methionine and glutathione with what is known for cisplatin. The other objective was to see if we could explain the short oxaliplatin half-life of 14 minutes *in vivo* (Ehrsson et al., 2002) by comparing the degradation in PUF with the degradation in a mix of physiologically relevant concentrations of the sulphur-containing compounds and chloride ions.

Oxaliplatin was more prone than cisplatin to react with the three tested sulphur-containing compounds and the reaction rates were concentration dependent. This could be of importance especially for the largely unexplored intracellular events that take place during platinum drug therapy. The intracellular pool of sulphhydryl groups can reach levels of 10 mM (Bose, 2002), which would imply a half-life of oxaliplatin of less than 15 minutes in the cell. This fact, together with the knowledge that the reaction with DNA is very slow *in vitro* (Butour et al., 1985), raises the question of how DNA adducts are formed. There are studies indicating that platinum-methionine complexes may slowly rearrange to platinum-guanosine 5-monophosphate complexes because of trans labilisation and more favourable thermodynamics (Barnham et al., 1995; van Boom et al., 1999; Soldatović and Bugarčić, 2005) or that histone methionine residues could serve as cisplatin or oxaliplatin reservoirs (Wu et al., 2008). However, this does not seem to happen for platinum-sulphhydryl (glutathione and cysteine) complexes (Bose et al., 1995; van Boom et al., 1999; Bugarčić et al., 2004) and may also not be valid for oxaliplatin because of the more stable diaminocyclohexane ligand (Strickmann et al., 2002).

The higher reactivity of oxaliplatin than cisplatin with the sulphur-containing compounds may also explain why fewer DNA adducts are formed, even though the cellular uptake of platinum compounds with a diaminocyclohexane carrier should be higher than that of cisplatin (Zou et al., 1998). It is also evident from our studies that the reaction can occur by direct displacement in accordance with what is previously known for cisplatin, carboplatin and Pt(dach)(malonate) (Dedon and Borch, 1987; Mauldin et al., 1988).

Glutathione has been used to ameliorate the neurotoxic side effects of oxaliplatin treatment, apparently without affecting the response rate (Cascinu et al., 2002). However, in view of the reactivity of oxaliplatin towards sulphur-containing compounds it is important to study the pharmacokinetic alterations in detail. For example, a study on reducing the ototoxicity of cisplatin in guinea pig by intravenous infusion of methionine resulted in a lower systemic exposure of intact cisplatin (Ekborn et al., 2002).

The fact that presence or absence of chloride in the culture medium did not affect the cytotoxicity of oxaliplatin (paper III) and that for example a Pt-methionine complex is not taken up by cells (Mauldin et al., 1988) implies that the toxic effects at the cellular

level are mediated by intracellular transformations. An interesting observation with respect to the intracellular environment, is that a glutathione-cisplatin adduct has been shown to be able to inhibit thioredoxin reductase (Arnér et al., 2001).

In our cocktail of sodium chloride, cysteine, methionine and glutathione, we could see a correlation with the degradation of oxaliplatin in PUF during the first three hours. We suggest that this can explain the basic non-enzymatic reactions that occur in PUF in the *in vivo* situation.

4.5 EFFECTS ON POTASSIUM CHANNELS (PAPER V)

Some investigators have indicated that oxaliplatin modifies voltage-gated ion channels (Adelsberger et al., 2000; Grolleau et al., 2001; Webster et al., 2005; Benoit et al., 2006). Since the acute neurotoxic side effects appear quickly after the administration of oxaliplatin to patients, we hypothesised that $[Pt(dach)oxCl]^-$ could affect the ion channels of the peripheral nervous system. The fact that this biotransformation product is negatively charged was interesting, because charged compounds can shift the channel's voltage dependence (Elinder and Århem, 2003; Börjesson, Hammarström, Elinder, manuscript submitted for publication).

Despite repeated experiments, we could not detect any effect of either oxaliplatin itself or $[Pt(dach)oxCl]^-$ in our system. If there is a general ion channel effect we would have been able to detect this in our experiments. Therefore we sought for specific effects. Since oxaliplatin is highly reactive towards sulphur-containing compounds and in particular cysteine (Jerremalm et al., 2006), we investigated if ion channels with cysteines at critical positions were affected by oxaliplatin. However, oxaliplatin did not alter the function of these cysteine-mutated channels.

Our experiments were designed to find effects on voltage-gated K^+ channels. The well-established oocyte experimental system makes it possible to study the channels with a low background from endogenous membrane transport systems (Wagner et al., 2000). Previous research groups have used animal preparations (for example neurons) to study changes in currents (Adelsberger et al., 2000; Grolleau et al., 2001; Webster et al., 2005; Benoit et al., 2006). Animal preparations can be unspecific with interference of other transport or receptor signals. Thus, there may be alternative explanations for the neurotoxic symptoms.

4.6 OXALIPLATIN AND APOPTOSIS (PAPER VI)

As described in 1.6.2, ROS and calcium can have an impact on the apoptotic response. We studied the apoptosis of oxaliplatin and cisplatin on a colon cancer cell line (HCT116) by the M30-Apoptosense® assay (measuring a fragment of cytokeratin 18, cleaved by caspases). The apoptotic response was similar for both drugs at concentrations of 10-30 μM. Exposing the cells to the superoxide ($O_2^{\bullet-}$) scavenger tiron, led to repressed apoptosis in the cells treated by cisplatin. For oxaliplatin, the apoptotic response remained constant. Tiron is an efficient scavenger of $O_2^{\bullet-}$; at equimolar concentrations in the millimolar range the half-life of the reaction is a few microseconds (Greenstock and Miller, 1975). In paper VI, it was also shown that the apoptosis of cisplatin, but not oxaliplatin, was inhibited by a calcium chelator (BAPTA-AM). This implies a difference in apoptotic routes for cisplatin and oxaliplatin, which may explain some of the diversities seen in the effects and side effects of the platinum drugs.

5 CONCLUSIONS

We have studied the reaction of oxaliplatin with a number of nucleophiles (water, chloride and sulphur-containing compounds), reactions that are likely to occur *in vivo*, thereby affecting the results of the antineoplastic therapy.

Oxaliplatin is "semi-stable" in aqueous solutions, constantly opening and closing the oxalato ring. The acid dissociation constant of the ring-opened intermediate is 7.23, favouring the ring-closing reaction at physiological pH. This can explain the apparent stability in a pharmaceutical glucose preparation.

The degradation of oxaliplatin in a chloride solution gives a ring-opened monochloro intermediate at a fast initial rate, followed by the formation of the dichloro complex. In our cytotoxicity assay, the monochloro intermediate was not more cytotoxic than oxaliplatin itself. The rapid initial degradation seen in a chloride solution is also observed in plasma ultrafiltrate, indicating the presence of the monochloro intermediate. The rate of biotransformation of oxaliplatin in plasma ultrafiltrate was in good agreement with the degradation of oxaliplatin in a cocktail of chloride, glutathione, methionine and cysteine at physiological concentrations.

The acute neurotoxic side effects do not seem to be mediated via a general effect on voltage-gated ion channels of either oxaliplatin or the negatively charged monochloro intermediate.

The intracellular actions of oxaliplatin are complicated. Platinum and other metal-based drugs affect numerous cellular systems including DNA, proteins and apoptotic pathways. Which one is *the* target system is difficult, if even possible, to evaluate. Still, in view of the different effects of cisplatin and oxaliplatin treatment, it is interesting to search for intracellular differences. We found that oxaliplatin induced apoptosis is independent of superoxide and calcium, while the apoptotic response of cisplatin treatment is both superoxide and calcium dependent.

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