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CISPLATIN, A PLATINUM-CONTAINING ANTINEOPLASTIC DRUG: PERSPECTIVES ON ANALYTICAL CHEMISTRY AND PREVENTION OF OTOTOXICITY

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ABSTRACT

The platinum-containing drug cisplatin plays a key role in the curative and palliative treatment of many solid malignancies. Unfortunately, the treatment can lead to sensorineural hearing loss, which limits the use of the drug. High single and cumulative dose levels are risk factors, but there is a large interindividual variability in the susceptibility to the ototoxic effects. The mechanisms behind the ototoxicity have not been fully elucidated, but one hallmark is oxidative stress. Moreover, the ototoxicity is dependent on the exposure of cisplatin and/or its biotransformation product MHC in the perilymphatic compartment of the cochlea. The aim of the research presented in this thesis was to contribute to the development of treatment strategies against cisplatin-induced ototoxicity.

Sulfur-containing nucleophiles are attractive candidate compounds against cisplatin-induced hearing loss since they are prone to chemically interact with cisplatin and MHC and could potentially reduce the exposure of these platinum species in the cochlea. A second possible mechanism may be relief of oxidative stress. The aim of the in vitro study described in Paper I was to investigate how quickly the concentrations of cisplatin and MHC can be reduced in the presence of five sulfur-containing nucleophiles. The results showed that thiosulfate was a promising candidate for future studies in vivo, since it reacted fast with cisplatin and, in particular, with MHC. This conclusion was further supported by the fact that thiosulfate is an endogenous ion, is well tolerated, and has been used clinically for decades against e.g. cyanide poisoning. Systemic administration of thiosulfate has earlier been investigated in several in vitro and in vivo studies against cisplatin-induced ototoxicity. However, it has been unknown whether thiosulfate at all reaches the cochlea. In the study described in Paper II, it was demonstrated that the distribution of thiosulfate to the perilymphatic compartment was quick and extensive after an i.v. bolus injection in guinea pigs. Unfortunately, this way of administration of thiosulfate in connection with systemic cisplatin delivery is risky, since it may lead to decreased antitumoral effects due to inactivation of cisplatin and MHC not only in the cochlea but also in tumor tissues. In the studies on which Paper III is based, it was found that the ototoxicity in cisplatin-treated guinea pigs was reduced by a local administration strategy employing a thiosulfate-containing hyaluronan gel administered into the middle ear cavity three hours prior to the systemic cisplatin injection.

When quantifying cisplatin, unselective methods are almost always used, which may confound the results. In the final study, on which Paper IV is based, a sensitive, robust, and fast method using liquid chromatography and UV detection for the selective analysis of cisplatin in blood was developed. This method will be a valuable instrument in future studies exploring the role of pharmacokinetic parameters of cisplatin for the ototoxic effects.

Keywords: cisplatin, liquid chromatography, local administration, ototoxicity, perilymph, thiosulfate
LIST OF PUBLICATIONS

The thesis is based on the following original papers and manuscripts, which will be referred to in the text by their Roman numerals.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Concentration of the analyte at time $t$</td>
</tr>
<tr>
<td>$A_0$</td>
<td>Initial concentration of the analyte</td>
</tr>
<tr>
<td>ABR</td>
<td>Acoustically-evoked auditory brainstem response</td>
</tr>
<tr>
<td>ATP7B</td>
<td>Copper transporting ATPase</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance unit</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
</tr>
<tr>
<td>BUF</td>
<td>Blood ultrafiltrate</td>
</tr>
<tr>
<td>B.w.</td>
<td>Body weight</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Initial concentration of the drug</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Ctr1</td>
<td>Copper transporter 1</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DDTC</td>
<td>Sodium $\text{N},\text{N}$-diethyldithiocarbamate</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Molar absorptivity</td>
</tr>
<tr>
<td>Et al.</td>
<td>And others</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HYA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>IHC</td>
<td>Inner hair cell</td>
</tr>
<tr>
<td>I.d.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>I.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>I.v.</td>
<td>Intravenous, intravenously</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wave length</td>
</tr>
<tr>
<td>$\lambda_{ex}$</td>
<td>Excitation wave length</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>Emission wave length</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>$k_1$</td>
<td>Pseudo first-order rate constant(s)</td>
</tr>
<tr>
<td>$k_{Nu}$</td>
<td>Second-order rate constant(s) in the presence of a specific sulfur-containing nucleophile</td>
</tr>
<tr>
<td>$k_{obs}$</td>
<td>Observation rate constant(s)</td>
</tr>
<tr>
<td>MHC</td>
<td>Monohydrated cisplatin, i.e. the monoaqua monochloro cisplatin and monochloro monohydroxy cisplatin complexes</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>$Nu$</td>
<td>Nucleophile</td>
</tr>
<tr>
<td>$[Nu]$</td>
<td>Concentration of nucleophile</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>OHC</td>
<td>Outer hair cell</td>
</tr>
<tr>
<td>OHC1, 2, and 3</td>
<td>First, second, and third outer hair cell rows</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PGC</td>
<td>Porous graphitic carbon</td>
</tr>
<tr>
<td>PLT</td>
<td>Perilymph of the scala tympani</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>$Q$</td>
<td>Amount of drug released per unit area</td>
</tr>
<tr>
<td>RWM</td>
<td>Round window membrane</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine B isothiocyanate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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</table>
1 INTRODUCTION

1.1 CANCER

About half a million people are living with cancer in Sweden and the number of new cases per year is approximately 50,000 (Engholm et al., 2010). Cancer is a group of diseases caused by accumulation of mutations of DNA, leading to uncontrolled cell proliferation due to dysfunctional gene expressions (Weinberg, 2007). Dysfunctional gene expressions are commonly occurring, and humans as well as other organisms are armored with several lines of defense, such as the immune system, DNA repair systems, and/or programmed cell death, in order to render them harmless (Weinberg, 2007). Failure of these lines of defense may lead to cancer (Weinberg, 2007).

1.2 ANTITUMORAL DRUG THERAPY

Treatment of cancer often involves a combination of surgical, radiation, and drug therapy. Antitumoral drug therapy springs from the research on war gases during the World Wars (Einhorn, 1985). Mustard gas was used in World War I and in the interim between the two wars, nitrogen mustard, a mustard gas derivative, was synthesized (Einhorn, 1985). Studies on experimental animals showed that the effects of nitrogen mustard was particularly significant in lymphoid tissue, bone marrow, and the epithelium of the gastrointestinal tract, tissues with a large fraction of renewal cell populations (Gilman, 1963). Subsequent investigations on experimental lymphoma showed remarkable results (Gilman, 1963). In 1942, the first patient, a male with lymphosarcoma, was treated, which resulted in a dramatic remission of the disease (Gilman, 1963).

Most clinically used chemotherapy treatment today is based on “traditional” antitumoral drugs, which were developed several decades ago, at a time when the development of therapeutics was not yet informed by the genetic and biochemical mechanisms of cancer pathogenesis (Weinberg, 2007). These drugs interfere with cellular mechanisms that are common for normal as well as malignant cells, such as DNA synthesis and cell division. The chemotherapy is therefore a balance act between killing as many malignant cells as possible without causing unacceptable side-effects due to the excessive killing of normal cells. One of these “traditional” antitumoral drugs is cisplatin, which is the focus of this thesis.

1.3 THE DISCOVERY OF CISPLATIN

In the middle of the 1960s, Barnett Rosenberg and his colleagues studied the possible effects of an electric field on growth processes in Escherichia coli using platinum (Pt) electrodes (Rosenberg et al., 1965). Compounds formed by reaction of Pt from the electrodes with ammonium chloride in the buffer stopped cell division and induced filamentous growth in the bacteria (Rosenberg et al., 1965). Some years later, the
antitumoral activity of these compounds was discovered when studying sarcoma 180 and leukaemia L1210 in mice (Rosenberg et al., 1969). One of the more successful species, cis-diamminedichloroplatinum(II) \((\text{cis}^{-}\left\{\text{Pt}\left(\text{Cl}_2\right)\left(\text{NH}_3\right)\_2\right\})\); Figure 1), later on known as cisplatin, entered clinical trials in 1971 (Higby et al., 1974). It was approved for the treatment of testicular and ovarian cancers in 1978 (Wong and Giandomenico, 1999). Today, several decades later, cisplatin is used in the curative and/or palliative treatment of a range of solid malignancies, e.g. of the testes, ovaries, bladder, lungs, and head and neck region (Go and Adjei, 1999). Moreover, it is a cornerstone of the chemotherapeutic therapy of some pediatric malignancies, such as osteosarcoma and neuroblastoma (Kolinsky et al., 2010).

1.4 CHEMISTRY OF CISPLATIN

Cisplatin is a square planar Pt molecule (Figure 1). The chlorides are reactive ligands sensitive to nucleophilic attack and hydrolysis, whereas the Pt ammine linkage is stable. These features are fundamental for the biological actions of cisplatin, especially since no enzymes are involved in its transformation. As cisplatin is not metabolized, the fate of the drug \textit{in vivo} is referred to as its biotransformation.

1.4.1 Biotransformation

Cisplatin is rapidly non-enzymatically biotransformed; its elimination half-life in patients is approximately 30 minutes (Andersson et al., 1996; Reece et al., 1989). Cisplatin easily undergoes ligand-exchange reactions with nucleophiles, resulting in Pt complexes with e.g. albumin, globulin (Gullo et al., 1980; Nagai et al., 1996), cysteine (Andrews et al., 1984; Dedon and Borch, 1987), glutathione (Andrews et al., 1984; Dedon and Borch, 1987; Nagai et al., 1996), and methionine (Andrews et al., 1984; Dedon and Borch, 1987; Ekborn et al., 2002; Shirazi et al., 1996). The biotransformation products have in most cases shown less activity than the parent compound (Andrews et al., 1984; Daley-Yates and McBrien, 1984; Dedon and Borch, 1987; Muldoon et al., 2001; Shirazi et al., 1996). For example, high levels of the ubiquitous antioxidant glutathione is associated with cisplatin resistance (Hall et al., 2008). However, the results from cisplatin studies with the thioether methionine have been contradictory (Alden and Repta, 1984; Korver et al., 2002; Shirazi et al., 1996; Vrana and Brabec, 2002). The reason for the conflicting results have been approached in incubation studies, which have shown that the monosubstituted Pt-methionine complex can be even more active than cisplatin, in contrast to polysubstituted Pt-methionine complexes (Andrews et al., 1984; Barnham et al., 1995; Daley-Yates and McBrien, 1982; van Boom et al., 1999). This has been explained by labilization of the Pt ammine linkage trans to the sulfur atom of the incoming methionine, resulting in a more reactive Pt complex (Barnham et al., 1995; Daley-Yates and McBrien, 1982).
Cisplatin undergoes spontaneous hydrolysis reactions, which are some of the most fundamental reactions of the drug, both in vitro and in vivo. The most important contribution to the understanding of cisplatin hydrolysis can be attributed to the scientists S. E. Miller and D. A. House (Miller et al., 1991; Miller and House, 1989a; Miller and House, 1989b; Miller and House, 1990; Miller and House, 1991). They showed that depending on the pH and chloride concentration of the aqueous solution of cisplatin, various products are formed (Figure 1). The hydrolysis products are more active than the parent compound as demonstrated in vitro (Daley-Yates and McBrien, 1982; Kohl et al., 1979; Shirazi et al., 1996; Zheng et al., 1997) and in vivo (Daley-
Yates and McBrien, 1984; Jones et al., 1991; Litterst, 1981; Zheng et al., 1997). However, in these studies, a mixture of hydrolysis products has been used, which tells little about the in vivo situation, since monohydrated cisplatin (MHC, i.e. the monochloro monohydroxy and monoaqua monochloro cisplatin complexes (pKa ~6.6 (Andersson et al., 1994); Figure 1) is the prevailing hydrated species at physiological conditions (Bancroft et al., 1990; Miller and House, 1990; Segal and Le Pecq, 1985). The few studies that have investigated isolated MHC have shown that its area under the blood concentration-time curve (AUC) is about 15% of that of cisplatin in cisplatin-treated patients (Andersson et al., 1996) and about 10% of that of cisplatin in cisplatin-treated experimental animals (Ekborn et al., 2002; Ekborn et al., 2003b). The higher reactivity of MHC compared to that of cisplatin has been demonstrated in vitro (Yachnin et al., 1998) and in experimental animals (Ekborn et al., 2003b). Increasing the sodium chloride (NaCl) concentration of the cisplatin vehicle decreases the toxicity in cisplatin-treated animals (Daley-Yates and McBrien, 1985; Litterst, 1981; Wondergem et al., 1988), indicating the significance of hydrolysis of cisplatin in vivo. A correlation between lower pH and increased cisplatin-induced cytotoxicity has been demonstrated in vitro (Atema et al., 1993; Herman et al., 1988; Murakami et al., 2001), suggesting the importance of the monoaqua monochloro cisplatin complex. The pH dependence may have clinical significance, since the microenvironment in tumors is generally more acidic than in normal tissues (Tannock and Rotin, 1989).

The diaquo species (Figure 1) is highly toxic, as shown in experimental animals (Cleare and Hoeschele, 1973). Dihydrated cisplatin (Figure 1) may form polymers (Erickson et al., 1987). Even though probably not formed in vivo, the dihydrated species might play a role when conducting in vitro studies.

1.5 EFFECTS OF CISPLATIN TREATMENT

1.5.1 Antitumoral effects

![Figure 2. The cisplatin analog transplatin has much less anticancer activity than cisplatin and is not used clinically.](image)

Early studies on mice bearing well developed tumors showed that cisplatin treatment inhibited DNA synthesis (Howle and Gale, 1970). Despite the high and broad reactivity of cisplatin, it is generally assumed that nuclear DNA is the most important target for its antitumoral actions (Cohen and Lippard, 2001; Siddik, 2003). Both cisplatin and its analog transplatin (Figure 2), which has much less anticancer activities (Cleare and Hoeschele, 1973), bind almost exclusively at the N7 nitrogen atom in purine bases.
There is a preference for guanine (Figure 3A) over adenine (Figure 3B). Several types of DNA adducts are formed, including monofunctional adducts, intrastrand crosslinks, and interstrand crosslinks. Cisplatin forms predominantly 1,2-intrastrand crosslinks (Figure 3C) and, to a lesser extent, 1,3-intrastrand and interstrand crosslinks. Transplatin forms predominantly 1,3-intrastrand and interstrand crosslinks. This suggests that the toxicity of cisplatin originates from the 1,2-intrastrand crosslinks, which the geometry of transplatin does not allow (Cohen and Lippard, 2001). Cisplatin is cytotoxic also to enucleated cells (Mandic et al., 2003), showing that there are other potential cellular targets of cisplatin, e.g. mitochondrial DNA and sulfur-containing peptides, proteins, and enzymes.

![DNA Adducts](image)

**Figure 3.** Nuclear DNA is assumed to be the most important target for the antitumoral actions of cisplatin. Platinum-DNA adducts are formed almost exclusively via the N7 nitrogen atom in guanine (A) and, to a smaller extent, adenine (B). The N7 nitrogen atom is indicated with an arrow (A and B). Several types of platinum-DNA adducts are formed, the most important being 1,2-intrastrand crosslinks (C).

### 1.5.2 Side-effects

As for all “traditional” anticancer drugs, treatment with cisplatin induces a variety of side-effects. Two of the most important dose-limiting side-effects are renal damage and ototoxicity. The severity of the renal damage is effectively reduced by hydration therapy. The ototoxicity is the only dose-limiting side-effect without any clinically available treatment and will be discussed in detail in the next section.
Air-conducted sound travels through the external auditory canal (Figure 4) to the tympanic membrane (Figure 4), which starts to vibrate. The vibrations are conducted through the bones of the ossicular chain, i.e. the malleus, incus, and stapes (Figure 4), via the oval window (Figure 4) at the base of the cochlea (Figure 4) to the cochlear fluids. (Guyton and Hall, 1997)

The cochlea is composed of three different scalae coiled side by side: scala vestibuli and scala tympani (Figure 5), which are filled with perilymph, and scala media (Figure 5), which is filled with endolymph. The ion content of perilymph resembles that of plasma, whereas endolymph has a high potassium concentration and a low sodium concentration (Sterkers et al., 1988). The Reissner’s membrane and the basilar membrane separate the scala media from the scala vestibuli and the scala tympani, respectively (Figure 5). On the surface of the basilar membrane lies the organ of Corti with the inner and outer hair cells (IHCs and OHCs, respectively; Figure 6). The IHCs generate nerve impulses in response to movements of the cochlear fluids transmitted through the basilar membrane. The OHCs are amplifiers of this mechanotransduction. Due to the distance from the oval window and the stiffness of the basilar membrane, which decreases from the base to the apex, the resonance of the basilar membrane for high frequencies is highest at the base, whereas for low frequencies, it is highest at the apex. The nerve impulses generated by the inner hair cells travel via spiral ganglion cells (Figure 5) to the VIII cranial nerve and the auditory cortex. (Guyton and Hall, 1997)
1.6.1 Cisplatin-induced ototoxicity

Cisplatin-induced ototoxicity is a clinical problem. In pediatric patients, the ototoxicity may hamper the speech, cognitive, and social development of the patient. The hearing loss may even lead to discontinuation of the cisplatin therapy (de Jongh et al., 2003; Ekbom et al., 2004). Changing from cisplatin to the cisplatin analog carboplatin
reduces the risk of hearing loss but possibly also the chance of cure or, more common, the time to disease progression (Go and Adjei, 1999).

The fact that treatment with cisplatin can cause hearing loss was discovered shortly after the clinical introduction of the drug (Higby et al., 1974; Rossof et al., 1972). In general, the ototoxicity is manifested as bilateral high frequency sensorineural hearing loss, which rarely affects the perception of hearing of the patient (Blakley et al., 1994; Coupland et al., 1991; Higby et al., 1974; Laurell and Borg, 1988; Schaefer et al., 1985). The damage develops progressively over days (Grau et al., 1996). In more severe cases, lower frequencies are affected as well, involving the speech frequency range (Blakley et al., 1994; Coupland et al., 1991; Ekbom et al., 2004; Laurell and Jungnelius, 1990; Schaefer et al., 1985). Tinnitus is common (de Jongh et al., 2003; Ekbom et al., 2004; Higby et al., 1974; Laurell and Jungnelius, 1990) (Higby et al., 1974). Complete or partial recovery of the hearing may occur (de Jongh et al., 2003; Ekbom et al., 2004; Higby et al., 1974; Laurell and Borg, 1988; Laurell and Jungnelius, 1990). Risk factors for developing cisplatin-induced hearing loss are high single (Laurell and Jungnelius, 1990) and cumulative cisplatin dose levels (Coupland et al., 1991; Laurell and Jungnelius, 1990; Oldenburg et al., 2007; Schaefer et al., 1985), radiation to the head concomitantly with the cisplatin therapy (Kolinsky et al., 2010; Miettinen et al., 1997), and young age in pediatric patients (Coupland et al., 1991). Less well-established risk factors are preexisting hearing loss (Kolinsky et al., 2010), older age in elderly patients (Laurell and Borg, 1988; Laurell and Jungnelius, 1990), and decreased levels of serum albumin (Blakley et al., 1994), hemoglobin (Blakley et al., 1994; de Jongh et al., 2003), red blood cells (Blakley et al., 1994), and hematocrit (Blakley et al., 1994). When taken these risk factors into account, there is still a large interindividual variability of the severity of the ototoxic side-effect (Coupland et al., 1991; Ekbom et al., 2004; Laurell and Jungnelius, 1990; Miettinen et al., 1997; Schaefer et al., 1985). The reason remains to be elucidated. Interestingly, in a recent study on testicular cancer survivors, long-term hearing impairment was correlated with certain genotypes of glutathione S-transferases, components of the glutathione antioxidant system (Oldenburg et al., 2007).

How is cisplatin-induced ototoxicity evaluated? In humans, the most common method is assessment of the hearing with pure tone audiometry (Ekbom et al., 2004; Laurell and Borg, 1988; Laurell and Jungnelius, 1990), which involves frequencies within the hearing range, usually from 125 Hz up to 8,000 Hz (Anniko et al., 2001). When using this method for hearing evaluation of 186 gynecological cancer patients receiving a cisplatin dose of 50 mg/m² body surface every four weeks, 22% of the patients had high frequency hearing loss after completion of the therapy, but in no single case was a significant change observed in the speech frequency range of 500 to 2,000 Hz (Laurell and Borg, 1988). When using the same method for hearing assessment of 54 patients with metastatic cancer receiving a cisplatin dose of 100-120 mg/m² body surface, 81% of the patients showed significant changes in hearing thresholds after completion of the therapy and 13% sustained a significant hearing handicap (Laurell and Jungnelius, 1990).
The hearing can also be assessed with a method that does not require the cooperation of the subject, which is particularly convenient when examining small children and experimental animals. In cisplatin-treated experimental animals, electrophysiologic examination of the hearing thresholds using acoustically-evoked auditory brainstem response (ABR) is common (Church et al., 1995; Ekborn et al., 2000; Ekborn et al., 2002; Ekborn et al., 2003b; Hellberg et al., 2009; Mukherjea et al., 2010; Nader et al., 2010; Otto et al., 1988; Tanaka et al., 2004). When using this method, the subject is exposed to a defined sound stimulus, which creates nerve impulses that travel from the hair cells to the auditory cortex. The nerve impulses are recorded. The resulting electroencephalogram has characteristic peaks corresponding to certain neuroanatomic regions (Kennelly, 2006). In experimental research, the hearing threshold is usually defined as the lowest level where a reproducible response can be recorded (Church et al., 1995; Ekborn et al., 2000; Ekborn et al., 2002; Ekborn et al., 2003b; Mukherjea et al., 2010; Nader et al., 2010; Otto et al., 1988; Tanaka et al., 2004).

Cisplatin-induced ototoxicity can also be evaluated histologically. However, this can only be performed post mortem, since it otherwise would cause irreversible injury to aural sensory structures. In experimental animals, commonly guinea pigs in hearing research, cisplatin typically induces sensorineural ototoxicity by causing loss of OHCs in the basal turn of the cochlea (Böheim and Bichler, 1985; Fleischman et al., 1975; Laurell and Bagger-Sjöbäck, 1991a; Laurell and Bagger-Sjöbäck, 1991b). This has also been demonstrated in humans (Schaefer et al., 1985). The histological damage develops over a few days (Laurell and Bagger-Sjöbäck, 1991b) and is proportional to the dose (Böheim and Bichler, 1985; Laurell and Bagger-Sjöbäck, 1991a); in more severe cases, the OHC loss progresses towards the apical part of the cochlea (Böheim and Bichler, 1985; Fleischman et al., 1975; Laurell and Bagger-Sjöbäck, 1991a; Laurell and Bagger-Sjöbäck, 1991b). The OHCs in the first row are more sensitive than those of the second and third rows (Figure 6) (Böheim and Bichler, 1985; Laurell and Bagger-Sjöbäck, 1991b). A considerable interindividual variation in susceptibility to loss of OHCs can be seen (Ekborn et al., 2002; Laurell and Bagger-Sjöbäck, 1991b). Quantification of OHC loss is one of the most common methods for histological evaluation of cisplatin-induced ototoxicity in experimental animals. However, other changes can be seen as well, such as damage to the supporting cells (Figure 6) and the stria vascularis (Figure 5), predominantly in the basal turn of the cochlea (Laurell and Bagger-Sjöbäck, 1991a; Laurell et al., 2007).

Similar to the underlying mechanisms of the antitumoral effects of cisplatin, those of the ototoxic effects are obscure, although one hallmark is generation of oxidative stress (Lautermann et al., 1997; Lee et al., 2004; Mukherjea et al., 2006). Significantly lower levels of the antioxidant glutathione in OHCs in the basal part of the cochlea compared to those in the apical part have been shown and may explain, at least in part, the differential cochleotoxicity of cisplatin (Sha et al., 2001). Another explanation to the differential toxicity might be that the cisplatin concentration in the cochlear base is higher than in the apex (Hellberg et al., 2010).
1.7 TRANSPORT MECHANISMS OF CISPLATIN

The distribution of cisplatin in vivo has been considered to be governed simply by passive diffusion due to early studies demonstrating that the cellular uptake of cisplatin was linear and not saturable against either time or drug concentration (Hall et al., 2008). However, according to a recently published review article (Hall et al., 2008), cellular resistance to cisplatin, which is a big clinical problem, is often caused by reduced accumulation of the drug, which implicates that active uptake and efflux mechanisms are at play. For example, overexpression of a copper transporter protein, the copper transporting ATPase (ATP7B), has been associated with poor prognosis in ovarian carcinoma patients receiving cisplatin-based chemotherapy due to enhanced cisplatin efflux (Nakayama et al., 2004), and knockdown of copper transporter 2 (CTR2) greatly increased the therapeutic efficacy of cisplatin in a mouse model (Blair et al., 2010).

The discovery of the involvement of specific transporters in drug-induced nephrotoxicity (Ciarimboli et al., 2005; Pabla et al., 2009; Yonezawa et al., 2005) was intriguing for hearing researchers, since the most common ototoxic drugs in clinical practice, aminoglycoside antibiotics, cisplatin, and loop diuretics, are also nephrotoxic (Humes, 1999). The implication of specific transporters in the distribution of cisplatin to the inner ear has recently been demonstrated. For example, high expression of the organic cation transporter 2 (OCT2) was shown in OHCs, IHCs, and the stria vascularis of mice, and in cisplatin-treated OCT2 knockout mice, there were no signs of ototoxicity and only mild nephrotoxicity (Ciarimboli et al., 2010). Involvement of the copper transporter 1 (Ctr1) in cisplatin-induced ototoxicity has also been suggested (More et al., 2010).

A problem with many of the studies on transporter-mediated distribution of cisplatin is the use of transport inhibitors or competitors that are strong nucleophiles. They may confound the results by chemically interacting with cisplatin (Ehrsson and Wallin, 2010). Two examples are copper sulfate (More et al., 2010) and the thioether cimetidine (Ciarimboli et al., 2010; Ciarimboli et al., 2005; Pabla et al., 2009; Yonezawa et al., 2005). Therefore, caution is warranted when interpreting the results of such studies.

1.8 DRUG THERAPY AGAINST CISPLATIN-INDUCED OTOTOXICITY

Compounds that might neutralize mustard gas and nitrogen mustard were studied intensively during World War II and shortly thereafter (Philips, 1950). One of the compounds was the endogenous sulfur-containing ion thiosulfate (Philips, 1950), which was shown to increase the survival rate of mice and dogs receiving lethal doses of nitrogen mustard (Hatiboglu et al., 1962). Thiosulfate was probably the first candidate compound to be studied in vivo against cisplatin-induced toxicities, primarily nephrotoxicity, due to its ability to react with cisplatin (Howell and Taetle, 1980; Ishizawa et al., 1981). Studies were performed in which thiosulfate and other sulfur-containing nucleophiles were administered systemically in connection with cisplatin, aiming at inducing differential concentrations of cisplatin in tumor and normal tissues (Howell et al., 1982; Howell and Taetle, 1980; Ishizawa et al., 1981; Kuroiwa et al.,
Otoprotection studies on systemically administered thiosulfate in connection with cisplatin treatment has been conducted without knowing whether thiosulfate reaches the cochlea at all (Church et al., 1995; Kaltenbach et al., 1997; Otto et al., 1988; Saito et al., 1997; Zuur et al., 2007). Today, the knowledge of the complexity of cancer pathogenesis is immensely greater and local administration of the protecting agent in order to selectively reach the inner ear is for most initiated researchers the administration way of choice due to the smaller risk of interfering with the antitumoral activities of systemically administered cisplatin.

A large number of in vivo studies have shown that cisplatin-induced hearing loss may be reduced by antioxidant therapy (Choe et al., 2004; Li et al., 2001; Nader et al., 2010; Saliba et al., 2010; Wang et al., 2003; Wimmer et al., 2004), indicating an important role of oxidative stress. Conveniently, many antioxidants contain sulfur, such as methionine and N-acetyl-L-cysteine, and may thus exert a protective effect also by chemically interacting with cisplatin and/or MHC. In a recent in vivo study, cisplatin-induced otoxicity was reduced by targeting the generation of superoxide through local administration of short interfering RNA inhibiting a cochlear NADPH oxidase, NOX3 (Mukherjea et al., 2010).

Lately, otoprotection studies have often focused on inhibition of specific transporters involved in the cochlear distribution of cisplatin. As discussed previously, cimetidine (Ciarimboli et al., 2010) and copper sulfate (More et al., 2010) might prevent cisplatin-induced otoxicity by interfering with the transport of cisplatin via organic cation transporters and copper transporters, respectively, and/or by chemical interacting with cisplatin and/or MHC. Whether administration of cimetidine locally to the inner ear may reduce cisplatin-induced hearing loss remains to be established.

Administration of a compound directly to the inner ear is not a clinical option due to the risk of damaging aural end structures. Instead, middle ear application can be used for drug delivery to the inner ear. In patients, this can be performed easily by an injection through the tympanic membrane into the tympanic cavity (Figure 4). From there, the drug can distribute to the inner ear, e.g. via the round window membrane (Figure 4) (Hahn et al., 2006; Husmann et al., 1998; Mikulec et al., 2009; Parnes et al., 1999; Saber et al., 2009). Other distribution ways may be involved and the importance of these can be different in different species, complicating the interpretation of the significance of experimental animal data for humans (Mikulec et al., 2009).

A compound administered into the middle ear cavity can be quickly eliminated through the eustachian tube (Laurent et al., 1986). The elimination rate can be reduced by increasing the viscosity of the drug vehicle, e.g. with the aid of a gel (Borden et al., 2010). Viscous formulations are commercially available for ocular application (Doughty and Glavin, 2009), but not for treatment of middle or inner ear pathologies. The high viscosity of the vehicle increases the contact time of the drug with the surrounding tissues (Doughty and Glavin, 2009) and thus the possible distribution to the inner ear when applied in the middle ear cavity (Borden et al., 2010; Hoffer et al., 2001; Husmann et al., 1998). How long elevated cochlear levels of an otoprotector are needed in connection with cisplatin treatment remains to be established. The ototoxic
effects develop gradually over a few days after treatment with cisplatin (Laurell and Bagger-Sjöbäck, 1991a), indicating that less than a week of otoprotector therapy is sufficient.

There are many compounds that can be used to increase the viscosity of a drug formulation. One example is hyaluronan. A major advantage with hyaluronan is that it occurs naturally in the extracellular matrix of practically all human tissues and is already in clinical use, making general toxicological considerations redundant (Fraser et al., 1997).

1.9 QUANTITATIVE DRUG ANALYSIS

When a drug enters the body, it is often metabolized or biotransformed, resulting in a range of either active or inactive metabolites or biotransformation products. The vast majority of the biological studies on cisplatin found in the literature employs analytical methods with poor selectivity, such as atomic absorption spectrometry (Bosch et al., 2008; Riley, 1988), which detect all Pt complexes indiscriminately. Unfortunately, using bioanalytical methods with poor or varying selectivity can confound the results of pharmacokinetic investigations of cisplatin (Ekborn et al., 2003b; Hanada et al., 2001). In order to analyze a drug or metabolite/biotransformation product selectively, liquid chromatography (LC) can be used prior to detection with e.g. atomic absorption spectrometry.

1.9.1 Liquid chromatography

LC is a standard technique for separation of components in a liquid by distribution of the components between two phases, one that is stationary, the column, and one that moves, the mobile phase. For separation of cisplatin in biological samples, the column is often an anion exchange column (Andersson and Ehrsson, 1994; Farrish et al., 1994; Kinoshita et al., 1990; Kizu et al., 1995), i.e. a stationary phase that is predominately positively charged and therefore retains components of the mobile phase that have a high electron density, such as the chlorides of the cisplatin molecule. For isolation of cisplatin in simple aqueous solutions, a porous graphitic carbon (PGC) column can be employed (Ehrsson et al., 1995). The stationary phase of a PGC column is composed of spherical, PGC particles organized in flat sheets of hexagonally arranged carbon atoms. It is compatible with all solvent systems, it is stable throughout the entire pH range 1-14, and it is not affected by aggressive mobile phases (Thermo Scientific, 2007). The retention on a PGC column is achieved by dispersive interactions, by which the retention increases as the hydrophobicity of the analyte increases, and by charge-induced interactions of a polar analyte with the polarizable surface of graphite (Thermo Scientific, 2007).

1.9.2 UV and fluorescence spectrometry

After LC separation, detection of the analyte can be performed with ultraviolet (UV) spectrometry. UV spectrometry is one of the most widely used detection techniques,
and a UV detector is therefore standard equipment in most pharmaceutical laboratories. In UV spectrometric methods, the sample is irradiated with UV light and the power of the beam of light before and after interaction with the sample is compared. The amount of absorbed energy is related to the molecular structure and the concentration of the UV absorbing component(s) of the sample. Often, a molecule with good UV absorbing properties, i.e. with a high molar absorptivity ($\varepsilon$), is aromatic or has multiple conjugated bonds (Christian, 1994). Cisplatin absorbs very little UV light and a UV detection method is therefore not sensitive enough for direct quantification of the low concentrations of cisplatin that are present in biological samples. However, UV detection can be utilized for bioanalysis if the $\varepsilon$ is improved by allowing cisplatin to react with e.g. $N,N$-diethyldithiocarbamate (Figure 7A) (Andersson and Ehrsson, 1994; Andrews et al., 1984; Bannister et al., 1979; Borch et al., 1979; Drummer et al., 1984; Reece et al., 1984).

When a molecule has absorbed UV light, this energy is usually lost as heat, as the molecule is deactivated by collisional processes (Christian, 1994). With certain molecules, some of the energy is lost by photon emission, which is called photoluminescence (Christian, 1994). One type of photoluminescence is fluorescence. Many compounds that are aromatic, heterocyclic, or contain multiple conjugated double bonds fluoresce, especially if they contain electron-donating groups, such as -OH, -NH$_2$, and -OCH$_3$. (Christian, 1994). Similar to UV spectrometry, fluorescence spectrometry can be combined with LC for bioanalysis of drugs, e.g. thiosulfate. Thiosulfate does not fluoresce, but can be conjugated with monobromobimane (Figure 7B) to form a fluorescent derivative (Kosower and Kosower, 1987). Fluorescence can also be used for visualization of OHCs. The hair cells can be made highly fluorescent by utilizing the fluorochrome TRITC (tetramethylrhodamine B isothiocyanate; Figur 7C) attached to phalloidin, a compound with high affinity to actin filaments (Sigma-Aldrich, 2009), which are protein filaments that can be found in the cytoskeleton of e.g. OHCs (Anniko et al., 2001). The fluorescent hair cells can be detected with a fluorescence microscope.
The overall objective of the research presented in this thesis was to contribute to the development of treatment strategies against cisplatin-induced ototoxicity. The specific objectives of the studies presented in each individual paper were:

I. to investigate how quickly the concentration of cisplatin and its active biotransformation product MHC can be reduced in the presence of each of five sulfur-containing compounds aimed for middle ear administration against cisplatin-induced ototoxicity.

II. to investigate the pharmacokinetics of thiosulfate in the perilymphatic compartment and blood after systemic administration in guinea pigs.

III. to investigate the liberation of thiosulfate from a hyaluronan gel formulation in vitro, its pharmacokinetics after middle ear administration of the gel formulation in guinea pigs, and, if promising enough, to investigate if this local administration strategy can prevent cisplatin-induced ototoxicity in a guinea pig model.

IV. to develop a sensitive, robust, and fast LC-UV detection method for selective analysis of intact cisplatin in blood.
3 MATERIALS AND METHODS

For a more detailed description of the materials and methods used in this thesis, please see the materials and methods section of each individual paper (I-IV).

3.1 LABORATORY ANIMALS (PAPERS II AND III)

All experimental animal studies were performed with permission from the local ethical committee for experiments on laboratory animals.

Albino guinea pigs of both sexes from a local breeder were used in the in vivo studies on which Papers II and III are based. They were maintained on a 12:12 hours light/dark cycle with unrestricted access to food and water.

3.2 CHEMICALS (PAPERS I-IV)

In the studies described in Papers I and III, commercially available formulations of cisplatin without the diuretic mannitol were used (Platinol, Bristol-Myers Squibb Company, and Cisplatin Meda, Meda, respectively). In the study described in Paper IV, cisplatin powder was utilized to prepare aqueous solutions of cisplatin with more precise concentrations than that of commercial formulations. Cisplatin powder was also used to prepare MHC (Papers I and IV).

MHC was prepared by dissolving cisplatin in pure water to a concentration of about 4 mM (Papers I and IV). The solution was left to hydrolyze at room temperature for two days. It was then alkalized in order to obtain the less reactive uncharged form of MHC. MHC was isolated by collecting the MHC fraction after separation on a PGC column using sodium hydroxide as mobile phase. For quantification, comparison was made with a reference solution containing 1.1 mM MHC as determined with LC and atomic absorption spectrometry.

Sodium thiosulfate pentahydrate was utilized in the studies described in Papers I and III. In the study described in Paper II, the animals were administered a specially compounded thiosulfate solution containing thiosulfate (0.20 M), sodium ions (0.40 M), and water (Apoteket Production & Laboratories) with an osmolality of 304 mOsm/kg, similar to that of blood.

A thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel was used in the studies on which Paper III is based. It was composed of sodium thiosulfate pentahydrate, sodium hyaluronan (Advanced Medical Optics), and NaCl (9 mg/ml) containing phosphate buffer (~pH 7.2). Its osmolality was about 340 mOsm/kg, thus slightly higher than that of blood and inner ear fluids (Anniko et al., 2001; Sterkers et al., 1984a). A control gel without sodium thiosulfate was also prepared.
N-acetyl-L-cysteine, L-cysteine methyl ester, 1,3-dimethyl-2-thiourea, D-methionine, and sodium thiosulfate were dissolved in pure water immediately prior to each incubation experiment (Paper I).

### 3.3 REACTION OF CISPLATIN AND MHC WITH SULFUR-CONTAINING COMPOUNDS (PAPER I)

In the *in vitro* study described in Paper I, the reaction rates of cisplatin and MHC with five sulfur-containing nucleophiles aimed for middle ear administration against cisplatin-induced ototoxicity were explored. The reaction rate constants were evaluated by following the decrease of the cisplatin and MHC peak areas, respectively, in the presence of a large excess of a specific nucleophile using LC and UV detection. The incubations were performed in HEPES-buffer (10 mM; pH 7.4) at 37 °C. One incubation per analyte was performed at five different concentrations of each nucleophile.

When the nucleophile is in large excess over the Pt compound, the substitution kinetics of square-planar Pt(II) compounds proceed by the general rate law for pseudo first-order kinetics:

\[
A = A_0 e^{-k_{\text{obs}} t} \quad \text{(Equation 1)}
\]

\(A\) is the concentration of the analyte at time \(t\), \(A_0\) is the initial concentration of the analyte, and \(k_{\text{obs}}\) is the pseudo first-order rate constant of the analyte. \(k_{\text{obs}}\) can be expressed by Equation 2:

\[
k_{\text{obs}} = k_1 + k_{Nu} [Nu] \quad \text{(Equation 2)}
\]

\(k_1\) is the sum of the pseudo first-order rate constants of the analyte with the solvent, \(k_{Nu}\) is the second-order rate constant of the analyte with a specific sulfur-containing nucleophile \((Nu)\), and \([Nu]\) is the concentration of that nucleophile. (Dedon and Borch, 1987)

The concentrations of cisplatin and MHC, represented by their peak areas, were plotted *versus* time. The curves were fitted to the equation of one phase exponential decay according to Equation 1. By determining \(k_{\text{obs}}\) at a variety of different nucleophile concentrations, \(k_{\text{obs}}\) can be plotted versus \([Nu]\), and linear regression analysis will yield a straight line with \(k_{Nu}\) being the slope and \(k_1\) the y-intercept according to Equation 2. The slopes were compared using regression analysis.
3.4 ADMINISTRATION OF CISPLATIN AND THIOSULFATE (PAPERS II AND III)

Systemic administration of thiosulfate (0.20 M; 3.3 ml/kg b.w.; mean infusion time: 22 s; Paper II) and cisplatin (1 mg/ml, 8 mg/kg b.w., infusion time: ~3 minutes; Paper III) was performed through a catheter inserted in the internal jugular vein of the animals to ensure a reliable, reproducible, and relevant drug administration.

Local administration of the hyaluronan (0.5% w/w) gel formulation without or with thiosulfate (0.10 M) was performed by injection into the auditory bulla through the skin of the auricle with the aid of a fine needle (mean injection volume: 0.17 ml; BD Microlance 30G; external diameter 0.3 mm; Paper III). Prior to the injection, a small opening was made in the tympanic membrane to prevent rupture of the round window membrane during the injection of the gel. The animals were kept on their stomach with the head in a vertical position between the injection and the aspiration of the gel, which was performed one hour or three hours after the gel injection in the pharmacokinetic study (Paper III) and after three hours in the otoprotection study (Paper III).

3.5 SAMPLING IN VIVO (PAPERS II AND III)

The concentration of thiosulfate (Papers II and III) in the inner ear was evaluated using samples of perilymph aspirated from the scala tympani. Sampling was made through a drilled hole in the basal turn of the cochlea, which was accessed by opening of the bulla of the animal. With the aid of a micromanipulator, a 1 µl syringe was lowered into the drilled hole as quickly as possible to avoid significant leakage of perilymph. Aspiration was performed gently.

Blood (~0.35 ml) was sampled through a catheter inserted in the internal jugular vein (Papers II and III). In Paper II, blood sampling was performed from the vein contralaterally to the vein that was used for drug administration. After each blood sampling, the animals were given an equal volume of NaCl (9 mg/ml) to rinse the catheter and to replace lost fluid.

Samples of perilymph aspirated from the basal turn of the cochlea may be contaminated with cerebrospinal fluid (CSF) (Hara et al., 1989). To estimate how this may have influenced the results of the quantitative analysis of thiosulfate, CSF sampling were performed immediately after each perilymph sampling (Papers II and III). CSF was aspirated from the cisterna magna, using a suboccipital approach. Puncture of the cisterna magna was performed after exposure of the dura mater (Paper II) or percutaneously (Paper III).

In the pharmacokinetic study described in Paper III, samples of the thiosulfate-containing hyaluronan gel formulation was aspirated with a syringe one hour or three hours after middle ear administration of the gel in order to determine the concentration of thiosulfate. The gel was accessed by opening of the bulla of the animal.
3.6 HANDLING AND STORAGE OF CISPLATIN AND MHC (PAPERS I, III, AND IV)

All solutions containing cisplatin (Papers I, III, and IV) and MHC (Papers I and IV) were protected from light.

In the incubation study (Paper I), cisplatin samples were stored on ice until analysis, which was performed the same day. Stock and working solutions of cisplatin were prepared in acidified NaCl (HCl (0.1 M)-NaCl (0.15 M) (1:100, v/v)) and were stored at room temperature (Paper IV). The stock solution was used within two months, whereas working solutions were used within a few hours after their preparation. After spiking of blood with cisplatin, the blood samples were promptly transferred to cold centrifuge tubes (10 kDa cut-off filter; Paper IV). They were kept on ice until subjected to centripetal ultrafiltration (4000 g; 20 minutes; 4 °C) within 30 minutes. The resulting blood ultrafiltrate was rapidly transferred to cold Eppendorf tubes and frozen on dry ice. The tubes were stored at -80 °C until analyzed within approximately three weeks.

Stock solutions of isolated MHC were stored on ice or, if not used within one day, at -80 °C (Papers I and IV). Samples of MHC were quenched with ice-cold sodium hydroxide immediately after collection (Paper I). They were stored on ice for a maximum of 10 minutes, then frozen and stored at -80 °C until analyzed within 24 hours.

3.7 LC ANALYSIS (PAPERS I-IV)

3.7.1 Cisplatin and MHC (Papers I and IV)

Different LC-UV detection methods were employed for preparative, bioanalytical, or non-bioanalytical chromatographic analysis of cisplatin and MHC.

Cisplatin in non-biological samples was separated with a self-packed strong anionic exchanger column of Nucleosil SB with a mobile phase of succinate buffer (55 mM; pH 5.0) and methanol (2:3, v/v) prior to UV detection at 303 nm (Paper I).

In Paper IV, a method for the analysis of cisplatin in blood was developed. Blood samples with cisplatin were ultrafiltrated centripetally prior to injection (loop volume: 5 µl) into the chromatographic system consisting of a PGC column and a mobile phase of HEPES-buffer (20 mM; pH 9.3; flow rate: 0.25 ml/min). Prior to UV detection (wave length: 344 nm), the eluate was mixed on-line with the derivatization agent DDTC (1.3 mM in methanol; flow rate: 0.25 ml/min; Figure 7A). Microwave heating (115 °C) was used to increase the derivatization reaction rate. The instrumental set-up is shown in Figure 8.

Preparative chromatography with a PGC column and a mobile phase of sodium hydroxide (0.5 mM) was employed for isolation of MHC (Papers I and IV). UV detection was performed at 283 nm. When studying the degradation of MHC (Paper I),
MHC was isolated on a PGC column with a mobile phase of sodium hydroxide (1.0 mM) prior to microwave-assisted post-column derivatization with DDTC at 115 °C and UV detection at 344 nm).

Figure 8. Instrumental set-up of the LC-UV detection method used for analysis of cisplatin in blood.

3.7.2 Thiosulfate (Papers II and III)

Thiosulfate in biological and non-biological samples (Papers II and III) was derivatized with the reagent monobromobimane (Figure 7B) prior to LC separation on a self-packed strong anionic exchange column with a mobile phase of succinate buffer (36 mM; pH 5.0) and acetonitrile (2:1, v/v) and fluorescence detection (λex=excitation wave length: 396 nm; emission wave length: 476 nm). The method was adapted from a study found in the literature (Togawa et al., 1992).

3.8 EVALUATION OF HEARING (PAPER III)

In the in vivo studies described in Paper III, normal tympanic membranes were verified otoscopically in all animals before treatment. In the otoprotection study (Paper III), the electrophysiological hearing thresholds of the animals were verified as being normal before treatment by ABR recording. The electrophysiological hearing thresholds were determined by varying the intensity of the stimulus in 5 dB steps around the threshold while observing the resulting curves. The electrophysiological hearing threshold was defined as the lowest level where a reproducible response could be recorded.
3.8.1 Histology

In the otoprotection study described in Paper III, cisplatin-induced ototoxicity was evaluated by counting missing hair cells using surface preparations. The animals were decapitated immediately after euthanization four days after administration of cisplatin. The cochleae were harvested at once. They were perfused with paraformaldehyde (4%) in phosphate-buffered sodium chloride (PBS) in order to preserve the cellular and subcellular architectures via formation of intermolecular bridges (Sigma-Aldrich, 2010). Then, the bone was removed and the remaining modiulus was incubated with phalloidin-TRITC in order to stain the hair cells. Since the staining is not selective to hair cells, as much as possible of the surrounding tissues (bone, Reissner’s membrane, tectorial membrane) must be removed in order to reduce the background noise as much as possible. With the aid of a fluorescence microscope, missing hair cells were counted manually. Counting of intact hair cells is unnecessary, since the amount of hair cells in the guinea pig cochlea is already well documented.

3.9 STATISTICS

To compare more than two groups, Kruskal-Wallis and Friedman tests were employed for independent and dependent groups, respectively (Paper III). Dunn’s Multiple Comparison Test was employed to see which groups differed from which other groups. For additional information of the statistical calculations, please see the data analysis and statistics sections of each individual paper.
4 RESULTS AND DISCUSSION

For a more detailed description of the results of each individual paper, please see the results section of Papers I-IV.

Hearing loss due to cisplatin treatment is an important clinical problem, since it limits the use of the drug (de Jongh et al., 2003; Ekborn et al., 2004). The most well-established risk factors for developing cisplatin-induced hearing loss are high single (Laurell and Jungnelius, 1990) and cumulative cisplatin dose levels (Coupland et al., 1991; Laurell and Jungnelius, 1990; Oldenburg et al., 2007; Schaefer et al., 1985).

When accounting for this and less solid risk factors, such as younger age in pediatric patients (Coupland et al., 1991) and radiation to the head concomitantly with the cisplatin therapy (Coupland et al., 1991; Kolinsky et al., 2010; Miettinen et al., 1997), the interindividual variability of the ototoxic effects is still large (Coupland et al., 1991; Ekborn et al., 2004; Laurell and Jungnelius, 1990; Miettinen et al., 1997; Schaefer et al., 1985). There is obviously a need for an otoprotective treatment to be used in cisplatin-treated patients. Today, local otoprotection therapy appears to be the most promising clinical alternative in order to avoid interfering with the antitumoral activity of systemically administered cisplatin.

Many studies in which experimental animals have been exposed to presumptive otoprotector agents in conjunction with cisplatin are described in the literature. Most of the otoprotector candidates have been sulfur-containing antioxidants intended to reduce the cisplatin-induced oxidative stress in the cochlea either directly or indirectly by forming less ototoxic complexes with cisplatin and its active biotransformation product MHC (Choe et al., 2004; Li et al., 2001; Nader et al., 2010; Saliba et al., 2010; Wang et al., 2003; Wimmer et al., 2004). The recent discovery of the involvement of specific transporters in the distribution of cisplatin to the inner ear has turned much of the research in a new direction, with hopes of salvation by targeting e.g. organic cation transporters (Ciarimboli et al., 2010) or copper transporters (More et al., 2010).

![Figure 9](image1.png)  
**Figure 9.** The molecular formulas of N-acetyl-L-cysteine (A), L-cysteine methyl ester (B), 1,3-dimethyl-2-thiourea (C), D-methionine (D), and thiosulfate (E) at pH 7.4.

Most of the research described in this thesis has focused on sulfur-containing nucleophiles as candidate drugs against cisplatin-induced ototoxicity. The idea to use nucleophiles as otoprotector agents is highly attractive since it does not require detailed
knowledge about the mechanisms behind the damaging effects in the inner ear; suffice is to know that cisplatin reaches the inner ear. In the in vitro study described in Paper I, the rates of the disappearance of cisplatin and MHC in the presence of each of five sulfur-containing nucleophiles aimed for local otoprotective administration were explored. Figure 9 shows the molecular formulas of the nucleophiles at pH 7.4. The pKa values are given in Table 1.

The concentration of MHC decreased rapidly in thiosulfate-containing solutions; the $k_{Nu}$ was 3.9 M$^{-1}$s$^{-1}$, much higher than the other rate constants (Table 1). As expected from the discussion in the introduction section, the $k_{Nu}$ of MHC were in most cases higher than that of cisplatin. The $k_{Nu}$ of cisplatin were similar with all nucleophiles studied with the exception of N-acetyl-L-cysteine, which reacted slowly with both cisplatin and MHC (Table 1).

<table>
<thead>
<tr>
<th>N-Acetyl-L-cysteine</th>
<th>L-Cysteine methyl ester</th>
<th>1,3-Dimethyl-2-thiourea</th>
<th>D-Methionine</th>
<th>Thiosulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>1.2± 0.18</td>
<td>6.7± 0.51</td>
<td>7.4± 0.47</td>
<td>6.9± 0.83</td>
</tr>
<tr>
<td>MHC</td>
<td>4.4± 1.4</td>
<td>24± 1.8</td>
<td>16± 0.46</td>
<td>7.0± 0.39</td>
</tr>
<tr>
<td>pKa</td>
<td>1.7 (COOH)$^{-1}$</td>
<td>8.4 (NH$_3^+$)$^{-1}$</td>
<td>2.7 (COOH)$^{-1}$</td>
<td>0.6$^1$</td>
</tr>
</tbody>
</table>

*pKa values for l-cysteine.

1(Aylward and Findlay, 1994)

The existence of MHC in the inner ear remains to be demonstrated. However, its presence is very likely since the chloride concentrations and pH of perilymph and endolymph are similar to that of blood (Sterkers et al., 1988; Sterkers et al., 1984b), in which MHC has been detected in cisplatin-treated subjects (Andersson et al., 1996; Ekborn et al., 2002; Ekborn et al., 2003b). MHC has been found to be about twice as ototoxic as cisplatin when administered to guinea pigs (Ekborn et al., 2003b). There are indications of a role of MHC in ototoxicity induced also by administration of cisplatin. First, knockout of the cation transporter OCT2 protected against cisplatin-induced ototoxicity in mice (Ciaramboli et al., 2010), possibly caused by a decreased transport of the cation monoaqua monochloro cisplatin complex, the acid form of MHC. Second, application of alkaline (pH 10.2 and pH 9.0) PBS to the round window reduced cisplatin-induced ototoxicity in two experimental animal studies, whereas application of acidic (pH 6.5 and pH 6.0) PBS exacerbated it (Tanaka et al., 2003; Tanaka et al., 2004). These results suggest that MHC may be involved in the ototoxic effects, since its
reactivity is lower above pH 7.4 than below (Atema et al., 1993; Herman et al., 1988; Murakami et al., 2001) due to its pKa of 6.6 (Andersson et al., 1994). However, it can not be excluded that the ototoxicity of cisplatin in those studies was altered by pH-effects on the charges of endogenous protective compounds, resulting in e.g. deprotonation of sulfhydryl groups at alkaline pH, which increases their nucleophilicity and thus reactivity with cisplatin and/or MHC.

An important advantage with thiosulfate is that it is an endogenous ion, it is already used clinically (Koschel, 2006), and it is well tolerated even in very high doses (Ivankovich et al., 1983; Neuwelt et al., 1998; Shea et al., 1984). Moreover, it appears to have an antioxidative role (Chauncey et al., 1987) and its systemic application has been studied extensively both in vitro (Abe et al., 1986; Dickey et al., 2005; Elferink et al., 1986; Harned et al., 2008; Kovacs and Cinatl, 2002; Muldoon et al., 2001), in experimental animals (Aamdal et al., 1988; Church et al., 1995; Dickey et al., 2005; Harned et al., 2008; Inoue et al., 1991; Kaltenbach et al., 1997; Otto et al., 1988; Saito et al., 1997; Zheng et al., 1997), and in humans (Goel et al., 1989; Howell et al., 1982; Markman et al., 1985; Pfeifle et al., 1985; Robbins et al., 2000; Rohde et al., 2005; Shea et al., 1984; Tegeder et al., 2003; Zanon et al., 2004; Zuur et al., 2007) in order to find a way to reduce cisplatin-induced side-effects. None of these studies have investigated the distribution of thiosulfate to the inner ear. Therefore, the kinetics of thiosulfate in blood and perilymph of the scala tympani after i.v. administration was investigated using the guinea pig as an in vivo model (Paper II). The results showed that thiosulfate was rapidly and extensively distributed to perilymph of the scala tympani (Figure 10). The median AUC for the entire sampling period was 6300 µM×min and 3100 µM×min in blood and perilymph of the scala tympani, respectively. The transport mechanisms remain to be elucidated. Interestingly, many known ototoxic substances are organic acids (Rybak, 1987).

About 15 minutes after i.v. administration of thiosulfate, the concentration of thiosulfate in the blood sample with the highest concentration was 360 µM (Figure 10). If using the $k_{Nu}$ with thiosulfate given in Table 1, the half-lives of cisplatin and MHC can be calculated to 5.9 hours and 8.2 minutes, respectively, at 37 ºC and pH 7.4. These calculations assume that the concentration of thiosulfate was at least ten times higher than that of cisplatin and MHC, which is not unlikely considering what prevously has been found in guinea pigs (Ekborn et al., 2000). Thus, there is a risk that the concentration of MHC in the blood compartment is reduced by i.v. administration of thiosulfate concomitantly with that of systemic cisplatin administration, which may lead to decreased antitumoral effects.

The elimination of thiosulfate from perilymph of the scala tympani was slower than from blood, resulting in higher concentrations of thiosulfate in perilymph than in blood at the end of the observation period (Figure 10). This suggests that systemic administration of thiosulfate several hours prior to that of cisplatin may be an alternative way to obtain otoprotection without compromising the antitumoral effects. However, this strategy is risky, as mentioned in the introduction section, since the pharmacokinetics of thiosulfate in cancer tissues is impossible to predict given the complex pathogenesis of the disease.
Can the problem with systemic interaction with cisplatin be circumvented by local administration of thiosulfate to the middle ear cavity? An effort to answer this question was made in the studies on which Paper III is based. To increase the residence time of the thiosulfate formulation in the middle ear cavity and thereby the chance of distribution of thiosulfate to the inner ear, the endogenous polymer hyaluronan was used as a viscosity-enhancer of the formulation. The results showed that \textit{in vitro}, all thiosulfate had left the formulation after a few hours; the mean diffusion coefficient ± standard deviation of thiosulfate was $9.57 \cdot 10^{-6} \pm 0.21 \cdot 10^{-6}$ cm$^2$×s$^{-1}$.

\textbf{Figure 10.} Concentrations of thiosulfate in blood (closed circles) and perilymph of the scala tympani (open circles) of guinea pigs after a bolus injection of thiosulfate (0.20 M; mean injection volume: 1.28 ml, i.v.). Each symbol represents one sample. The lines connect the median concentration of thiosulfate at each sampling occasion. Thiosulfate was quantified with LC and fluorescence detection as described in the materials and methods section.
The situation was quite different in the *in vivo* model, as shown in a pharmacokinetic investigation. Guinea pigs were administered the thiosulfate-containing hyaluronan gel formulation intratympanically. After one hour (1-h gel group) or three hours (3-h gel group), the gel was removed and its thiosulfate content was quantified. In both groups, most thiosulfate was remaining in the gel; the median concentrations of thiosulfate were 98.5 mM (range 31.0 mM-121 mM, n=8) and 89.6 mM (range 75.2 mM-107 mM, n=6) in the 1-h and 3-h gel groups, respectively. The difference between the two groups was not statistically significant. Neither was there a statistically significant difference in the concentrations of thiosulfate in perilymph of the scala tympani from the 1-h gel group, the 3-h gel group (Paper III), and the group treated with thiosulfate.

**Figure 11.** Concentrations of thiosulfate in perilymph of the scala tympani of guinea pigs after a bolus injection of thiosulfate i.v. (0.20 M; mean injection volume: 1.28 ml; filled triangles) or intratympanically (0.10 M in a hyaluronan (0.5% w/w) gel; mean injection volume: 0.17 ml; open and closed circles). The gel was removed after one hour (closed circles) or three hours (open circles) prior to perilymph sampling. Each circle and triangle represents one sample. The thiosulfate concentrations in perilymph were statistically higher (indicated with a star) in the gel groups compared to the i.v. group 120 and 180 minutes after the i.v. administration. The broken lines connect the median concentrations of thiosulfate at each sampling occasion in the i.v. group as well as in the gel groups.
i.v. (Paper II) are given in Figure 11. The results showed that by using a local administration strategy (Paper III), higher concentrations of thiosulfate in perilymph of the scala tympani was reached than after i.v. administration (Paper II), despite the fact that the amount of thiosulfate given locally was only 7% of the i.v. dose. Moreover, it appeared that the high concentrations of thiosulfate in perilymph of gel-treated guinea pigs maintained for a prolonged period of time, whereas a rapid decrease was seen in i.v. treated guinea pigs.

What about the concentrations of thiosulfate in blood when using the middle ear administration strategy described in Paper III, which was the main question to be answered? The results are shown in Figure 12. The concentrations of thiosulfate were about the same as those found in blood three hours after i.v. administration of thiosulfate (comparing Figures 12A and 12B to Figure 10). There was no statistically significant difference between the 1-h gel group and the 3-h gel group. Neither was there a trend of successively increasing concentrations of thiosulfate within the two groups. The reason for the large inter- and intraindividual variability of the data shown
in Figures 12A and 12B remains to be explored. In most cases, the concentrations of thiosulfate in the gel groups were as low as what has been found previously in guinea pigs treated with NaCl (9 mg/ml) i.v (Paper II), which is indicated with solid horizontal lines in Figures 12A and 12B. However, in some cases, the concentrations were much higher and reached 15 µM in the most extreme case. If using the $k_{\text{Nu}}$ of MHC with thiosulfate given in Table 1, the half-life of MHC with 15 µM thiosulfate can be calculated to 3.3 hours at 37 °C and pH 7.4, if assuming that the thiosulfate concentration was at least ten times higher than that of MHC, which is not unlikely (Ekborn et al., 2000). Thus, the risk of decreased anticancer effects due to inactivation of cisplatin and MHC in the blood compartment seem negligible when using the local administration strategy described in Paper III. However, the pH in tumor tissues can be significantly lower than 7.4 (Tannock and Rotin, 1989), which will reduce the half-life of MHC due to an equilibration shift towards the more reactive protonated form of MHC.

In conclusion, the results obtained in the studies presented in Papers I, II, and III supported our hypothesis that by using a local administration strategy for thiosulfate (Paper III) instead of i.v. administration (Paper II), high concentrations of thiosulfate could be obtained in perilymph of the scala tympani (Figure 11) while low concentrations in blood were maintained (Figure 12). Moreover, the local administration strategy resulted in higher concentrations of thiosulfate in perilymph of the scala tympani than i.v. administration, and it seemed to offer a continuous distribution of thiosulfate to the inner ear (Figure 11). However, the most fundamental question is unanswered: can cisplatin-induced ototoxicity be prevented by using the local administration strategy for thiosulfate described in Paper III?

![Figure 13](image-url)

Figure 13. Cytocochleograms for cisplatin-treated guinea pigs (8 mg/kg b.w., i.v.) showing loss of outer hair cells (OHCs) in control ears (A) and ears treated with a thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel (B). The gel was injected into the middle ear cavity three hours prior to administration of cisplatin. Inner hair cells (IHCs) are represented by open triangles and OHCs in the first row by filled circles, in the second row by open circles, and in the third row by closed triangles. Data are expressed as median values.
In the final study described in Paper III, we explored the efficacy of thiosulfate as a protector against cisplatin-induced ototoxicity when using the thiosulfate-containing hyaluronan gel formulation administered to the middle ear cavity in a guinea pig model. As a control, a gel formulation without thiosulfate was administered to the contralateral middle ear cavity. The pharmacokinetic investigation described in Paper III had shown that the concentrations of thiosulfate in perilymph of the scala tympani did not differ between the 1-h and 3-h gel groups. However, since the samples of perilymph with the lowest concentrations of thiosulfate were higher in the 3-h gel group than in the 1-h gel group, an exposure time of three hours was used in the otoprotection study. Thus, three hours after middle ear application of the thiosulfate-containing hyaluronan gel formulation, the guinea pigs were administered cisplatin i.v. Four days later, they were killed, the cochleae were harvested, and the possible otoprotection was evaluated by counting missing hair cells using surface preparations. The results for control and thiosulfate-treated ears are shown in Figures 13A and 13B, respectively.

The difference in OHC loss between the control and thiosulfate-treated ears of each animal was calculated. The median values of these differences are shown in Figure 14. The differences were statistically significant in the basal turn of the cochlea where the
approximate 95% confidence interval (CI) did not overlap y=0. Thus, middle ear application of thiosulfate (0.10 M) in a hyaluronan (0.5% w/w) gel formulation three hours prior to cisplatin administration (8 mg/kg b.w., i.v.) protected against ototoxicity in guinea pigs. It remains to be established whether this local administration strategy can prevent cisplatin-induced hearing loss in humans as well.

Animal models play a crucial role in the development of otoprotective drugs. The *in vivo* experiments described in Papers II and III rely on sampling techniques and histopathological investigations that are invasive and technically not possible in humans. However, interspecies differences may complicate extrapolation of the results found in experimental animals to humans. For example, the passage of drugs from the middle ear to the inner ear via the round window membrane can be hindered in the human by the presence of extraneous membranes or by fibrous or fat tissue plugs. These obstructions are reported to occur singly or in combination in about one third of human temporal bones (Alzamil and Linthicum, 2000). Such obstructions were never found in the guinea pigs used in the studies described in Papers II and III. The distribution way(s) to the cochlear fluids of a drug applied in the middle ear cavity may also differ between species. For example, a pharmacological substance can reach the perilymph through the bone of the cochlea in the guinea pig, which is less likely to occur in humans, who have a much thicker bone (Mikulec et al., 2009).

Animal studies are often performed in rodents. Guinea pigs have been used for a long time in hearing research. It has a large cochlea (Thorne et al., 1999) that is accessible for both physiological and histological studies as most of it is protruding in the middle ear. Early in the clinical history of cisplatin, it was shown that cisplatin induces a cumulative ototoxicity in the guinea pig progressing from high to low frequencies (Fleischman et al., 1975). It was also found that the latency for hearing loss was inversely dose-related (Fleischman et al., 1975). The surface preparations revealed that cisplatin caused hair cell loss in the organ of Corti, in particular in the lower turns of the cochlea (Fleischman et al., 1975). The guinea pig has become one of the most employed animal species in studies on cisplatin-induced ototoxicity (eg. (Choe et al., 2004; Ekborn et al., 2000; Ekborn et al., 2003a; Ekborn et al., 2002; Ekborn et al., 2003b; Hellberg et al., 2009; Nader et al., 2010; Otto et al., 1988; Saliba et al., 2010). Compared to other rodents, the guinea pig is more sensitive to the ototoxic effects of cisplatin (Poirrier et al., 2010; Sockalingam et al., 2000), which is essential in order to keep the mortality rate as low as possible. Due to the lack of pigmented guinea pigs, albinos were used in the *in vivo* studies described in Papers II and III. Albino guinea pigs are reported to be even more sensitive to the ototoxic effects of cisplatin than pigmented guinea pigs (Schweitzer, 1993). Albino animals have a normal distribution of melanocytes, but essentially no melanin pigment is formed (Tolleson, 2005). Some functions that are associated with melanin are sequestering reactive oxygen species, metal ions, and organic as well as inorganic cations, which are believed to provide protective effects for melanocytic tissues, such as the stria vascularis in the cochlea (Figure 5) (Tolleson, 2005). Interestingly, in pigmented guinea pigs, cisplatin caused a lower density of melanin content in the stria vascularis in the basal part of the cochlea, whereas there were no changes in melanin content in the middle or apical cochlear regions (Laurell et al., 2007).
A cisplatin dose of 8 mg/kg b.w. i.v. was used in the otoprotection study described in Paper III, since it has induced ototoxic effects without unacceptably high deterioration of the general condition of the guinea pigs in several previous studies performed by members of our group (Ekborn et al., 2000; Ekborn et al., 2003a; Ekborn et al., 2002; Ekborn et al., 2003b; Laurell and Bagger-Sjöbäck, 1991a; Laurell and Bagger-Sjöbäck, 1991b; Laurell and Engström, 1989). In cancer patients, cisplatin is most often infused i.v. over at least one hour (e.g. (de Jongh et al., 2003; Ekborn et al., 2004)). In a guinea pig study comparing short time infusion (15 s) of cisplatin (8 mg/kg b.w., i.v.) with one hour infusion, it was found that the interindividual variability in the susceptibility to ototoxicity was far greater than the variability in pharmacokinetics (Ekborn et al., 2000).

In most animal studies on cisplatin-induced ototoxicity, cisplatin is not administered i.v. but intraperinoneally (i.p.) by a single injection (e.g. (Blair et al., 2010; Ciarimboli et al., 2010; Sockalingam et al., 2000) or by multiple injections (e.g. (Choe et al., 2004; Church et al., 1995; Nader et al., 2010; Saliba et al., 2010). An injection i.p. is very easy to perform and to repeat compared to i.v. However, i.p. injections have been compared to injections into a black box; there are absorption, tolerance, and misplacement issues to be taken into consideration (Svendsen, 2005). The i.p. injection is made through the abdominal wall into the peritoneal cavity, which is a potential, rather than an actual, cavity, since the abdominal contents occupy the space. There is, therefore, a risk of inadvertent administration into the urinary bladder, intestine, caecum or other organs, or into fat or muscle (Gaines Das and North, 2007). One of the major consequences of i.p. injection failure may be a substantial increase in the apparent variability of the measured responses resulting in an increased requirement of number of animals to achieve the power desired (Gaines Das and North, 2007). Since there is already a large interindividual variability in the sensibility to cisplatin-induced ototoxicity (Coupland et al., 1991; Ekborn et al., 2004; Ekborn et al., 2000; Ekborn et al., 2002; Laurell and Bagger-Sjöbäck, 1991a; Laurell and Jungnelius, 1990; Miettinen et al., 1997; Schaefer et al., 1985; Sockalingam et al., 2000), i.v. administration is to prefer. In the otoprotection study described in Paper III, the guinea pigs were their own controls, which reduce the influence of interindividual variability.

In all in vivo studies presented in this thesis, the concentration of thiosulfate in the inner ear was quantified based on the levels in perilymph of the scala tympani (Figure 5) aspirated with a 1 µl syringe from the basal turn of the cochlea (Papers II and III). This technique was employed since it is well established in our research group. Compared to sampling from scala vestibuli or scala media (Figure 5), sampling from scala tympani at the cochlear base is more feasible. One drawback is the risk of contamination with cerebrospinal fluid (CNS) (Salt et al., 2003); the cochlear aqueduct, which connects the inner ear with the CNS, is located near the round window at the base of the cochlea (Ghiz et al., 2001). Therefore, it is essential to sample not too close to the round window, to sample quickly to avoid spillover of perilymph, and to sample a small volume (Hara et al., 1989). When aspirating 1 µl as described in Papers II and III, approximately 20% of the sample is expected to consist of CSF (Hara et al., 1989). Therefore, the concentration of thiosulfate in CSF was also determined to verify that contamination with CSF would not lead to overestimation of the concentrations of
thiosulfate in perilymph of the scala tympani. A second drawback with the perilymph aspiration technique is that it is impossible to discover accidental aspiration of air. Only when transferring the perilymph sample to a vial was it practicable to make a rough ocular estimation of the sample volume. However, differences in sample volumes were never accounted for when performing the quantitative analysis, unless the syringe was empty. On those few occasions, the sampling process was repeated, the second time always with success. In order to better control the sample volume, sampling with a small capillary instead of a syringe may possibly be an alternative method in future studies. A capillary is used when sampling perilymph from the apex of the cochlea (Salt et al., 2006). Apical sampling reduces the risk of contamination with CSF from the cochlear aqueduct (Salt et al., 2006). A disadvantage of the apical sampling technique is that it is more traumatic to the animals, since it requires more extensive surgery. Our research group has recently performed a comparative study of the two sampling techniques for determination of the concentration of cisplatin (Hellberg et al., 2010). The results will show which technique fits the best with our guinea pig model of cisplatin-induced ototoxicity.

ABR recordings were performed to obtain the normal electrophysiological hearing thresholds of the animals before treatment (Paper III). The guinea pigs were anesthetized with ketamine and xylazine during all the recordings. The method has been employed in several studies on guinea pigs by members of our group (e.g. (Ekborn et al., 2000; Ekborn et al., 2003a; Ekborn et al., 2002; Ekborn et al., 2003b; Hellberg et al., 2009). Anesthetics can have important effects on the auditory response (Harel et al, 1997), but this seems not to be the case for the combination of ketamine and xylazine (Goss-Sampson and Kriss, 1991; Smith and Mills, 1989; Smith and Mills, 1991).

Being composed of entirely endogenous species, the thiosulfate-containing hyaluronan gel used in the study described in Paper III is attractive. However, the transient conductive hearing loss and possible discomfort that follow the treatment will most certainly have a negative impact on the quality of life of the patient. A drawback with hyaluronan is that it is probably hard to control its elimination from the middle ear cavity. Preliminary data from guinea pigs indicate that it might reside in the middle ear for at least a week (Engmér Berglin, 2010), which is probably longer than necessary when used as a vehicle for a protector drug against cisplatin-induced ototoxicity. In that respect, other viscosity-enhancing compounds may appear more convenient than hyaluronan, such as “environmentally responsive” or “smart” gels, the elimination of which can be controlled by an external stimulus. For example, by using an intratympanic injection of an alkaline solution, the pH of such a “smart” gel residing in the middle ear cavity can be altered, which will lead to a greatly reduced viscosity and thereby most likely an increased elimination rate. A major obstacle with this approach is toxicity. Since the cisplatin-treated patient “only” runs the risk of developing hearing loss, there is a very low acceptance for an otoprotection therapy with other side-effects than a transient conductive hearing loss. Even if a completely safe otoprotection formulation is found, the transient conductive hearing loss may lead to a reduced compliance. Such compliance issues can be complicated to resolve, since the fact that a
patient forgoes the local otoprotection therapy does not mean that his or hers perception of hearing will surely be affected by the ototoxic effects of cisplatin.

When using middle ear application of a drug formulation, defining the formulation as much as possible is important in order to avoid confounding factors when interpreting the results. The pH of the formulation may affect the ototoxicity of cisplatin, as discussed previously (Tanaka et al., 2003; Tanaka et al., 2004). Furthermore, the osmolality of the formulation may influence the permeability of the round window membrane, which can lead to increased permeability when the osmolality is higher than that of the perilymph (Mikulec et al., 2008). Moreover, if the osmolality of the perilymph is altered, e.g. by using a formulation that is far from isoosmotic to the perilymph, the cochlear function can be affected (Choi and Oghalai, 2008). The osmolality was also considered when designing the study described in Paper II, which is why a thiosulfate solution with an osmolality similar to that of blood was used. Several studies can be found in the literature where much higher thiosulfate doses have been administered to guinea pigs by use of hypertonic solutions (Leitao and Blakley, 2003; Otto et al., 1988). However, increasing the osmolarity of the blood can alter the composition of the inner ear fluids (Juhn et al., 1976; Ueda et al., 1987).

![Figure 15](image_url)

**Figure 15.** Unacceptably high baseline noise and drifting were frequent problems when developing a LC-UV detection method for the selective analysis of cisplatin in blood.

Little is known about the importance of different pharmacokinetic parameters of cisplatin for the ototoxic effects of cisplatin, since few studies have been performed with analysis methods that are selective for cisplatin (Andersson et al., 1996; Ekborn et al., 2004). Unselective bioanalytical methods can confound the results of pharmacokinetic investigations of cisplatin (Ekborn et al., 2003b; Hanada et al., 2001). The initial aim of the study described in Paper IV was to develop a selective method for simultaneous determination of cisplatin and MHC using LC separation, post-column derivatization, and UV detection. The efforts turned out unsuccessful. One problem was the divergent
reactivity of cisplatin and MHC with the derivatization agent DDTC (data not shown). Typically, the derivatization of cisplatin was initially slower than that of MHC. When the appropriate derivatization conditions for cisplatin were finally found, these proved to be less suitable for MHC. At the same time, the options to optimize the post-column derivatization conditions were limited by the chromatographic conditions. Using a pre-column derivatization method was not an alternative, since the derivatives of cisplatin with DDTC are similar to those of MHC (Andersson and Ehrsson, 1994) and other low molecular weight Pt-containing molecules, e.g Pt-glutathione (Dedon and Borch, 1987) and Pt-methionine (Andrews et al., 1984).

After having turned the focus to bioanalysis of cisplatin only, there were still some major obstacles to overcome. Often, the efforts to find appropriate analysis conditions ended in a baseline appearance as exemplified in Figure 15, i.e. with an unacceptably high noise level.

The first critical step towards the final success was to switch the buffer component of the mobile phase from succinic acid to HEPES. Use of the former caused problems with precipitations, resulting in irregular pressure and baseline noise. The second and final critical step was to start to elute with phosphoric acid at the end of a day’s run in order to destroy remains of DDTC in the system. These remains caused baseline noise and
drifting, which were particularly troublesome when continuously altering the LC-UV detection conditions.

The end result turned out sensitive, robust, and reproducible. Cisplatin eluted as a Pt-DDTC complex after approximately 11.8 min, as seen in Figure 16 (upper curve), which shows the elution profile of a blood sample with a cisplatin concentration of $2.5 \times 10^{-7}$ M, the lowest concentration of the study. For comparison, the elution profile of the blood sample matrix is also shown (Figure 16, lower curve). The injection loop volume of the method is only 5 µl, which means that sample volumes can be very small. This may be of fundamental importance when performing pharmacokinetic studies on pediatric cancer patients and experimental animals. However, a blood sample volume of at least 200 µl is usually required when employing the technique of centripetal ultrafiltration prior to the analysis.

The peak area of the cisplatin-DDTC complex increased when the hematocrit was increased, which is illustrated by a graph of the slopes of the linear regression line versus the hematocrit (Figure 17). This agrees with the results of a previous cisplatin study (Andersson et al., 1996).

The sensitivity of the method described in Paper IV is high enough to allow quantification of cisplatin in blood samples from cisplatin-treated patients and...
experimental animals, although only demonstrated in blood samples spiked with cisplatin. Therefore, the method will be a valuable instrument in future pharmacokinetic studies of the drug. Unfortunately, MHC eluted after approximately 8.8 min (data not shown) and was impossible to analyze in blood ultrafiltrate with the present method due to interfering endogenous peaks (Figure 16). However, this can be done if altering the pH of the mobile phase to 8.2 (Wallin, 2010), although that method remains to be validated.

A pre-column derivatization method was employed for quantification of thiosulfate (Papers II and III). The method was adapted from a study found in the literature (Togawa et al., 1992). One important modification of the method was to perform the derivatization reaction at lower pH, pH 5. The derivatization reagent monobromobimane (Figure 7B) reacts primarily with thiolate ions, but can also react with other nucleophiles, e.g. amines, carboxylates, and phosphate (Kosower and Kosower, 1987). At pH 5, many of the competing nucleophiles, e.g. the sulfur atom of cysteine (pKa 10.8 (Aylward and Findlay, 1994)), are uncharged and less prone to react with monobromobimane, while thiosulfate is still highly reactive due to its low pKa (Table 1). Moreover, amines are largely in the ammonium ion form at pH 5, which reduces their reactivity with monobromobimane (Fahey and Newton, 1987; Kosower and Kosower, 1987). However, some interference may occur even with weak nucleophiles when present in high concentrations, which may be the case of buffer ions (Fahey and Newton, 1987; Kosower and Kosower, 1987).

Correct handling of cisplatin- and MHC-containing solutions is important considering the high and broad reactivity of the species. The solutions should be protected from light, since UV light has been shown to increase the degradation rate of cisplatin (Zieske et al., 1991). The stability of cisplatin and MHC in blood samples has been investigated in a previous study (Andersson and Ehrsson, 1995). The results showed that the mean half-lives of cisplatin and MHC in blood samples were 1.43 hours and 0.36 hours, respectively, at 37 °C and pH 7.4 (Andersson and Ehrsson, 1995). At 0 °C, cisplatin was stable for at least four hours, whereas the mean half-life of MHC was 8.8 hours (Andersson and Ehrsson, 1995). Intriguingly, the mean half-lives of cisplatin in plasma and plasma ultrafiltrate at -25 °C were 52 hours and 31 days, respectively (Andersson and Ehrsson, 1995). Storage of cisplatin ultrafiltrate samples for three weeks at -80 °C results in insignificant degradation (Wallin, 2010).

Stock and working solutions of cisplatin were prepared in saline that was acidified (Paper IV), since it has been shown to decrease the degradation rate of cisplatin (Zieske et al., 1991). Another important aspect to consider is the low solubility of Pt species (Cleare and Hoeschele, 1973). To reduce the risk of precipitation, strong solutions of cisplatin were stored at room temperature (Paper IV).
5 CONCLUSIONS

1. Thiosulfate decreased the concentrations of cisplatin and, in particular, MHC rapidly \textit{in vitro} (Paper I). Thiosulfate is an endogenous ion, is well tolerated in experimental animals and humans, and has been used clinically for decades against cyanide poisoning. Therefore, thiosulfate was considered an interesting candidate compound for further studies \textit{in vivo} against cisplatin-induced ototoxicity, which has been suggested to be caused by high concentrations of cisplatin and MHC in the cochlea.

2. Thiosulfate was rapidly and extensively distributed to the perilymphatic compartment of the cochlea after an i.v. bolus injection in guinea pigs (Paper II). Its elimination from perilymph was slower than from blood, resulting in higher concentrations in perilymph at the end of the observation period, three hours after the injection (Paper II). However, systemic administration of thiosulfate against cisplatin-induced ototoxicity is risky since it may reduce the antineoplastic effects of the systemic cisplatin therapy by decreasing the concentrations of cisplatin and MHC in tumor tissues and not only in the cochlea.

3. Hyaluronan did not inhibit the release of thiosulfate as shown \textit{in vitro} (Paper III).

4. Almost all thiosulfate remained in the gel vehicle three hours after administration of a thiosulfate-containing hyaluronan gel formulation into the middle ear cavity of guinea pigs (Paper III), suggesting that a sustained-release formulation is not necessary in order to maintain high concentrations of thiosulfate in the middle ear cavity for a prolonged period of time.

5. Compared to i.v. administration (Paper II), local administration of thiosulfate into the middle ear cavity by using the thiosulfate-containing hyaluronan gel formulation resulted in higher concentrations of thiosulfate in perilymph while low concentrations in blood were maintained (Paper III), indicating a low risk of interfering with the antitumoral effects of systemic cisplatin therapy.

6. Compared to i.v. administration (Paper II), local administration of thiosulfate into the middle ear cavity by using the thiosulfate-containing hyaluronan gel formulation seemed to offer a continuous distribution of thiosulfate to the perilymphatic compartment of the cochlea (Paper III). This may be of importance in order to prevent cisplatin-induced ototoxicity, since the ototoxicity develops progressively during the days following the cisplatin administration.

7. Middle ear administration of thiosulfate by using the thiosulfate-containing hyaluronan gel formulation prevented cisplatin-induced OHC loss in a guinea
pig model (Paper III). It remains to be investigated whether this local administration strategy for thiosulfate can prevent cisplatin-induced ototoxicity also in humans, in which the distribution to the perilymphatic compartment may be hindered by a thicker cochlear bone and by the presence of extraneous membranes or fibrous or fat tissue plugs.

8. A method for selective, sensitive, and fast quantification of cisplatin in blood was developed (Paper IV). It consists of separation with centripetal ultrafiltration and a PGC column prior to on-line microwave-assisted derivatization with DDTC and UV detection. The injection loop volume of 5 µl means that sample volumes can be very small. The method will be a valuable tool in future studies aiming at exploring the pharmacokinetics of cisplatin.
6 POPULÄRVETENSKAPLIG SAMMANFATTNING


Ämnen som innehåller svavel är heta kandidatläkemedel mot hörselskada orsakad av cisplatin, eftersom de lätt reagerar med cisplain och MHC och därmed möjligen kan minska mängden av dessa i hörselsnäckan. En del av svavelämnen kan kanske också skydda mot hörselskada genom minska den oxidativa stressen. Målet med avhandlingens första studie (Paper I) var att undersöka hur snabbt mängden cisplatin och MHC minskar i närvaro av fem svavelinnehållande ämnen i provrörs. Resultaten visade att tiosulfat var en lovande kandidat för framtid studier på försöksdjur, eftersom det reagerade snabbt med cisplatin och, i synnerhet, MHC. Denna slutsats stöddes också av att tiosulfat är ett kroppet ämne med få biverkningar även i höga halter och att det i flera decennier har använts i människa för att behandla bl.a. cyanidförgiftning.

Systemisk (till skillnad från lokal) tillförsel av tiosulfat har undersöks i flera tidigare studier, i såväl provrörs som försöksdjur och människa, med mål att förhindra cisplatinorskad hörselskada. Det har dock aldrig varit känt huruvida tiosulfat alls når hörselsnäckan. I avhandlingens andra studie (Paper II) visades att tiosulfat nådde hörselsnäckan både snabbt och i hög utsträckning efter systemisk tillförsel i försöksdjur. Dessvärre är denna typ av tillförsel av tiosulfat riskfyld i samhåll med systemisk administrering av cisplatin. Orsaken är att den kan leda till att behandlingen av själva cancersjukdomen blir mindre effektiv p.g.a. att tiosulfat reagerar med cisplatin och MHC i cancervävnad och inte bara i hörselsnäckan. Resultaten av de studier som är beskrivna i den tredje artikeln (Paper III) visade att lokal tillförsel av tiosulfat till örat tre timmar före cisplatin kunde minska hörselskadan i försöksdjur. Huruvida detta behandlingssätt är effektivt också i människa återstår att undersöka.

När mängden cisplatin mäts i försöksdjur och människa används nästan alltid metoder med otillräcklig selektivitet, d.v.s. metoder som samtidigt mäter andra platininnehållande ämnen som kan vara aktiva, såsom MHC, men som i de flesta fall inte är aktiva. Det är därför svårt att dra några slutsatser av vad sådana resultat har för betydelse för den hörselskadande sidoeffekten. I avhandlingens sista arbete (Paper IV) utvecklades en enkel metod för att snabbt och selektivt mäta cisplatin i blod. Denna metod kommer att vara ett värdefullt instrument i framtida studier med mål att kartlägga hur cisplatin fördelas sig i kroppen och vad det har för betydelse för utvecklandet av hörselskada.
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Många personer har på olika sätt bidragit till denna avhandling, vilket jag är oerhört glad och tacksam för. Följande personer vill jag tacka extra mycket:


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**Inger Wallin**, medförfattare och före detta laborativ huvudhandledare, nu främst intresserad av ost och barnbarn. Utan dig hade det inte blivit någon avhandling, i alla fall inte än på 10 år! Du har mer än jag arbetat med att utveckla en analysmetod för cisplatin och MHC och det var du som satte upp analysmetoden för tiosulfat. Det har inneburit att jag har kommit framåt i mitt arbete, trots att jag har varit på BB eller hemma med småbarn som varit nyfödda, sjuka, trötta eller hemma p.g.a. stängt dagis. Om ostaffärerna visar sig olönsamma: starta egen konsultfirma för labbutrustning- du behöver ingen utbilden!

Blivande otorhinolaryngolog **Cecilia Engmér Berglin**, medförfattare. Du har opererat marsvin, medan jag har hanterat proverna- det har fungerat toppen! Det har varit både kul och lärorikt att jobba med dig!

**Tobias Bramer** och **Katarina Edsman**, medförfattare och gelexperter. Också detta samarbete har fungerat ypperligt!

**Anette Fransson**- tack för all hjälp på GV och att du stod ut med att räkna mina söndertrasade ytpreparat!

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Elin Jerremalm, min före detta rumskompis. Jag har aldrig träffat någon som är lika (eller mer) generös!

Professor Sharon Stone-Elander. Du har varit till stor hjälp när jag har haft problem med amerikanskan. Dessutom har du bjudit på goda kakor och gott skratt i lunchrummet!


Nuvarande och tidigare kollegor på Karolinska Apoteket. Extra stort tack till alla blodgivare- utan er inget Paper IV!

Nuvarande och tidigare medarbetare på GV- tack för att ni gör GV till en trevlig arbetsplats!

Karin Gustafsson- tack för att du är en fin vän!

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Magnus- tack för att du kom hem från USA, så att jag fick göra den här avhandlingen. Jag ser fram emot fortsättningen… ; )


