The cochlear targets of cisplatin

ISBN: 90-393-3939-2

Layout en drukwerk: Febodruk BV, Enschede

The cochlear targets of cisplatin

De aangrijpingspunten van cisplatine
in de cochlea

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op

vrijdag 18 februari 2005 des middags te 14.30 uur

door

Marjolein Wilhelmina Margaretha van Ruijven
Geboren op 16 september 1977 te Delft

Promotor : Prof. Dr. G.F. Smoorenburg

Co-promotor : Dr. J.C.M.J. de Groot

The studies presented in this thesis were performed in the Hearing Research Laboratories of the Department of Otorhinolaryngology, University Medical Center Utrecht, as a part of the research program "Analysis of Inner Ear Disorders". Marjolein van Ruijven was a Heinsius-Houbolt Research Fellow.

Printing of this thesis was financially supported by:
Carl Zeiss BV, CoSta-Keukens, Gyrus Medical BV, GN ReSound BV, Harlan Nederland BV, J.E. Jurriaanse Stichting, Multi Finance & Insurance BV, Nederlandse Vereniging Voor Slechthorenden, MediTop BV, Oticon Nederland BV, Parmachemie BV Haarlem, Schoonenberg Hoorcomfort, Stichting Atze Spoor Fonds, Stichting ORLU, SEN; Stichting ter bevordering van de Elektronenmicroscopie in Nederland, Veenhuis Medical Audio BV.

Contents

Chapter 1

Introduction

Some introductory remarks

In its recent report "Advies Gehooronderzoek. Gehoor voor het Gehoor", the Dutch Advisory Council on Health Research estimates that 1 in 10 of the Dutch population is hearing impaired to some extent (RGO, 2003). Because this disorder is widespread and hearing-impaired (especially elderly) people often have social and psychological problems, the consequences of hearing impairment should not be underestimated.

In general, peripheral hearing loss is divided into two types: conductive and sensorineural hearing loss. Conductive hearing loss occurs when the outer and/or middle ear is damaged, as a consequence of which sound waves cannot be carried all the way to the cochlea - which itself may still function properly. This blockage may be caused by middle ear infections, perforation of the eardrum or otosclerosis, a disorder in which the ossicles become immobilized due to excessive growth of bone. The causes of conductive hearing loss can be treated by medical or surgical intervention. Sensorineural hearing loss, on the other hand, is caused by damage to or malfunction of the cochlea, the auditory nerve or the brain. It can be induced by aging, noise, infections and chemical agents (including drugs), or it can be congenital. Sensorineural hearing loss usually is permanent and can only be partly compensated for by hearing aids or cochlear implants. Well-known examples of clinically applied ototoxic agents (agents having a toxic side effect on the auditory system) are aminoglycosides, a class of antibiotics, and the anticancer drug cisplatin. Since these compounds have such a critical role in the treatment of serious, life-threatening diseases, the risk of drug-induced ototoxicity is often considered to be of minor importance.

Whereas the incidence of ototoxicity in industrialized countries may reach 22%, it is assumed to be considerably higher in developing countries, especially since in these countries the use of drugs is less stringently controlled (Arslan et al., 1999). To reduce the incidence of drug-induced ototoxicity, effective rescue therapies and preventive treatment strategies should be developed. However, for such an approach to be successful, it is essential to obtain a better understanding of the mechanisms underlying the toxic action of these drugs upon the peripheral auditory system.

The peripheral auditory system

Auditory information reaches the brain through the peripheral auditory system, which can be divided into three parts: the outer ear, the middle ear, and the inner ear. The outer ear consists of: (1) the auricle (pinna), which is important in spatial focusing of sound; and (2) the external ear canal (external auditory meatus), which

ends at the eardrum (tympanic membrane). The tympanic membrane acts as the interface between the outer ear and the middle ear. Sound (air pressure waves) sets the tympanic membrane in motion, whereupon the vibration is transferred to three tiny bones (ossicles) in the tympanic cavity. These ossicles, known as the hammer (malleus), anvil (incus) and stirrup (stapes), are linked together as a system of levers. This system and, mainly, the ratio of the surface areas of the tympanic membrane and oval window compensate for the huge difference in acoustic impedance between air and water. The ossicles efficiently convert the air vibrations of the sound, via the stapes to the oval window membrane, into fluid waves in the cochlea. The inner ear is comprised of two main sections: (1) the semicircular canals and otolithic organs, which make up the vestibular system and are involved in balance; and (2) the cochlea, which is responsible for the transduction of the mechanical vibration of sound into electrical signals. These signals are transported along the auditory nerve to the brain, where further processing of the auditory input takes place.

The cochlea

The cochlea is shaped like a snail's shell and is divided into three fluid-filled compartments, which are enclosed within a bony capsule. The three compartments are known as the scala vestibuli, the scala tympani and the scala media. Together, these scalae form a tube that spirals around a central bony axis (the modiolus) along the entire length of the cochlea and which diminishes in diameter towards the apex (Fig. 1). The scala vestibuli ends at the oval window membrane, to which the stapes is attached. At the apex of the cochlea, the scala vestibuli and the scala tympani are in open connection with one another through the so-called helicotrema. The scala tympani ends at the round window membrane. The scala vestibuli and scala tympani contain a fluid called perilymph.

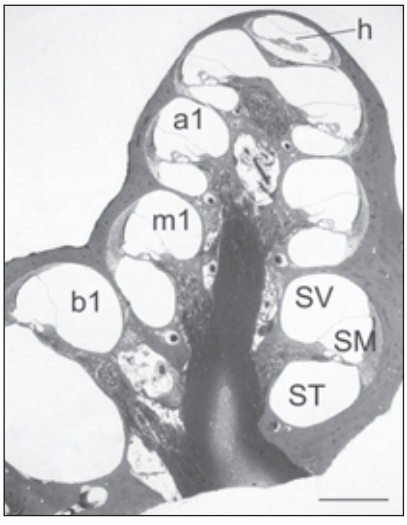


Figure 1.
Midmodiolar section (1 μm) of a guinea pig cochlea. SV, scala vestibuli; SM, scala media; ST, scala tympani; b1, lower basal turn; m1, lower middle turn; a1, lower apical turn; h, helicotrema. Scale bar represents 550 μm.

The scala media is situated between the two other scalae (Figs. 1 and 2). The scala media is separated from the scala vestibuli by Reissner's membrane, which consists of only two cell layers which are separated from one another by a basement membrane. Its vestibular surface is lined by mesothelial cells, while the side facing the scala media is lined by epithelial cells. The basilar membrane separates the scala media from the scala tympani and is composed of collagen fibers, arranged in a radial and longitudinal plane. The part of the basilar membrane facing the scala tympani is lined by a single layer of mesothelial cells. On top of the basilar membrane, facing the scala media, rests the organ of Corti, the sensory epithelium.

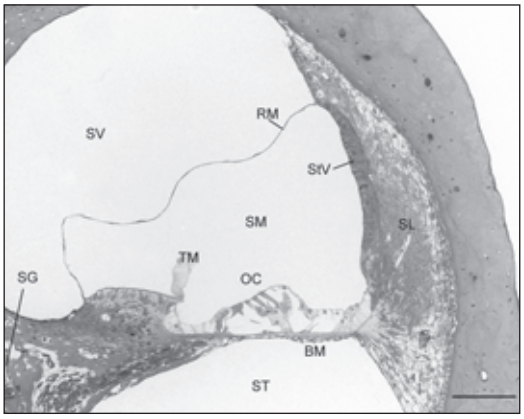


Figure 2.
Transection (1 μm) of the basal turn of a guinea pig cochlea. SV, scala vestibuli; SM, scala media; ST, scala tympani; RM, Reissner's membrane; StV, stria vascularis; OC, organ of Corti; SL, spiral ligament; BM, basilar membrane; SG, spiral ganglion. The tectorial membrane (TM) is shrunken as a result of aldehyde fixation and has detached from the organ of Corti. Scale bar represents 100 μm.

Perilymph (in the scalae vestibuli and tympani) resembles extracellular fluid in its electrolyte composition, i.e., low levels of potassium ions (K^+) and high levels of sodium ions (Na^+). In contrast to perilymph, endolymph (in the scala media) resembles intracellular fluid and has high levels of K^+ and low levels of Na^+. K^+ is the major charge carrier for the mechano-electric transduction process, which takes place in the sensory cells of the organ of Corti. The electrolyte composition of the endolymph as well as the endocochlear potential (+80 mV) are regulated by the stria vascularis, which is located in the lateral wall of the cochlea (Fig. 2). The stria vascularis is a multilayered epithelium, containing different cell types. Facing the scala media are the marginal cells, which are characterized by numerous basolateral infoldings that are rich in mitochondria. The middle layer consists of capillaries and intermediate cells, which are actually melanocytes. The third layer is composed of multiple layers of flat, interleaved basal cells and is delimited by a basement membrane, which separates the stria vascularis from the fibrocytes-containing spiral ligament.

The sensory epithelium of the cochlea, the organ of Corti, is supported by the basilar membrane (Figs. 2 and 3). The organ of Corti is composed of sensory cells (hair cells) which are involved in the mechano-electric transduction process, and a variety of non-sensory (supporting) cells. The sensory cells can be divided into two populations, which are morphologically and functionally distinct: the inner hair cells (IHCs) and the outer hair cells (OHCs). The IHCs are positioned in a single row along the medial aspect of the basilar membrane. They are pear-shaped and enclosed by supporting cells. From the apical part of the IHC protrudes a bundle of regularly arranged hair-like extensions into the scala media, which are called stereocilia. The stereocilia are arranged in almost a straight line, not touching the tectorial membrane.

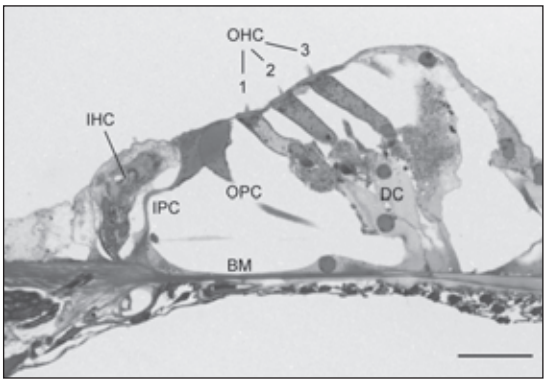


Figure 3.
Detail of the organ of Corti (1 μm). OHC, outer hair cells 1, 2, and 3; IHC, inner hair cell; IPC, inner pillar cell; OPC, outer pillar cell; BM, basilar membrane; DC, Deiters' cells. Scale bar represents 25 μm.

The OHCs, in contrast, are shaped as a cylinder and arranged in three rows along the basilar membrane. They bath in a fluid called Cortilymph, which is similar in electrolyte composition to perilymph. The apical part of the OHCs is surrounded by the phalangeal processes of the Deiters' cells. The basal part of the OHCs is supported by the Deiters' cells. The stereocilia of the OHCs are arranged in a W-configuration and the tips of the longest stereocilia insert into the tectorial membrane, which covers but does not touch the organ of Corti. The IHCs and OHCs are separated from one another by the inner and outer pillar cells, which together form the tunnel of Corti.

Sound-induced vibrations enter the cochlea via the oval window, and force the basilar membrane to move up and down because of differences in fluid pressure between scala vestibuli and scala tympani. This traveling wave moves from the base of the cochlea toward its apex, where the basilar membrane is more elastic, and peaks at a point along the basilar membrane that is related to the frequency

of the sound. Variations in the mechanical properties of the basilar membrane account for the fact that each frequency component evokes maximal displacement at a particular position. The characteristic frequencies are arranged in a monotonic order along the basilar membrane, in such a way that high-frequency sounds reach a maximal vibration at the basal part of the basilar membrane and low-frequency sounds at the apical part.
These movements of the basilar membrane result in a shearing motion between the organ of Corti and the tectorial membrane, causing a deflection of the stereocilia which evokes the opening of mechanosensitive ion channels (transduction channels) in the stereocilia. The influx of K^+ results in depolarization of the hair cell and causes the opening of voltage-gated calcium (Ca^{2+}) channels that are located in the baso-lateral membranes. The influx of Ca^{2+} subsequently causes synaptic vesicles to fuse with the synaptic membrane, upon which neurotransmitter is released into the synaptic cleft. This, in turn, initiates nerve impulses in a nerve fiber that synapses with the hair cell. The electric signals are generated in both IHCs and OHCs, but the neural information predominantly originates from the IHCs, whereas the OHCs are responsible for enhanced cochlear sensitivity and frequency selectivity. The enhancement originates with unique electro-motile properties of the OHCs.

The afferent nerve fibers, which convey auditory information from the cochlea to the central nervous system, have their cell bodies (perikarya) in the spiral ganglion (Fig. 2), which is located in Rosenthal's canal in the modiolus. The cells are bipolar, with one axon projecting to the base of a hair cell and a second axon to the cochlear nucleus in the brainstem. Approximately 80-95% (depending on the species) of the afferent nerve fibers connect directly with the IHCs, and are called type-I fibers. Type-I fibers contact the IHCs directly opposite the habenula perforata and originate from the large, myelinated type-I spiral ganglion cells. Each IHC is connected to approximately 20 type-I fibers. The remaining 5-20% of the afferent nerve fibers are called type-II fibers. They originate from the small, unmyelinated type-II spiral ganglion cells. These afferent fibers spiral basally after entering the organ of Corti and branch to connect to several OHCs.
In addition to the afferent innervation, efferent nerve fibers are present in the organ of Corti. The efferent innervation pattern complements the afferent pattern, in the sense that there is multiple innervation of the IHCs by one efferent fiber and single innervation of the individual OHCs.

The cochlear potentials

Electrocochleography (EcochG) is a method to study cochlear function by recording the stimulus-related potentials of the cochlea and auditory nerve. When a transient stimulus is delivered to the cochlea, an electrode placed on the cochlea can record these electric responses, which can be separated into three components.

The cochlear microphonics (CM) is a stimulus-related alternating current (AC) potential that closely follows the frequency of the stimulus. The CM originates with the receptor potential of the hair cells in response to sound-induced motion of the basilar membrane. Consequently, the CM reflects primarily the condition of the hair cells. However, it is important to keep in mind that damage to other structures, e.g. the stria vascularis that gives rise to the endocochlear potential, might also affect the CM.

The compound action potential (CAP) is a transient response that is generated by the eight cranial nerve. It represents the summed result of the synchronous firing of a group of auditory nerve fibers at a transient in the stimulus, usually the onset. Evaluating the CAP is a way to assess the function of the auditory periphery up to and partially including the eight cranial nerve.

The third cochlear potential is the summating potential (SP) which represents a nonlinear component in cochlear transduction. This direct current (DC) potential is only mentioned here because we used it as a baseline to measure the CAP.

Cisplatin

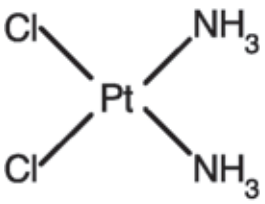


Figure 4.
Chemical structure of cisplatin.
Molecular weight=300.03.

Cisplatin (generic name: cis-diamminedichloro-platinum (II); Fig. 4) was first synthesized by Peyrone in 1844 and is also known as Peyrone's Chloride. Its chemical structure was first elucidated by Werner in 1893. In the early 1960s, Rosenberg and co-workers at Michigan State University found some curious results. Experiments designed to measure the effect of electric currents on cell cultures of the bacterium Escherichia coli, yielded cells that were 300-times their normal length. This effect was not due to the electric fields themselves, but to a compound that was formed during the electrolytic reaction between the platinum electrodes and the ammonium chloride in the nutrient medium. This compound appeared to be cisplatin and proved to prevent cell division, but not the cellular processes involved in bacterial growth (for a review, see Rosenberg

et al., 1999). This effect prompted several groups to test cisplatin against tumors in mice and they found it to be highly effective in killing tumor cells. Clinical trials were successful and cisplatin was approved for use in 1978. Cisplatin is now widely used in the treatment of solid tumors, such as testicular and ovarian carcinoma, advanced bladder carcinoma, head-and-neck carcinoma, lung carcinoma, and several childhood malignancies (O'Dwyer et al., 1999; Bertolini et al., 2004).

Cytotoxic mechanisms of cisplatin

It is generally accepted that, in tumor cells, nuclear DNA is the critical subcellular target of cisplatin and that its cytotoxicity is due to the formation of so-called cisplatin-DNA adducts (Reed et al., 1996; Martin, 1999; Eastman, 1999; Bose, 2002; Fuertes et al., 2003). Cisplatin enters the cell by diffusion, where, due to the lower chloride concentration, the chloride atoms of the cisplatin molecule are substituted by water molecules, resulting in the formation of positively charged mono- and diaquated species (Fig. 5). These charged species move into the cell nucleus and react with the DNA molecules. A monofunctional adduct is formed when the reactive molecule binds to a single nitrogen on a DNA nucleotide (guanine or adenine). The resulting structure is likely to bind to a second nucleotide on the same DNA strand (intrastrand cross-link) or to the complementary DNA strand (interstrand cross-link). Quantitative studies have shown that intrastrand cross-links between two adjacent guanine residues or between adenine and an adjacent guanine residue account for about 65% and 25%, respectively, of the cisplatin-DNA adducts formed in vitro. Intrastrand cross-links produce a severe local distortion in the DNA double helix, which may interfere with transcription and replication, eventually leading to mitotic arrest. The exact mechanism, however, that leads to tumor cell death following treatment with cisplatin is not known, but several models have been proposed to explain the role of adduct-binding proteins in mediating cisplatin cytotoxicity (Eastman, 1986; Zamble, 1995; Reed et al., 1996; Kartalou and Essigmann, 2001; Gonzalez et al., 2001; Bose, 2002; Fuertes et al., 2003).

In addition to its nuclear target, cisplatin also binds to other targets in the cytoplasm (Bose, 2002; Fuertes et al., 2003). Charged species of cisplatin rapidly react with glutathione and other sulfhydryl-containing molecules, such as metallothioneins, which leads to lipid peroxidation and mitochondrial damage (oxidative stress). Also, charged species of cisplatin bind to mitochondrial DNA, interact with phospholipids in membranes, alter microtubule assembly and disrupt the cytoskeleton (Gonzalez et al., 2001; Fuertes et al., 2003).

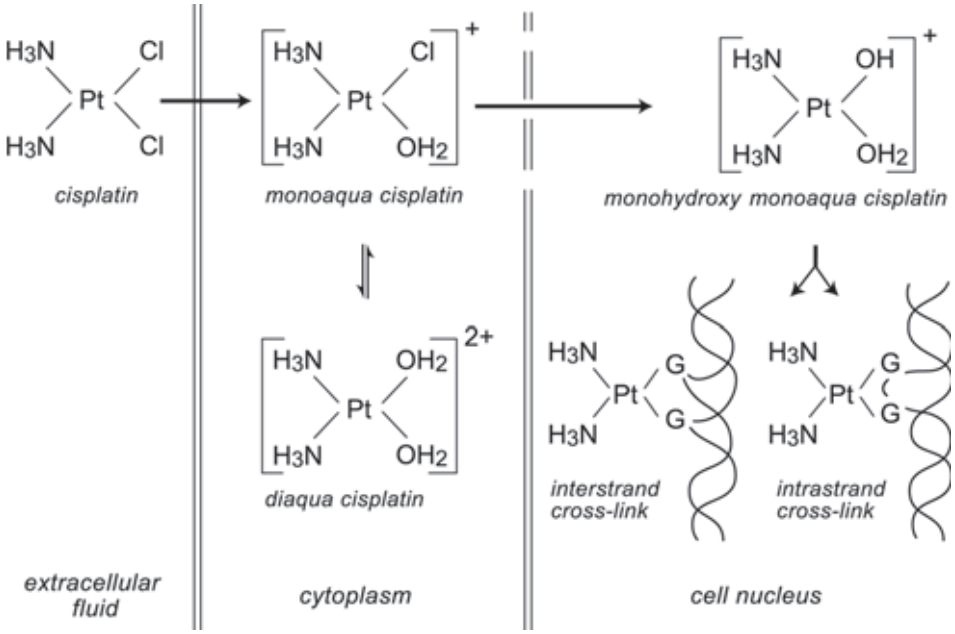


Figure 5.

The cisplatin molecule crosses the plasma membrane and is transformed within the cytoplasm into several positively charged species. Especially the monoaqua and monohydroxy-monoaqua species are reactive and interact with nuclear DNA, resulting in the formation of interstrand and intrastrand cross-links. (Adapted from Kartalou and Essigmann, 2001).

Side effects of cisplatin chemotherapy

Cisplatin can induce serious side effects, such as: (1) tumor resistance, (2) myelosuppression, (3) nausea and vomiting, (4) nephrotoxicity, (5) neurotoxicity, and (6) ototoxicity. Nephrotoxicity, neurotoxicity and ototoxicity are clinically the major dose-limiting toxicities, in that the toxic side effects are cumulative and, in general, are only partially reversible with discontinuation of therapy.

Clinically, cisplatin-induced nephrotoxicity is characterized by a reduction in glomerular filtration rate, excessive mineral excretion or acute renal failure (Reed et al., 1996; Cvitkovic, 1998). Morphologically, degeneration and loss of epithelial cells is evident in the proximal and distal tubules. Experimental studies indicate that the major effect of cisplatin is the impairment of reabsorption in the proximal tubules, with some effect also seen in the function of the distal tubules (i.e., concentration). Renal injury may be worsened by the concomitant administration of other nephrotoxic drugs, such as aminoglycosides. Nephrotoxicity may be minimized by continuous intravenous hydration before and following the

infusion of cisplatin, forced diureses with hypertonic saline or diuretic drugs, and the use of so-called rescue agents, such as sodium thiosulphate, amifostine and BNP7787 (Reedijk and Teuben, 1999; Boven et al., 2002; Verschraagen et al., 2003). Cisplatin-induced peripheral neurotoxicity is of a purely sensory nature and is dose dependent. It is clinically manifested by numbness, paresthesias and tingling of the limbs. Even after cessation of chemotherapy, the neuropathy may continue to progress for several weeks to months (Grunberg et al., 1989). Histological studies have shown sensory root ganglia disruption, axonal and myelin sheath degeneration and loss of large myelinated fibers (Roelofs et al., 1984; Thompson et al., 1984; Gregg at al., 1992). It has been suggested that cisplatin-induced peripheral neuropathies can be attenuated or prevented in rodents and humans by melanocortins, without adversely affecting the anticancer effect of cisplatin (De Koning et al., 1987; Gerritsen Van Der Hoop et al., 1990, 1994).

Cisplatin-induced ototoxicity

Ototoxicity remains a potentially serious long-term impairment in cisplatin-based chemotherapy. Clinically, it is characterized by a bilateral, usually permanent sensorineural hearing loss which is usually associated with tinnitus and vestibular dysfunction (Moroso and Blair, 1983). Hearing deficits start at the higher frequencies and, with increasing doses or prolonged treatment, progressively extend to lower frequencies involved in speech perception (De Oliveira, 1989; Schweitzer, 1993).

Organ of Corti

The most striking histological feature seen in cochleas of cisplatin-treated animals is loss of the OHCs and degeneration of the organ of Corti. This loss typically starts at the first row of OHCs in the basal turn of the cochlea followed by the other rows and, with increasing doses or prolonged administration, progresses towards the more apically located cochlear turns and, eventually, to the single row of IHCs. Hair cell loss is accompanied by protrusion of the supporting cells into Nuel's space and the tunnel of Corti, resulting in a disturbed microarchitecture of the organ of Corti and eventually in complete replacement of the sensory epithelium by a single layer of epithelial cells (Estrem et al., 1981; Nakai et al., 1982; Tange et al., 1982; Konishi et al., 1983; Tange, 1984; Laurell and Bagger-Sjöbäck, 1991; Fernández-Cervilla et al., 1993; Saito and Aran, 1994; Saito et al., 1995; De Groot et al., 1997; Heijmen et al., 1999; Cardinaal et al., 2000a, b). These findings correlate well with the permanent frequency-dependent elevation in the CAP thresholds and the irreversible suppression of hair-cell related potentials

and acoustic emissions, such as CM and distortion-product otoacoustic emissions (Komune et al., 1981; Konishi et al., 1983; Hamers et al., 1994; Sie and Norton, 1997; Stengs et al., 1998; Heijmen et al., 1999; Wang et al., 2003).

The exact mechanism by which cisplatin causes OHC degeneration and cell loss remains unknown. Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in the organ of Corti of mongolian gerbil cochleas after cisplatin administration, suggesting the involvement of apoptosis in cisplatin-induced OHC degeneration. Furthermore, it has been suggested that cisplatin administration results in oxidative stress leading to apoptotic OHC death (Huang et al., 2000; Lefebvre et al., 2002). Comis et al. (1986) observed that cisplatin administration leads to increased levels of intracellular Ca^{2+} in the OHCs and suggest that this is due to interference with enzymes involved in the regulation of intracellular Ca^{2+} concentrations. Moreover, electrophysiological studies have demonstrated that cisplatin, when applied directly into the cochlea or to dissociated cochlear OHCs, interferes indirectly with the mechano-electric transduction process by blocking the voltage-gated Ca^{2+} channels located in the lateral membranes of the OHCs (McAlpine and Johnstone, 1990; Saito et al., 1991; Kimitsuki et al., 1993; Yamamoto et al., 1994; Ernst and Zenner, 1995).

Stria vascularis

In addition to its detrimental effect upon the organ of Corti, cisplatin also affects the stria vascularis by interfering with strial function and morphology. At the morphological level, changes in strial volume, signs of apoptosis, intermediate cell atrophy, and swelling and blebbing of the marginal cells as well as vacuolation of the latter's cytoplasm have been reported (Nakai et al., 1982; Tange, 1984; Tange and Vuzevski, 1984; Kohn et al., 1988; Laurell and Bagger-Sjöbäck, 1991; Saito and Aran, 1994; Meech et al., 1998; Campbell et al., 1999; Cardinaal et al., 2000a, b; Alam et al., 2000; Sluyter et al., 2003).

However, there are also reports in which no morphological changes were observed in the stria vascularis after cisplatin administration (Fleischman et al., 1975; Böheim and Bichler, 1985; De Groot et al., 1997). Cisplatin-induced reduction of the endocochlear potential (EP) – which is generated and maintained by the stria vascularis – has been described after application of single high-dose injections (Komune et al., 1981; Laurell and Engstrom, 1989; Komune et al., 1995; Ravi et al., 1995), following repeated systemic administration (Konishi et al., 1983; Klis et al., 2000, 2002; O'Leary and Klis, 2002; Hamers et al., 2003; Sluyter et al., 2003) and during perilymphatic administration (Ford et al., 1997; Tsukasaki et al., 2000; O'Leary et al., 2001; Wang et al., 2003). Klis et al. (2000, 2002) showed

that the EP may recover after termination of cisplatin administration. Moreover, endolymphatic hydrops – which may be regarded as a histological indication of strial dysfunction – has occasionally been reported in cochleas after cisplatin administration (De Groot et al., 1997; Cardinaal et al., 2000a, b). Also, collapse of Reissner's membrane onto the organ of Corti has been observed (Komune et al., 1981; Laurell and Bagger-Sjöbäck, 1991).

Spiral ganglion

Experimental evidence of a direct effect of cisplatin on the spiral ganglion cells (SGCs) or auditory neurons in the adult mammalian cochlea is rather limited. However, cisplatin-induced neuronal death and degeneration of SGCs is a common finding in organotypic explant cultures of the postnatal rodent cochlea and dissociated spiral ganglia cultures (Zheng et al., 1995; Zheng and Gao, 1996; Gabaizadeh et al., 1997; Feghali et al., 2001; Bowers et al., 2002). Cardinaal et al. (2000b) observed cytoplasmic vacuolation of the SGCs in adult guinea pigs treated with low doses (≤1.5 mg/kg/day) of cisplatin. Ultrastructurally, this vacuolation is due to mitochondrial swelling (Cardinaal et al., 2004). In addition, cisplatin-induced shrinkage of the cell body (O'Leary et al., 2001) and the cell nucleus (Hamers et al., 2003) have been described in SGCs. Moreover, Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in SGCs of mongolian gerbil cochleas after cisplatin administration, suggesting the involvement of apoptosis in cisplatin-induced SGC degeneration.

Objectives of this thesis

Cisplatin is used extensively in cancer treatment. Therefore, its ototoxic side effects are of great concern. Numerous studies have already shown that cisplatin-induced ototoxicity is correlated with functional and morphological changes in the organ of Corti, the stria vascularis and the spiral ganglion. However, there is no consensus of opinion with regard to the question of whether there is a primary target of cisplatin in the cochlea or whether the changes run in parallel. This is mainly due to the fact that there is a considerable lack of data concerning the time sequence of the degeneration pattern in the cochlea. Furthermore, the sites of cisplatin uptake and distribution in cochlear tissues have not been properly identified.

In the **first study** (Chapter 2), the key tissues that are implicated in cisplatin ototoxicity were investigated within the time window during which degeneration starts, in order to determine whether or not the time sequence of the degeneration pattern in these tissues run in parallel. Guinea pigs were treated with cisplatin

for 4, 6 or 8 consecutive days and the cochleas were processed for histological examination. In semithin sections, morphological changes in the organ of Corti, the stria vascularis and the spiral ganglion were studied and quantified.

The **second study** (Chapter 3) was designed to differentiate between the individual contributions of the OHCs and SGCs in the deterioration of the cochlear response after cisplatin administration. For this, the individual electrophysiological data (CAP, CM) were compared with OHC loss and the number of affected SGCs.

In the **third study** (Chapter 4), an immunohistochemical protocol is described in order to localize cisplatin in the cochlea. Knowing the precise localization of the cellular sites of uptake and accumulation of cisplatin in the cochlea may eventually be helpful in the development and implementation of more sophisticated treatment protocols to prevent the ototoxic side effects of cisplatin. A polyclonal rabbit antiserum containing antibodies against cisplatin-DNA adducts was used to (indirectly) detect cisplatin in the organ of Corti and lateral wall of guinea pigs treated with cisplatin for 2x5 days.

The **fourth study** (Chapter 5) concerns the distribution of cisplatin in several cochlear tissues using a time-sequence study in order to determine the drug's primary target. For this purpose, guinea pigs were treated with cisplatin for either 2, 4, 6, 8, 10, 12, 14 or 16 consecutive days. Cisplatin-DNA adducts were detected in semithin cryosections of the guinea pig cochlea, using the method described in Chapter 4.

In this thesis, the guinea pig was used as an animal model in studying cisplatin ototoxicity, because the guinea-pig cochlea is well accessible for electrocochleography and easy to prepare for histology. Guinea pigs of the Dunkin Hartley strain were used since albino guinea pigs are less susceptible to systemic toxicity than pigmented strains (Schweitzer, 1993).

References

Alam, S.A., Ikeda, K., Oshima, T., Suzuki, M., Kawase, T., Kikuchi, T., Takasaka, T., 2000. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. Hear. Res. 141, 28-38.

Arslan, E., Orzan, E., Santarelli, R., 1999. Global problem of drug-induced hearing loss. Ann. N. Y. Acad. Sci. 28, 1-14.

Bertolini, P., Lassalle, M., Mercier G., Raquin, M.A., Izzi, G., Corradini, N., Hartmann, O., 2004. Platinum compound-related ototoxicity in children: Long-term follow-up reveals continuous worsening of hearing loss. J. Pediatr. Hematol. Oncol. 26, 649-655.

Böheim, K., Bichler, E., 1985. Cisplatin-induced ototoxicity: Audiometric findings and experimental cochlear pathology. Arch. Otorhinolaryngol. 242, 1-6.

Bose, R.N., 2002. Biomolecular targets for platinum antitumor drugs. Mini Rev. Med. Chem. 2, 103-111.

Boven, E., Verschraagen, M., Hulscher, T.M., Erkelens, C.A., Hausheer, F.H., Pinedo, H.M., Van Der Vijgh, W.J., 2002. BNP7787, a novel protector against platinum-related toxicities, does not affect the efficacy of cisplatin or carboplatin in human tumour xenografts. Eur. J. Cancer. 38, 1148-1156.

Bowers, W.J., Chen, X., Guo, H., Frisina, D.R., Federoff, H.J., Frisina, R.D., 2002. Neurotrophin-3 transduction attenuates cisplatin spiral ganglion neuron ototoxicity in the cochlea. Mol. Ther. 6, 12-18.

Campbell, K.C.M., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear. Res. 138, 13-28.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000a. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000b. Cisplatin-induced ototoxicity: Morphological evidence of spontaneous outer hair cell recovery in albino guinea pigs? Hear. Res. 144, 147-156.

Cardinaal, R.M., De Groot, J.C.M.J, Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh.) 124, 144-154.

Comis, S.D., Rhys-Evans, P.H., Osborne, M.P., Pickles, J.O., Jeffries, D.J., Pearse, H.A., 1986. Early morphological and chemical changes induced by cisplatin in the guinea pig organ of Corti. J. Laryngol. Otol. 100, 1375-1383.

Cvitkovic, E., 1998. Cumulative toxicities from cisplatin therapy and current cytoprotective measures. Cancer Treat. Rev. 24, 265-281.

De Groot, J.C.M.J., Hamers, F.P.T., Gispen, W.H., Smoorenburg, G.F., 1997. Co-administration of the neurotropic $ACTH_{(4-9)}$ analogue, ORG 2766, may reduce the cochleotoxic effects of cisplatin. Hear. Res. 106, 9-19.

De Koning, P., Neijt, J.P., Jennekens, F.G., Gispen, W.H., 1987. ORG2766 protects from cisplatin-induced neurotoxicity in rats. Exp. Neurol. 97, 746-750.

De Oliveira, J.A.A., 1989. Audiovestibular Toxicity of Drugs, Volume II. CRC Press, Boca Raton, FL, pp. 181-198.

Eastman, A., 1986. Reevaluation of interaction of cis-dichloro(ethylenediamine)platinum (II) with DNA. Biochemistry 25, 3912-3915.

Eastman, A., 1999. The mechanism of action of cisplatin: From adducts to apoptosis. In: Lippert, B. (Ed.) Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug. Wiley-VCH, Weinheim, pp. 111-133.

Ernst, A., Zenner, H.P., 1995. Acute hyperpolarization and elongation of cochlear outer hair cells on superfusion with cis-platinum. Eur. Arch. Otorhinolaryngol. 252, 163-166.

Estrem, S.A., Babin, R.W., Ryu, J.H., Moore, K.C., 1981. Cis-diamminedichloroplatinum (II) ototoxicity in the guinea pig. Otolaryngol. Head Neck Surg. 89, 638-645.

Feghali, J.G., Liu, W., Van De Water, T.R., 2001. L-N-acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. Laryngoscope 111, 1147-1155.

Fernández-Cervilla, F., Crespo, P.V., Ciges, M., Campos, A., 1993. Early morphofunctional alterations induced by cisplatin in the cochlea. A quantitative and electrocochleographic study. ORL 55, 337-340.

Fleischman, R.W., Stadnicki, S.W., Ethier, M.F., Schaeppi, U., 1975. Ototoxicity of cis-dichlorodiammine platinum (II) in the guinea pig. Toxicol. Appl. Pharmacol. 33, 320-332.

Ford, M.S., Zhongzhen, N., Whitworth, C., Rybak, L.P., Ramkumar, V., 1997. Up-regulation of adenosine receptors in the cochlea by cisplatin. Hear. Res. 111, 143-152.

Fuertes, M.A., Castilla, J., Alonso, C., Perez, J.M., 2003. Cisplatin biochemical mechanism of action: From cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. Curr. Med. Chem. 10, 257-266.

Gaibaizadeh, R., Staecker, H., Liu, W., Kopke, R.D., Malgrange, B., Lefebvre, P.P., Van De Water, T., 1997. Protection of both auditory hair cells and auditory neurons from cisplatin induced damage. Acta Otolaryngol. (Stockh.) 117, 232-238.

Gerritsen Van Der Hoop, R.G., Vecht, C.J., Van Der Burg, M.E., Elderson, A., Boogerd, W., Heimans, J.J., Vries, E.P., Van Houwelingen, J.C., Jennekens, F.G., Gispen, W.H., 1990. Prevention of cisplatin neurotoxicity with an $ACTH_{(4-9)}$ analogue in patients with ovarian cancer. N. Engl. J. Med. 322, 89-94.

Gerritsen Van Der Hoop, R., Hamers, F.P.T., Neijt, J.P., Veldman, H., Gispen, W.H., Jennekens, F.G. 1994. Protection against cisplatin induced neurotoxicity by ORG 2766: Histological and electrophysiological evidence. J. Neurol. Sci. 126, 109-115.

Gonzalez, V.M., Fuertes, M.A., Alonso, C., Perez, J.M., 2001. Is cisplatin-induced cell death always produced by apoptosis? Mol. Pharmacol. 59, 657-663.

Gregg, R.W., Molepo, J.M., Monpetit, V.J., Mikael, N.Z., Redmond, D., Gadia, M., Stewart, D.J., 1992. Cisplatin neurotoxicity: The relationship between dosage, time, and platinum concentration in neurologic tissues, and morphologic evidence of toxicity. J. Clin. Oncol. 10, 795-803.

Grunberg, S.M., Sonka, S., Stevenson, L.L., Muggia, F.M., 1989. Progressive paresthesias after cessation of therapy with very high-dose cisplatin. Cancer Chemother. Pharmacol. 2, 62-64.

Hamers, F.P.T., Klis, S.F.L., Gispen, W.H., Smoorenburg, G.F., 1994. Application of a neuroprotective $ACTH_{(4-9)}$ analog to affect cisplatin ototoxicity: An electrocochleographic study in guinea pigs. Eur. Arch. Otorhinolaryngol. 251, 23-29.

Hamers, F.P.T., Biessels, G.J., Van Dam, S., Gispen W.H., 1997. Neuroprotection in diabetic and toxic neuropathies. In: Bär, P.R., Flint Beal, M. (Eds.) Neuroprotection in CNS Diseases. Marcel Dekker Inc., New York, pp. 513-554.

Hamers, F.P.T, Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by α-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

Heijmen, P. S., Klis, S.F.L., De Groot, J.C.M.J., Smoorenburg, G.F., 1999. Cisplatin ototoxicity and the possibly protective effect of alpha-melanocyte stimulating hormone. Hear. Res. 128, 27-39.

Huang, T., Cheng, A.G., Stupak, H., Liu, W., Kim, A., Staecker, H., Lefebvre, P.P., Malgrange, B., Kopke, R., Moonen, G., Van De Water, T.R., 2000. Oxidative stress-induced apoptosis of cochlear sensory cells: Otoprotective strategies. Int. J. Dev. Neurosci. 18, 259-270.

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

Kimitsuki, T., Nakagawa, T., Hisashi, K., Komune, S., Komiyama, S., 1993. Cisplatin blocks mechano-electric transducer current in chick cochlear hair cells. Hear. Res. 71, 64-68.

Klis, S.F.L., O'Leary, S.J., Hamers, F.P.T., De Groot, J.C.M.J., Smoorenburg, G.F., 2000. Reversible cisplatin ototoxicity in the albino guinea pig. NeuroReport 11, 623-626.

Klis, S.F.L., O'Leary, S.J., Wijbenga, J., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2002. Partial recovery of cisplatin-induced hearing loss in the albino guinea pig in relation to cisplatin dose. Hear. Res. 164, 138-146.

Kohn, S., Fradis, M., Pratt, H., Zidan, J., Podoshin, L., Robinson, E., Nir, I., 1988. Cisplatin ototoxicity in guinea pigs with special reference to toxic effects in the stria vascularis. Laryngoscope 98, 865-871.

Komune, S., Asakuma, S., Snow, J.B.J., 1981. Pathophysiology of the ototoxicity of cis-diamminedichloroplatinum. Otolaryngol. Head Neck Surg. 89, 275-282.

Komune, S., Matsuda, K., Nakagawa, T., Kimitsuki, T., Hisashi, K., Inokuchi, A., Komiyama, S., Kobayashi, T., 1995. Disturbance of regulation of sodium by cis-diamminedichloroplatinum in perilymph of the guinea pig cochlea. Ann. Otol. Rhinol. Laryngol. 104, 149-154.

Laurell, G., Engström, B., 1989. The ototoxic effect of cisplatin on guinea pigs in relation to dosage. Hear. Res. 38, 27-34.

Lefebvre, P.P., Malgrange, B., Lallemend, F., Staecker, H., Moonen, G., Van De Water, T.R., 2002. Mechanisms of cell death in the injured auditory system: Otoprotective strategies. Audiol. Neurootol. 7, 165-170.

Martin, R.B., 1999. Platinum complexes: Hydrolysis and binding to N(7) and N(1) of purines. In: Lippert, B. (Ed.) Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug. Wiley-VCH, Weinheim, pp. 183-205.

McAlpine, D., Johnstone, B.M., 1990. The ototoxic mechanism of cisplatin. Hear. Res. 47, 191-203.

Meech, R.P., Campbell, K.C.M., Hughes, L.F., Rybak, L.P., 1998. A semiquantative analysis of the effects of cisplatin on the rat stria vascularis. Hear. Res. 124, 44-59.

Moroso, M.J., Blair, R.L., 1989. A review of cis-platinum ototoxicity. J. Otolaryngol. 12, 365-369.

Nakai, Y., Konishi, K., Chang, K.C., Ohashi, K., Morisaki, N., Minowa, Y., Morimoto, A., 1982. Ototoxicity of the anticancer drug cisplatin. An experimental study. Acta Otolaryngol. (Stockh.) 93, 227-232.

O'Dwyer, P.J., Stevenson, J.P., Johnson, S.W., 1999. Clinical status of cisplatin, carboplatin, and other platinum-based antitumor drugs. In: Lippert, B. (Ed.) Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug. Wiley-VCH, Weinheim, pp. 31-96.

O'Leary, S.J., Klis, S.F.L., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2001. Perilymphatic application of cisplatin over several days in albino guinea pigs: Dose-dependency of electrophysiological and morphological effects. Hear. Res. 154, 135-145.

O'Leary, S.J., Klis, S.F., 2002. Recovery of hearing following cisplatin ototoxicity in the guinea pig. Anticancer Res. 22, 1525-1528.

Ravi, R., Somani, S.M., Rybak, L.P., 1995. Mechanism of cisplatin ototoxicity: Antioxidant system. Pharmacol. Toxicol. 76, 386-394.

Reed, E., Dabholkar, M., Chabner, B.A., 1996. Platinum analogs. In: Chabner, B.A., Longo, D.L. (Eds.) Cancer Chemotherapy and Biotherapy, 2nd Edition. Lippincott-Raven Publishers, Philadelphia, pp. 357-378.

Reedijk, J., Teuber, J.M., 1999. Platinum-sulfur interactions involved in antitumour drugs, rescue agents and biomolecules. In: Lippert, B. (Ed.) Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug. Wiley-VCH, Weinheim, pp. 339-362.

RGO (Raad voor Gezondheidsonderzoek), 2003. Advies Gehooronderzoek. Gehoor voor het Gehoor. The Hague, the Netherlands, September 2003; http://www.rgo.nl/pdf/advies43.pdf.

Roelofs, R.I., Hrushesky, W., Rogin, J., Rosenberg, L., 1984. Peripheral sensory neuropathy and cisplatin chemotherapy. Neurology 34, 934-938.

Rosenberg, B., 1999. Platinum complexes for the treatment of cancer: Why the search goes on. In: Lippert, B. (Ed.) Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug. Wiley-VCH, Weinheim, pp. 3-27.

Saito, T., Moataz, R., Dulon, D., 1991. Cisplatin blocks depolarization-induced calcium entry in isolated cochlear outer hair cells. Hear. Res. 56, 143-147.

Saito, T., Aran, J.M., 1994. Comparative ototoxicity of cisplatin during acute and chronic treatment. ORL 56, 315-320.

Saito, T., Manabe, Y., Honda, N., Yamada, T., Yamamoto, T., Saito, H., 1995. Semiquantitative analysis by scanning electron microscopy of cochlear hair cell damage by ototoxic drugs. Scanning Microsc. 9, 271-281.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Sie, K.C.Y., Norton, S.J., 1997. Changes in otoacoustic emissions and auditory brain stem response after cis-platinum exposure in gerbils. Otolaryngol. Head Neck Surg. 116, 585-592.

Sluyter, S., Klis, S.F.L, De Groot, J.C.M.J., Smoorenburg, G.F., 2003. Alterations in the stria vascularis in relation to cisplatin ototoxicity and recovery. Hear. Res. 185, 49-56.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1998. Cisplatin ototoxicity. An electrophysiological dose-effect study in albino guinea pigs. Hear. Res. 124, 99-107.

Tange, R.A., Conijn, E.A.J.G., Van Zeijl, L.G.P.M., 1982. The cortitoxic effects of cis-platinum in the guinea pig. Arch. Otorhinolaryngol. 237, 17-26.

Tange, R.A., 1984. Differences in the cochlear degeneration pattern in the guinea pig as a result of gentamicin and cis-platinum intoxication. Clin. Otolaryngol. 9, 323-327.

Tange, R.A., Vuzevski, V.D., 1984. Changes in the stria vascularis of the guinea pig due to cis-platinum. Arch. Otorhinolaryngol. 239, 41-47.

Thompson, S.W., Davis, L.E., Kornfeld, M., Hilgers, R.D., Standefer, J.C., 1984. Cisplatin neuropathy. Clinical, electrophysiologic, morphologic, and toxicologic studies. Cancer 54, 1269-1275.

Tsukasaki, N., Whitworth, C.A., Rybak, L.P., 2000. Acute changes in cochlear potentials due to cisplatin. Hear. Res. 149, 189-198.

Verschraagen, M., Kedde, M.A., Hausheer, F.H., Van Der Vijgh, W.J., 2003. The chemical reactivity of BNP7787 and its metabolite mesna with the cytostatic agent cisplatin: Comparison with the nucleophiles thiosulfate, DDTC, glutathione and its disulfide GSSG. Cancer Chemother. Pharmacol. 51, 499-504.

Wang, J., Lloyd Faulconbridge, R.V., Fetoni, A., Guitton, M.J., Pujol, R., Puel, J.L., 2003. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacol. 45, 380-393.

Yamamoto, T., Kakehata, S., Saito, T., Saito, H., Akaike, N., 1994. Cisplatin blocks voltage-dependent calcium current in dissociated outer hair cells of guinea-pig cochlea. Brain Res. 648, 296-298.

Zamble, D.B., Lippard, S.J., 1995. Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem. Sci. 20, 435-439.

Zheng, J.L., Stewart, R.R., Gao, W.Q., 1995. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. J. Neurosci. 15, 5079-5087.

Zheng, J.L., Gao, W.Q., 1996. Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur. J. Neurosci. 8, 1897-1905.

Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study

Marjolein W.M. van Ruijven, John C.M.J. de Groot, Guido F. Smoorenburg

Hearing Research 2004; 197: 44-54.

Chapter 2

Abstract

We investigated the key tissues that are implicated in cisplatin ototoxicity within the time window during which degeneration starts. Guinea pigs were treated with cisplatin at a dose of 2 mg/kg/day for either 4, 6 or 8 consecutive days. Histological changes in the organ of Corti, the stria vascularis and the spiral ganglion were quantified at the light microscopical level. Outer hair cell (OHC) loss started between 4 and 6 days of cisplatin administration, but is only significantly different from the non-treated group after 8 days of treatment. Midmodiolar OHC counts were comparable to the cytocochleogram data. The cross-sectional area of the stria vascularis did not differ from the non-treated group, nor did an endolymphatic hydrops develop during the course of treatment. Spiral ganglion cell (SGC) densities did not decrease. After 6 days, however, detachment of the myelin sheath of the type-I SGCs was seen in the lower basal turn, whereas after 8 days it was also present in the more apically located turns. Myelin sheath detachment is the result of perikaryal shrinkage and swelling of the myelin sheath. The present study confirms that cisplatin at a daily dose of 2 mg/kg has a detrimental effect on the OHCs as well as on the type-I SGCs. These intracochlear effects occur simultaneously; OHC loss and SGC shrinkage start between the fourth and sixth day of cisplatin administration and appear to develop in parallel. At this dose, no histological effect on the stria vascularis could be observed, although previous electrophysiological experiments demonstrated a clear effect on the endocochlear potential (Klis et al., 2000, 2002).

1. Introduction

Cis-diamminedichloroplatinum (II) or cisplatin is a potent anticancer drug, which is widely used in the treatment of a variety of epithelial tumors. Cisplatin has, at doses normally used for clinical therapy, several disabling side effects, such as renal insufficiency, sensory peripheral neuropathies, and ototoxicity. Ototoxicity remains a major dose-limiting factor in cisplatin-based chemotherapy. Clinically, it is characterized by a cumulative, dose-related and usually permanent sensorineural hearing loss, which starts at the higher frequencies and, with increasing doses or prolonged treatment, progressively extends to frequencies involved in speech perception (De Oliveira, 1989; Schweitzer, 1993).

The most striking histological feature seen in cochleas of cisplatin-treated animals is degeneration and loss of the sensory cells in the organ of Corti. This loss typically starts at the first row of outer hair cells (OHCs) in the basal turn of the cochlea followed by the other OHC rows. With increasing doses or prolonged administration, it progresses towards the more apically located cochlear turns and, eventually, to the single row of inner hair cells (IHCs). Hair cell loss is accompanied by protrusion of the supporting cells into Nuel's space and the tunnel of Corti, resulting in a disturbed microarchitecture of the organ of Corti and eventually in complete replacement of the sensory epithelium by a single layer of epithelial cells (Estrem et al., 1981; Nakai et al., 1982; Tange et al., 1982; Konishi et al., 1983; Tange, 1984; Laurell and Bagger-Sjöbäck, 1991a; Fernández-Cervilla et al., 1993; Saito and Aran, 1994; Saito et al., 1995; De Groot et al., 1997; Heijmen et al., 1999; Cardinaal et al., 2000a, b).

In addition to its detrimental effect upon the organ of Corti, cisplatin also affects the stria vascularis by interfering with strial function and morphology (Schweitzer, 1993; Klis et al., 2000, 2002). On the morphological level, strial damage primarily involves the intermediate and marginal cells, and consists of intermediate cell atrophy and swelling and blebbing of the marginal cells as well as vacuolation of the latter's cytoplasm (Nakai et al., 1982; Tange, 1984; Tange and Vuzevski, 1984; Kohn et al., 1988; Laurell and Bagger-Sjöbäck, 1991a; Saito and Aran, 1994; Meech et al., 1998; Cardinaal et al., 2000a, b; Sluyter et al., 2003). Systemic cisplatin administration also interferes with strial volume. Meech et al. (1998) and Campbell et al. (1999) have observed that a single high-dose cisplatin injection causes an increase in strial volume in the basal turn of the rat cochlea within 3 days. In addition, Sluyter et al. (2003) reported that chronic systemic administration with cisplatin results in a decrease in the strial cross-sectional area in the basal cochlear turns of guinea pigs after 4 weeks or more. Moreover,

endolymphatic hydrops – which may be regarded as a histological indication of strial dysfunction – has occasionally been reported in cochleas after cisplatin administration (De Groot et al., 1997; Cardinaal et al., 2000a, b). Also, collapse of Reissner's membrane onto the organ of Corti has been observed (Komune et al., 1981; Laurell and Bagger-Sjöbäck, 1991a).

Cisplatin is known to affect peripheral nerves and to induce peripheral neuropathies in patients (for a review, see Hamers et al., 1997). Experimental evidence for a direct effect of cisplatin on the spiral ganglion cells (SGCs) or auditory neurons in the adult mammalian cochlea is rather limited. However, cisplatin-induced neuronal death and degeneration of SGCs is a common finding in organotypic explant cultures of the postnatal rodent cochlea and dissociated spiral ganglia cultures (Zheng et al., 1995; Zheng and Gao, 1996; Gabaizadeh et al., 1997; Feghali et al., 2001; Bowers et al., 2002). Cardinaal et al. (2000a) observed cytoplasmic vacuolation in the SGCs of adult guinea pigs treated with low doses (≤1.5 mg/kg/day) of cisplatin. Ultrastructurally, this vacuolation is due to mitochondrial swelling (Cardinaal et al., 2004). In addition, cisplatin-induced shrinkage of the cell body (O'Leary et al., 2001) and the cell nucleus (Hamers et al., 2003) have been described in SGCs. Moreover, Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in SGCs of mongolian gerbil cochleas after cisplatin administration, suggesting the involvement of apoptosis in cisplatin-induced SGC degeneration.

From these experimental findings it is evident that cisplatin ototoxicity is a three-fold phenomenon, involving injury to the organ of Corti, the stria vascularis, and the spiral ganglion. However, there is no consensus of opinion with regard to the primary target of cisplatin in the cochlea, especially since: (1) the sites of cisplatin uptake and distribution in cochlear tissues have not been properly identified; and (2) there is a considerable lack of data concerning the time sequence of the degeneration pattern in the cochlea. The present study aims to investigate the key tissues that are implicated in cisplatin ototoxicity within the time window during which degeneration starts, in order to resolve the important question of whether the drug's intracochlear effects run in parallel or are dependent on one another. For this purpose, albino guinea pigs were treated with cisplatin for either 4, 6 or 8 consecutive days. Histological changes in the organ of Corti, the stria vascularis and the spiral ganglion were studied and quantified. Electrophysiological results will be presented in a future paper.

2. Materials and methods

2.1. Experimental design

Healthy, female albino guinea pigs (strain: Dunkin Hartley; weighing 250-350 g.) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the Animal Care Facility of the Central Laboratory Animal Institute of Utrecht University. Animals had free access to both food and water and were kept under standard laboratory conditions. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC #89055). Animal care was under the supervision of the Central Laboratory Animal Institute of Utrecht University.

Cisplatin (Platinol®, containing 1 mg cisplatin per ml injection fluid; Bristol-Myers Squibb, Woerden, the Netherlands) was diluted with sterile physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml. This cisplatin solution was stored in the dark at room temperature. During the entire experiment it was made sure that the shelf life did not exceed the maximum limit of 3 days, as recommended by the Hospital Pharmacist.

Thirty-five animals were assigned at random to either a non-treated group or one of the three experimental groups. Five animals received no cisplatin and served as controls. The remaining animals (n=30) were treated with cisplatin (2 mg/kg/day) by daily intraperitoneal injections. Animals were weighed every morning and the injection volume was adjusted accordingly for each individual animal. The first experimental group received cisplatin for 4 consecutive days (total dose: 8 mg/kg; n=10), the second group for 6 consecutive days (total dose: 12 mg/kg; n=10), and the third group for 8 consecutive days (total dose: 16 mg/kg; n=10). At the end of the experiment all animals had survived. Electrocochleography was performed 24 h after the final application of cisplatin (as described previously by Stengs et al. [1997]), and followed by processing for histological examination.

2.2. Tissue preparation

Immediately after electrocochleography, the left and right cochleas were fixed by intralabyrinthine perfusion with a fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in 0.08 M sodium cacodylate buffer (pH 7.4), followed by immersion in the same fixative for 3 h at room temperature. Following several rinses (2x15 min) in 0.1 M sodium cacodylate buffer (pH 7.4), the cochleas were decalcified in 10% EDTA.2Na for 5 days at room temperature. Next, the specimens were postfixed in 1% OsO_4 containing 1% $K_4Ru(CN)_6$ in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Dehydration was performed

in a graded ethanol and propylene oxide series. The cochleas were embedded *in toto* in Spurr's low-viscosity resin and polymerized overnight at 70°C. The cochleas were divided into two halves along a standardized midmodiolar plane using the attachment point of the cochlea on the medial wall of the bulla and both the oval and round windows as anatomical landmarks. Next, the cochlear halves were re-embedded in the same resin. For histological examination and quantitative analyses, semithin (1 μm) sections were cut with a diamond knife on a Reichert-Jung 2050 microtome, collected on glass slides and stained with 1% methylene blue and 1% azur II in 1% sodium tetraborate. Sections of the left and right cochleas from the non-treated and experimental groups were examined with a Zeiss Axiophot light microscope equipped with a JVC TK-1281 colour videocamera.

2.3. Midmodiolar OHC counts

OHC counts were performed using 5-6 serial midmodiolar sections. The number of OHCs was counted in each individual transection of the respective half-turns of both the right and left cochleas from the non-treated group (n=10 cochleas) and the three experimental groups (n=20 cochleas, each group). In oblique transections of the organ of Corti, either one of the following features was used for the identification of OHCs: (1) the presence of the reticular lamina and/or stereocilia; (2) the degree of cytoplasmic staining characteristic for OHCs; and (3) the presence of the basal part of the hair cell. All remaining OHCs were counted irrespective of their histological appearance. Histopathological features such as cytoplasmic vacuolation, Hensen's bodies, shrinkage, absence of stereocilia or diminished cytoplasmic staining were not used as exclusion criteria. OHC counts were expressed as the mean percentage of remaining OHCs per individual transection of the respective half turn (two transections each for the basal [b1, b2] and middle [m1, m2] turns and three transections for the apical [a1, a2, a3] turn). All OHC counts were performed by two well-trained investigators, independently of one another, in a single-blind fashion.

2.4. Cytocochleograms

In order to verify whether the midmodiolar OHC samples are a reliable indication for overall OHC loss, total hair cell counts were subsequently obtained from block-surface preparations. Hair cells were counted in the 8-day group only, as these animals demonstrated the largest variability in midmodiolar OHC counts. Orthogonal cuts were made between the coils of the re-embedded halves of the right cochleas (n=10) – from which at an earlier stage midmodiolar sections

had been obtained – to separate them into 8-9 individual slices (per cochlea) according to the block-surface technique (Spoendlin and Brun, 1974; Jiang et al., 1993). The slices were mounted on glass slides with Spurr's low-viscosity resin. After polymerization at 70°C, the slices were thinned with sanding disks to an appropriate thickness to allow examination in a Zeiss *Axiophot* light microscope equipped with differential interference contrast (Nomarski) optics. The slices containing the individual half-turns were examined using a x40 objective lens with a long free-working distance. Images were captured with a colour videocamera and directed to a monitor for on-line measurement. The width of the monitor screen (at this magnification) corresponded to a distance of 150 μm in the specimen. The number of remaining OHCs (in each of the three rows) and IHCs as well as the number of phalangeal scars were counted in all 150-μm segments of each slice. Hair cells were counted if the stereociliary bundle was obvious and/or the entire cell body could be discerned. Next, the total number of 150-μm segments was counted, resulting in the total length of basilar membrane used for counting the hair cells. Cutting loss (i.e., the total length of basilar membrane lost due to slicing and midmodiolar sectioning) was calculated by subtracting the measured length of the basilar membrane by the actual length of the basilar membrane, which was assumed to be 18.4 mm for the resin-embedded guinea-pig cochlea (Jiang et al., 1993). The average basilar membrane loss per cut, which was estimated by dividing the cutting loss by the number of slices, amounted to 0.22 mm. The number of OHCs (for each row) and IHCs were averaged per mm distance of basilar membrane (including tissue lost per cut) and plotted into a cytocochleogram.

2.5. Determination of the strial cross-sectional area

In order to establish whether cisplatin induces volume changes in the stria vascularis, the strial cross-sectional area was determined in midmodiolar sections of the left and right cochleas from both the non-treated group (n=10 cochleas) and the three experimental groups (n=20 cochleas, each group). Images of the individual transections of the respective half turns were captured with a videocamera and digitally stored (TIFF format) for off-line processing. Digitized images were imported into the public-domain *NIH Image* program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Next, the boundaries of the stria vascularis were outlined with a pressure-sensitive stylus on a Wacom UD-0608-R digitizer interfaced to a Macintosh computer, and the strial cross-sectional area (in μm^2) was calculated.

2.6. Quantification of endolymphatic hydrops

In order to quantify the degree of distension of Reissner's membrane – which may be regarded as a histological indication of strial dysfunction – the ratio l_R/d was used as an objective measure of endolymphatic hydrops (Bouman et al., 1998). This ratio is calculated by dividing the actual length of Reissner's membrane, l_R, by the rectilinear distance, d, between its medial (i.e., the spiral limbus) and its lateral (i.e., the stria vascularis) attachment points. Images of the individual transections of the respective half turns were captured with a videocamera and digitally stored (TIFF format) for off-line measurements. The digitized images of the individual transections of the respective half turns were imported into the *NIH Image* program. The actual length of Reissner's membrane and the rectilinear distance between its attachment points were measured, and the l_R/d ratio was calculated for the left and right cochleas from the non-treated group (n=10 cochleas) and the three experimental groups (n=20 cochleas, each group).

2.7. Spiral ganglion analyses

2.7.1. Spiral ganglion cell densities

In order to resolve whether cisplatin results in loss of cells in the spiral ganglion, SGC densities were determined in all three experimental groups (n=20 cochleas, each group) and compared to those of the non-treated group (n=10 cochleas). Digitized images (TIFF format) of the spiral ganglia from each individual transection were imported into the *NIH Image* program. The bony boundaries of Rosenthal's canal were outlined using a pressure-sensitive stylus on a Wacom UD-0608-R digitizer, and its cross-sectional area (in mm^2) was calculated. The number of perikarya of the myelinated (type-I) SGCs and unmyelinated (type-II) SGCs was counted in each individual transection of Rosenthal's canal (Webster and Webster, 1981; Miller et al., 1997). SGC density was calculated by dividing the number of perikarya counted per transection by the cross-sectional area in each transection and expressed as the number of SGCs per mm^2.

2.7.2. Spiral ganglion cell shrinkage

During histological examination of the spiral ganglia in both non-treated and cisplatin-treated animals, it was observed that in some type-I SGCs the myelin sheath was detached from the perikaryon, leaving a void space between the cell body and the enveloping myelin sheath. The percentage of affected perikarya was determined for each individual transection of the respective half-turns in the left and right cochleas from the non-treated group (n=10 cochleas) and three

experimental groups (n=20 cochleas, each group). Digitized images (TIFF format) of the spiral ganglia from each individual transection were imported into the program *ColorIt!*™. The total number of the perikarya (both type-I and type-II SGCs) per transection and the number of affected perikarya (i.e., type-I SGCs) in that particular transection were counted. The percentage of affected perikarya was calculated as follows:

(number of affected perikarya ÷ total number of perikarya) * 100%

Detachment of the myelin sheath from the perikaryon could be the result of either perikaryal shrinkage or swelling of the myelin sheath. Therefore, we measured both the perikaryal area and the area delineated by the innermost layer of the myelin sheath in SGCs at the b1 location in a random selection of cochleas from both the non-treated group and the three experimental groups.
In normally appearing SGCs, i.e. cells without detachment of the myelin sheath, perikaryal area was identical to the area delineated by the innermost layer of the myelin sheath. Perikaryal area was measured in 177 SGCs from the non-treated group and in 304 SGCs from the three experimental groups (pooled data).
In affected cells, both the perikaryal area and the area delineated by the innermost layer of the myelin sheath were measured in the non-treated group (n=18 SGCs) and the three experimental groups (n=128 SGCs; pooled data).

2.8. Statistical analysis

Statistical analysis was performed by means of analysis of variance (ANOVA) using Statistica® software. The significance of differences was tested by the post-hoc Tukey's HSD test for unequal n. A *P*-value of <0.05 was considered to be the criterion for statistical significance.

3. Results

3.1. Hair cell counts

3.1.1.Midmodiolar OHC counts

Figure 1 shows the mean percentages of remaining OHCs per individual transection of the respective half-turns, comparing the non-treated group with the experimental groups. Statistical analysis by means of ANOVA showed

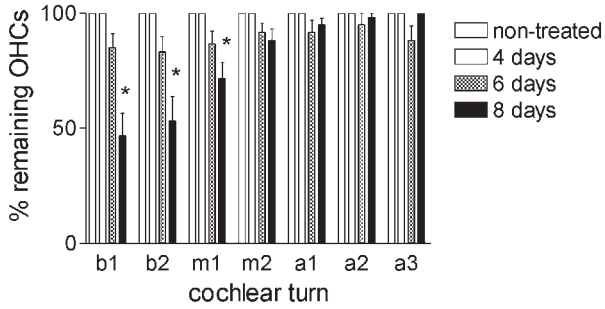


Figure 1.
Midmodiolar OHC counts represented as percentage of remaining OHCs (± standard error of the mean) at 7 different locations along the basilar membrane. Asterisks denote statistically significant differences compared to the non-treated group (P<0.005).

main effects of cisplatin treatment ($F_{(3,96)}$=16.44, P<0.0001), location ($F_{(6,576)}$=17.72, P<0.0001), and a significant interaction between both factors ($F_{(18,576)}$=15.94, P<0.0001). We did not observe loss of OHCs in the cochleas of the non-treated

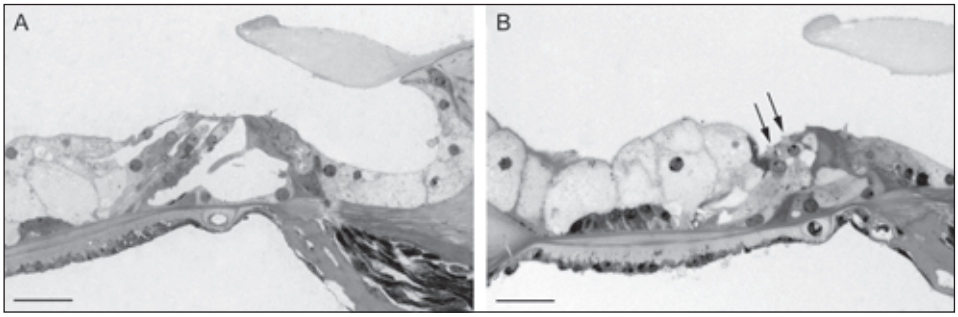


Figure 2.
Light micrographs of the organ of Corti (midmodiolar sections) in the basal turn of a non-treated animal (A) and an animal treated with cisplatin for 8 days (B). In the non-treated animal, both the OHCs and IHC are present. In the cisplatin-treated animal, OHC-1 is absent, OHCs 2 and 3 (arrows) are present but shrunken and the organ of Corti is collapsed. IHC is still present. Scale bar represents 25 μm.

animals (n=10 cochleas), nor in cochleas of the animals in the 4-day group (n=20 cochleas). In contrast, 11 out of 20 cochleas from the 6-day group and 15 out of 20 cochleas from the 8-day group demonstrated OHC loss, which was of a varying degree and restricted mainly to the basal and middle cochlear turns (Figs. 2A-B). OHC counts in animals treated for 6 days with cisplatin were not statistically significantly different from those in the non-treated group at the 5% level. However, for the 8-day group, the number of remaining OHCs in the lower basal turn (b1: 46.7%), the upper basal turn (b2: 53.3%), and the lower middle turn (m1: 71.7%) were significantly lower ($P<0.005$) than the OHC counts in the non-treated group at the 5% level. Loss of IHCs was not observed, neither in the non-treated group nor in the experimental groups.

3.1.2. Cytocochleograms

Figure 3 presents the averaged hair cell counts obtained from block-surface preparations of the right cochleas (n=10) for the 8-day group. There is a marked difference in susceptibility for cisplatin between the different rows of hair cells. OHCs are more vulnerable than the IHCs, as the latter did not demonstrate any loss, not even after 8 days of cisplatin administration. OHC loss is most severe in the first row, followed by the second and third rows. In addition, OHC loss is most prominent in the basal turn, whereas it is less pronounced in the middle turn and almost non-existent in the apical turn.

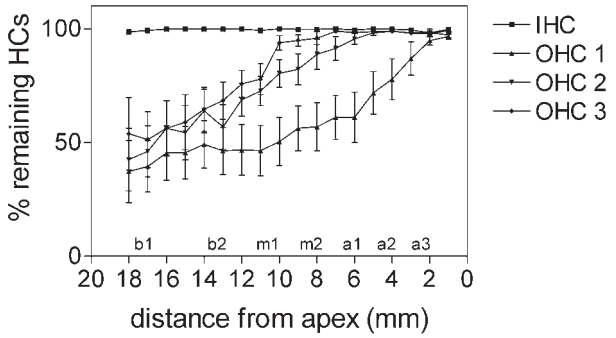


Figure 3.
Average cytocochleogram of the right cochleas of the 8-day group (n=10). Data are expressed as percentage of remaining OHCs and IHCs (± standard error of the mean).

In Fig. 4, the mean OHC counts obtained from block-surface preparations are compared to the midmodiolar OHC counts of the same 10 cochleas. To facilitate comparison of both data sets, the individual cytocochleogram data of the three OHC rows were averaged per cochlea, followed by averaging over the 10 right cochleas. There are no significant differences between the midmodiolar OHC counts and the averaged cytocochleogram data.

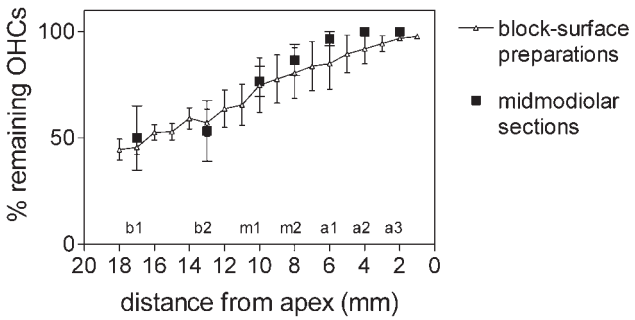


Figure 4.

Comparison of the average OHC loss (± standard error of the mean) in the right cochleas for the 8-day group using midmodiolar sections and block-surface preparations. In midmodiolar sections, the OHCs were counted at 7 different locations along the basilar membrane (upper horizontal axis). In block-surface preparations, OHCs were counted along the entire basilar membrane (lower horizontal axis). Hook region: 18.4-16.5 mm (from apex); b1: 16.5 mm; b2: 12.5 mm; m1: 9.5 mm; m2: 7.5 mm; a1: 5.5 mm; a2: 3.5 mm; a3: 1.5 mm; helicotrema region: 1.5-0 mm from apex.

3.2. Strial cross-sectional area

Strial cross-sectional area was measured in each individual transection of the respective half-turns in both the non-treated group (n=10 cochleas) and the experimental groups (n=20 cochleas, each group). The data are presented in Fig. 5. It is evident from these measurements that strial cross-sectional area decreases towards the more apically located cochlear turns (cf., Conlee et al., 1994), in both the non-treated group and the experimental groups. Significant changes in strial cross-sectional area were not observed after cisplatin administration, not even in the 8-day group. Also, there were no obvious differences in the gross morphology of the stria vascularis. Therefore, we did not consider it relevant to determine the relative areas of the different cell layers of the stria vascularis (cf., Sluyter et al., 2003).

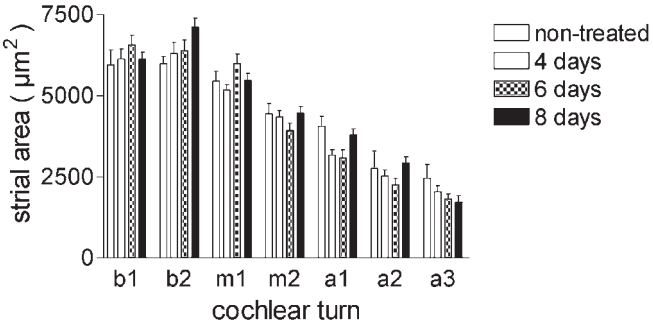


Figure 5.

Mean cross-sectional area of the stria vascularis (± standard error of the mean) determined at 7 different locations in midmodiolar sections in the non-treated and experimental groups.

3.3. Quantification of endolymphatic hydrops

The l_R/d values for the respective transections in the non-treated group (n=10 cochleas) and the experimental groups (n=20 cochleas, each group) are displayed in Fig. 6. In all groups, higher l_R/d values for the lower basal turn (b1) were observed, but this is a common finding using our fixation protocol (cf., Bouman et al., 1998; Cardinaal et al., 2000a). No significant changes in l_R/d values were observed between the non-treated group and the experimental groups, implying that cisplatin administration did not result in a distension of Reissner's membrane (i.e., endolymphatic hydrops). We could not corroborate our earlier finding that 8-day administration of cisplatin results in an endolymphatic hydrops (De Groot et al., 1997; Cardinaal et al., 2000a).

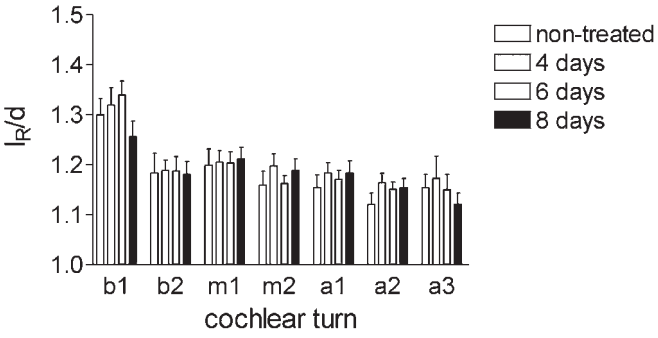


Figure 6.
The degree of distension of Reissner's membrane in the non-treated and experimental groups, expressed as the mean l_R/d ratio (± standard error of the mean), measured at the 7 different locations in midmodiolar sections.

3.4. Spiral ganglion analyses

3.4.1. Spiral ganglion densities

Figure 7 shows the SGC densities measured in the individual transections of the respective half-turns, comparing the non-treated group (n=10 cochleas) and the experimental groups (n=20 cochleas, each group). None of the experimental groups demonstrated a statistically significant decrease in the SGC densities in

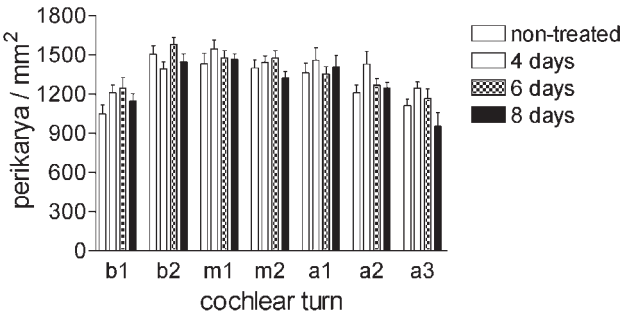


Figure 7.
SGC densities in the individual transections in the respective half-turns. SGC density is expressed as the number of perikarya (both type-I and type-II SGCs) per mm^2 (± standard error of the mean).

the different cochlear turns. These data suggest that administration of cisplatin (at a dose of 2 mg/kg/day) for as long as 8 days does not result in loss of SGCs.

3.4.2. Spiral ganglion cell shrinkage

In the myelinated (type-I) SGCs, both in non-treated and in cisplatin-treated animals, the myelin sheaths were occasionally detached from the perikarya, leaving a void space between the perikarya and the enveloping myelin sheaths. Since the number of affected cells seemed to be larger in the cisplatin-treated animals (Figs. 8A-B), we quantified the number of affected cells. in the non-treated group (n=10 cochleas) and the three experimental groups (n=20 cochlea, each group). The data, expressed as the percentage of affected perikarya, are presented in Fig. 9. Statistical analysis by means of ANOVA showed main effects of cisplatin treatment ($F_{(3,38)}$=6.20, P<0.002), location ($F_{(6,228)}$=9.96, P<0.0001), and a significant interaction between both factors ($F_{(18,228)}$=2.86, P<0.0002). In the non-treated group, affected cells were present in all cochlear turns of all animals, but the mean percentage affected cells never exceeded 10%. In the 4-day group, the number of affected cells was not statistically significantly different from that in the non-treated group. In the 6-day and 8-day groups, significantly more affected cells were observed in the lower basal turn (b1), as compared to the non-treated group (6 days: 35%, P<0.01; 8 days: 32%, P<0.03). In addition, the 8-day group exhibited significantly more affected cells in the upper basal turn (b2: 38%, P<0.002), the lower middle turn (m1: 21%, P<0.04) and the upper middle turn (m2: 18%, P<0.05), as compared to the non-treated group. Affected SGCs were also observed in the apical turns of animals treated for 8 days with cisplatin, but their number was not statistically significant increase.

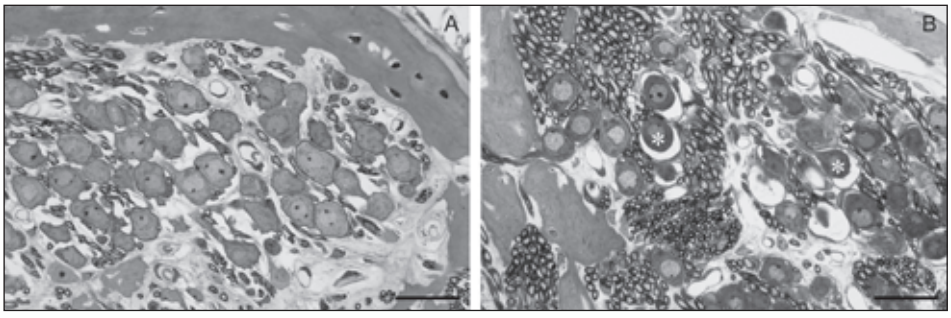


Figure 8.

Light micrographs of spiral ganglion in the basal turn of both a non-treated animal (A) and an animal treated with cisplatin for 8 days (B). Asterisks denote SGCs demonstrating detachment of their myelin sheaths. Scale bar represents 30 μm.

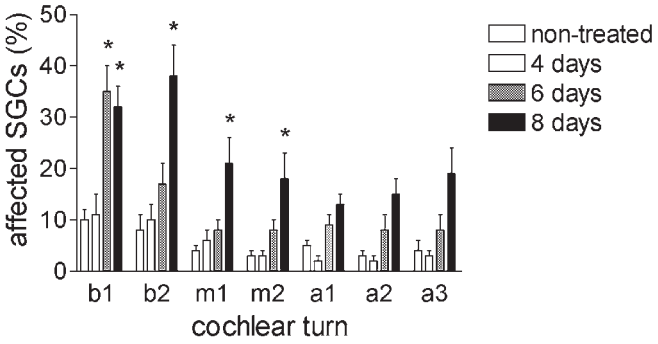


Figure 9.

Number of type-I SGCs demonstrating detachment of the myelin sheath. Data are expressed as the percentage of affected perikarya (± standard error of the mean) at the 7 different locations along the basilar membrane in the non-treated group and the experimental groups. Asterisks denote statistically significant differences compared to the non-treated group ($P<0.05$).

In order to determine whether detachment of the myelin sheath is the result of perikaryal shrinkage or swelling of the myelin sheath, the perikaryal area and the area delineated by the innermost layer of the myelin sheath were measured (Fig. 10). In the non-treated group, the mean area of SGCs with a normal appearance amounted to 262 μm^2. In the affected cells of this group, the mean perikaryal area (235 μm^2) was lower and the mean area delineated by the myelin sheath (278 μm^2) was larger than the mean area of normal cells, but statistically not significant.

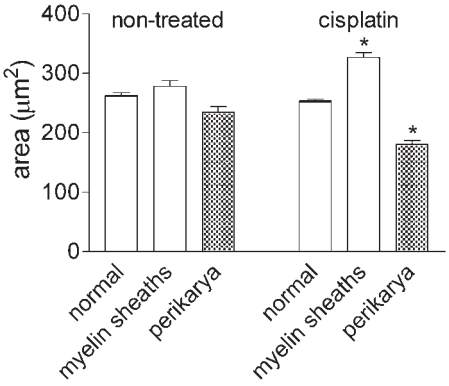


Figure 10.

Mean area (± standard error of the mean) of SGCs in non-treated and cisplatin-treated animals (pooled data of 4, 6, and 8 days). In normally appearing SGCs (normal; open bars), perikaryal area was identical to the area delineated by the myelin sheath. In affected cells (closed bars), both the area delineated by the myelin sheath (myelin sheaths; light grey bars) and the perikaryal area (perikarya; dark grey bars) were measured. Asterisks denote statistically significant differences compared to normal SGCs ($P<0.0001$).

In the cisplatin-treated groups (pooled data), the mean area of SGCs with a normal appearance was 253 μm^2, which was not statistically different from the normally appearing SGCs in the non-treated group. In affected cells, however, the mean perikaryal area (181 μm^2) was significantly lower than the normal SGCs in the non-treated group ($P<0.0001$) as well as in the cisplatin-treated groups ($P<0.0001$), suggesting shrinkage of the perikaryon. The mean area delineated by the myelin sheath was significantly increased to 327 μm^2, as compared to the normal SGCs in the non-treated group ($P<0.0001$) and the cisplatin-treated groups ($P<0.0001$). These data suggest that detachment of the myelin sheath is the result of both perikaryal shrinkage and swelling of the myelin sheath.

4. Discussion

Research aimed at elucidating the effects of cisplatin in the mammalian cochlea is complicated by the fact that the ototoxic effects of this cytotoxic agent are dose dependent, as demonstrated in adult cochleas (Laurell and Engström, 1989; Laurell and Bagger-Sjöbäck, 1991b; Schweitzer, 1993; Stengs et al., 1998; Cardinaal et al., 2000a), organotypic explant cultures (Zheng and Gao, 1996), dissociated cell cultures (Zheng et al., 1995; Gabaizadeh et al., 1997; Bowers et al., 2002), and immortalized cell lines (Bertolaso et al., 2001). An additional complicating factor is that cisplatin ototoxicity involves multiple targets: the organ of Corti, the stria vascularis, and the spiral ganglion (Cardinaal et al., 2000a, b; Alam et al., 2000; Hamers et al., 2003).
In the light of these findings it is not remarkable that there is an ongoing debate with regard to the primary target of cisplatin in the cochlea and the question arises whether or not there exists a causal relationship between the three targets. Furthermore, reliable histological data concerning the time sequence of the degeneration pattern of the various cochlear tissues are lacking. A time-sequence study may shed light on the question whether there is one primary target or whether cisplatin acts at the three targets in parallel. Therefore, we have investigated the time period during which degeneration starts in the different tissues using a fixed dose of the drug. Previous studies showed that cisplatin applied at a dose of 2 mg/kg/day during 4, 6 and 8 days would provide an adequate ototoxic effect (cf., Stengs et al., 1998; Cardinaal et al., 2000a).

In this study, OHC loss was observed after 6 days and 8 days of cisplatin administration. ANOVA showed main effects of cisplatin treatment, location and a significant interaction between both factors. OHC loss in the 6-day group was observed in the basal and lower middle turns, but it was not statistically significant. In the 8-day group, however, OHC losses in the basal and lower middle turns did reach statistical significance. In none of the experimental groups was loss of IHCs obvious, not even after 8 days of consecutive administration. These findings are in general agreement with earlier studies using cisplatin at a dose of 2 mg/kg/day during an 8-day treatment protocol (Nakai et al., 1982; De Groot et al., 1997; Heijmen et al., 1999; Cardinaal et al., 2000a). The response to cisplatin administration varied considerably between the animals, e.g., 11 of 20 cochleas in the 6-day group and 15 of 20 cochleas in the 8-day group demonstrated OHC loss in a varying degree. This high interanimal variability has been reported before by numerous authors (Tange et al., 1982; Hoeve et al., 1989; De Oliveira, 1989; Saito

and Aran, 1994; Barron and Daigneault, 1987; Konishi et al., 1983; Schweitzer, 1993; Heijmen et al., 1999).
In order to verify whether midmodiolar OHC counts are a reliable indication for actual OHC loss, the midmodiolar OHC counts from the 8-day group were compared with the corresponding cytocochleograms. Hair cells were counted if the entire cell body and/or the stereocilia could be discerned, in order to facilitate comparison of both methods. From Fig. 4 it is evident that the data obtained with both approaches do not differ significantly, suggesting that midmodiolar counts are a reliable, albeit semiquantitative, indication for actual hair cell loss (cf., De Groot et al., 2000). Major advantages of the midmodiolar approach are that counting hair cells in these sections is less time-consuming and that it not only gives a better impression of the gross morphology of the hair cells but also allows for morphological analyses of the other cochlear tissues in the corresponding cochlear turn(s).

To ascertain whether cisplatin at a daily dose of 2 mg/kg also affects strial volume, we determined the cross-sectional area of the stria vascularis in midmodiolar sections. In none of the experimental groups did the strial cross-sectional area differ from that in non-treated cochleas. This result corresponds well with the earlier finding of Tange and Vuzevski (1984) who found no strial changes in guinea pigs after 10 days of cisplatin administration (1.5 mg/kg/day). Furthermore, Cardinaal et al. (2000a) found that 8-day administration of cisplatin at a daily dose of 2 mg/kg does not result in any obvious histological changes in the stria vascularis. Moreover, Sluyter et al. (2003) reported that histological changes in the stria vascularis are not seen within 1-3 days after cessation of cisplatin administration (1.5 mg/kg/day for 5-18 days, until a CAP threshold shift of 40 dB occurred). It should be noted that the occurrence of histological changes in the stria vascularis not only depends on the total dose (Cardinaal et al., 2000), but also is determined by the treatment protocol used. Meech et al. (1998) and Campbell et al. (1999) reported an increase in strial volume in the basal turn of the rat cochlea, 3 days after a single high-dose (16 mg/kg) injection with cisplatin. In contrast, Sluyter et al. (2003) observed a decrease in strial cross-sectional area in the basal turn of the guinea pig cochlea after post-treatment survival of ≥4 weeks following treatment with cisplatin (1.5 mg/kg/day). This latter finding is in line with the recovery experiments of Cardinaal et al. (2000b), who found that, after 8-day administration of cisplatin at a daily dose of 1.5 mg/kg, strial atrophy is present in the basal and middle turns, and it progresses when the survival period is prolonged.

Another histological parameter to determine possible interference of cisplatin with strial function is the occurrence of endolymphatic hydrops. Endolymphatic hydrops is the net result of an imbalance between endolymph production and resorption, which are generally thought to take place in the stria vascularis and the endolymphatic sac, respectively. In this study, we again have used the l_R/d ratio as an objective measure of the degree of endolymphatic hydrops (cf., Bouman et al., 1998). The l_R/d values obtained in the experimental groups did not differ significantly from those obtained in the non-treated group, not even in the 8-day group. This result was quite unexpected, especially since Cardinaal et al. (2000a) measured significantly higher l_R/d values, suggesting an endolymphatic hydrops, in the basal and middle cochlear turns of guinea pigs treated with cisplatin at a daily dose of 2.0 mg/kg/day for 8 days. We do not have an explanation for this discrepancy, although it cannot be excluded that this is due to interanimal variability. The literature with regard to the endolymphatic hydrops after cisplatin administration is confusing. Although most studies have not described an effect on Reissner's membrane, Komune et al. (1981) and Laurell and Bagger-Sjöbäck (1991a) observed a collapse of Reissner's membrane onto the organ of Corti after cisplatin administration. The latter phenomenon can only be explained by an initial elongation of Reissner's membrane.
In the present study, at none of the studied time intervals was morphological damage of the stria vascularis obvious. However, it should be noted that cisplatin may have interfered with strial function, resulting in a depression of the endocochlear potential (Klis et al., 2000, 2002) and/or inhibition of membrane-bound enzyme systems (Bagger-Sjöbäck et al., 1980; Koch and Gloddek, 1991).

The reported effects of cisplatin on SGCs in adult cochleas are dose-dependent and varied. They consist of cell shrinkage (O'Leary et al., 2001), nuclear shrinkage (Hamers et al., 2003), and mitochondrial swelling (Cardinaal et al., 2004). In the present study, many of the myelinated (type-I) SGCs in cisplatin-treated animals demonstrated detachment of their myelin sheaths. This effect, although to a slight degree, was also present in the non-treated cochleas. The small effect most probably represents a fixation artefact due to less-than-optimal preservation. However, the observation that myelin sheath detachment in the experimental groups is progressive after 6 and 8 days of treatment, implies that in these groups it cannot be attributed to sub-optimal fixation alone. A similar observation was already reported by O'Leary et al. (2001) after intracochlear application of cisplatin with osmotic pumps, and they attributed this effect to shrinkage. Our analyses demonstrate that detachment of the myelin sheath is the result of both

perikaryal shrinkage and swelling of the myelin sheath.
In the present study, detachment of the myelin sheath of type-I SGCs was not induced by retrograde degeneration, since IHC loss was not observed after 6 and 8 days of cisplatin treatment. Ylikoski et al. (1974) and Spoendlin (1979) have found that retrograde degeneration of SGCs occurs only after IHC loss and is a slow and progressive process.
Perikaryal shrinkage and swelling of the myelin sheath might precede actual SGC loss. However, we have determined SGC densities in the different turns, and after 8 days of cisplatin treatment, there was still no loss of SGCs. In some affected cells the degree of perikaryal shrinkage was very severe, and we assume that within prolonged exposure or after post-treatment survival periods exceeding 24 hours, these cells may die.

The present study confirms that cisplatin at a daily dose of 2 mg/kg has a detrimental effect on the OHCs as well as on the type-I SGCs. Both OHC loss and SGC shrinkage are observed after 6 days of cisplatin administration. From the present results we infer that both processes occur simultaneously and run parallel to each other. Histological effects were not observed in the stria vascularis, nor did we find endolymphatic hydrops. However, it cannot be excluded that at this dose and within this time window cisplatin acts at a molecular level, e.g., interference with the endocochlear potential (Klis et al., 2000, 2002), the effects of which cannot be discerned in light microscopy.

Acknowledgements
This study has been supported by grants from the Heinsius-Houbolt Foundation, the Netherlands. The authors wish to thank E.G.J. Hendriksen for his technical assistance.
Marjolein W.M. van Ruijven is a Heinsius-Houbolt Research Fellow at the Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands.

References

Alam, S.A., Ikeda, K., Oshima, T., Suzuki, M., Kawase, T., Kikuchi, T., Takasaka, T., 2000. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. Hear. Res. 141, 28-38.

Bagger-Sjöbäck, D., Filipek, C. S., Schacht J., 1980. Characteristics and drug responses of cochlear and vestibular adenylate cyclase. Arch. Otorhinolaryngol. 228, 217-222.

Barron, S.E., Daigneault, E.A., 1987. Effect of cisplatin on hair cell morphology and lateral wall Na,K-ATP-ase activity. Hear. Res. 26, 131-137.

Bertolaso, L., Martini, A., Bindini, D., Lanzoni, I., Parmeggiani, A., Vitali, C., Kalinec, G., Kalinec, F., Capitani, S., Previati, M., 2001. Apoptosis in the OC-k3 immortalized cell line treated with different agents. Audiol. 40, 327-335.

Bouman, H., Klis, S.F.L., De Groot, J.C.M.J., Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 1998. Induction of endolymphatic hydrops in the guinea pig by perisaccular deposition of sepharose beads carrying and not carrying immune complexes. Hear. Res. 117, 119-130.

Bowers, W.J., Chen, X., Guo, H., Frisina, D.R., Federoff, H.J., Frisina, R.D., 2002. Neurotrophin-3 transduction attenuates cisplatin spiral ganglion neuron ototoxicity in the cochlea. Mol. Ther. 6, 12-18.

Campbell, K.C.M., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear. Res. 138, 13-28.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000a. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000b. Cisplatin-induced ototoxicity: Morphological evidence of spontaneous outer hair cell recovery in albino guinea pigs? Hear. Res. 144, 147-156.

Cardinaal, R.M., De Groot, J.C.M.J, Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh.) 124, 144-154.

Conlee, J.W., Gerity, L.C., Westenberg, I.S., Creel, D.J., 1994. Pigment-dependent differences in the stria vascularis of albino and pigmented guinea pigs and rats. Hear. Res. 72, 108-124.

De Groot, J.C.M.J., Hamers, F.P.T., Gispen, W.H., Smoorenburg, G.F., 1997. Co-administration of the neurotropic $ACTH_{(4-9)}$ analogue, ORG 2766, may reduce the cochleotoxic effects of cisplatin. Hear. Res. 106, 9-19.

De Groot, J.M.C.J., Hendriksen, E.G.J., Cardinaal, R.M., Veldman, J.E. Smoorenburg, G.F., 2000. Cisplatin-induced OHC loss and spontaneous recovery in the adult guinea pig cochlea: Cytocochleograms versus midmodiolar OHC counts. Abstractbook 37th Workshop on Inner Ear Biology, Uppsala, p. 46.

De Oliveira, J.A.A., 1989. Audiovestibular Toxicity of Drugs, Volume II. CRC Press, Boca Raton, FL, pp. 181-198.

Estrem, S.A., Babin, R.W., Ryu, J.H., Moore, K.C., 1981. Cis-diamminedichloroplatinum (II) ototoxicity in the guinea pig. Otolaryngol. Head Neck Surg. 89, 638-645.

Feghali, J.G., Liu, W., Van De Water, T.R., 2001. L-*N*-acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. Laryngoscope 111, 1147-1155.

Fernández-Cervilla, F., Crespo, P.V., Ciges, M., Campos, A., 1993. Early morphofunctional alterations induced by cisplatin in the cochlea. A quantitative and electrocochleographic study. ORL 55, 337-340.

Gaibaizadeh, R., Staecker, H., Liu, W., Kopke, R.D., Malgrange, B., Lefebvre, P.P., Van De Water, T., 1997. Protection of both auditory hair cells and auditory neurons from cisplatin induced damage. Acta Otolaryngol. (Stockh.) 117, 232-238.

Hamers, F. P. T., Biessels, G. J., Van Dam, S., Gispen W. H., 1997. Neuroprotection in diabetic and toxic neuropathies. In: Bär, P.R., Flint Beal, M. (Eds.) Neuroprotection in CNS diseases. Marcel Dekker, New York, Inc., pp. 513-554.

Hamers, F.P.T, Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by α-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

Heijmen, P.S., Klis, S.F.L., De Groot, J.C.M.J. Smoorenburg, G.F., 1999. Cisplatin ototoxicity and the possibly protective effect of alpha-melanocyte stimulating hormone. Hear. Res. 128, 27-39.

Hoeve, L.J., Mertens Zur Borg, I.R.A.M., Rodenburg, M., Brocaar, M.P., Groen, B.G.S., 1989. Correlations between cis-platinum dosage and toxicity in a guinea pig model. Arch. Otorhinolaryngol. 245, 98-102.

Jiang, D., Furness, D.N., Hackney, C.M., Lopez, D.E., 1993. Microslicing of the resin-embedded cochlea in comparison with the surface preparation technique for analysis of hair cell number and morphology. Br. J. Audiol. 27, 195-203.

Klis, S.F.L., O'Leary, S.J., Hamers, F.P.T., De Groot, J.C.M.J., Smoorenburg, G.F., 2000. Reversible cisplatin ototoxicity in the albino guinea pig. NeuroReport 11, 623-626.

Klis, S.F.L., O'Leary, S.J., Wijbenga, J., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2002. Partial recovery of cisplatin-induced hearing loss in the albino guinea pig in relation to cisplatin dose. Hear. Res. 164, 138-146.

Koch, T., Gloddek, B., 1991. Inhibition of adenylate-cyclase-coupled G protein complex by ototoxic diuretics and cis-platinum in the inner ear of the guinea pig. Eur. Arch. Otorhinolaryngol. 248, 459-464.

Kohn, S., Fradis, M., Pratt, H., Zidan, J., Podoshin, L., Robinson, E., Nir, I., 1988. Cisplatin ototoxicity in guinea pigs with special reference to toxic effects in the stria vascularis. Laryngoscope 98, 865-871.

Komune, S., Asakuma, S., Snow, J.B.J., 1981. Pathophysiology of the ototoxicity of cis-diamminedichloroplatinum. Otolaryngol. Head Neck Surg. 89, 275-282.

Konishi, T., Gupta, B.N., Prazma, J., 1983. Ototoxicity of cis-dichlorodiammine platinum (II) in guinea pigs. Am. J. Otolaryngol. 4, 18-26.

Laurell, G., Engström, B., 1989. The ototoxic effect of cisplatin on guinea pigs in relation to dosage. Hear. Res. 38, 27-34.

Laurell, G., Bagger-Sjöbäck, D., 1991a. Degeneration of the organ of Corti following intravenous administration of cisplatin. Acta Otolaryngol. (Stockh.) 111, 891-898.

Laurell, G., Bagger-Sjöbäck, D., 1991b. Dose-dependent inner ear changes after I.V. administration of cisplatin. J. Otolaryngol. 20, 158-167.

Meech, R.P., Campbell, K.C.M., Hughes, L.F., Rybak, L.P., 1998. A semiquantative analysis of the effects of cisplatin on the rat stria vascularis. Hear. Res. 124, 44-59.

Miller, J.M., Chi, D.H., O'Keeffe, L.J., Kruszka, P., Raphael, Y., Altschuler, R.A., 1997. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. Int. J. Devl. Neurosci. 15, 631-643.

Nakai, Y., Konishi, K., Chang, K.C., Ohashi, K., Morisaki, N., Minowa, Y., Morimoto, A., 1982. Ototoxicity of the anticancer drug cisplatin. An experimental study. Acta Otolaryngol. (Stockh.) 93, 227-232.

O'Leary, S.J., Klis, S.F.L., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2001. Perilymphatic application of cisplatin over several days in albino guinea pigs: Dose-dependency of electrophysiological and morphological effects. Hear. Res. 154, 135-145.

Saito, T., Aran, J.M., 1994. Comparative ototoxicity of cisplatin during acute and chronic treatment. ORL 56, 315-320.

Saito, T., Manabe, Y., Honda, N., Yamada, T., Yamamoto, T., Saito, H., 1995. Semiquantitative analysis by scanning electron microscopy of cochlear hair cell damage by ototoxic drugs. Scanning Microsc. 9, 271-281.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Sluyter, S., Klis, S.F.L, De Groot, J.C.M.J., Smoorenburg, G. F., 2003. Alterations in the stria vascularis in relation to cisplatin ototoxicity and recovery. Hear. Res. 185, 49-56.

Spoendlin, H., 1979. Anatomischen-pathologische Aspekte der Elektrostimulation des ertaubten Innenohres. Arch. Otorhinolaryngol. 223, 1-75.

Spoendlin, H., Brun, J.P., 1974. The block-surface technique for evaluation of cochlear pathology. Arch. Otorhinolaryngol. 208, 137-145.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1997. Cisplatin-induced ototoxicity: Electrophysiological evidence of spontaneous recovery in the albino guinea pig. Hear. Res. 111, 103-113.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1998. Cisplatin ototoxicity. An electrophysiological dose-effect study in albino guinea pigs. Hear. Res. 124, 99-107.

Tange, R.A., 1984. Differences in the cochlear degeneration pattern in the guinea pig as a result of gentamicin and cis-platinum intoxication. Clin. Otolaryngol. 9, 323-327.

Tange, R.A., Vuzevski, V.D., 1984. Changes in the stria vascularis of the guinea pig due to cis-platinum. Arch. Otorhinolaryngol. 239, 41-47.

Tange, R.A., Conijn, E.A.J.G., Van Zeijl, L.G.P.M., 1982. The cortitoxic effects of cis-platinum in the guinea pig. Arch. Otorhinolaryngol. 237, 17-26.

Webster, M., Webster, D.B., 1981. Spiral ganglion neuron loss following organ of Corti loss: A quantitative study. Brain Res. 212, 17-30.

Ylikoski, J., Wersäll, J. Björkroth, B., 1974. Correlative studies on the cochlear Pathology and hearing loss in guinea pigs after intoxication with ototoxic antibiotics. Acta Otolaryngol. (Stockh.) Supplement 326, 23-41.

Zheng, J.L., Stewart, R.R., Gao, W.Q., 1995. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. J. Neurosci. 15, 5079-5087.

Zheng, J.L., Gao, W.Q., 1996. Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur. J. Neurosci. 8, 1897-1905.

The cochlear targets of cisplatin. An electrophysiological and morphological time-sequence study

Marjolein W.M. van Ruijven, John C.M.J. de Groot, Sjaak F.L. Klis, Guido F. Smoorenburg

Hearing Research, manuscript in revision

Chapter 3

Abstract

Cisplatin ototoxicity has at least three major targets in the cochlea: the stria vascularis, the organ of Corti, and the spiral ganglion. This study aims to differentiate between these three targets. In particular, we address the question of whether the effects at the level of the organ of Corti and spiral ganglion are mutually dependent or whether they develop in parallel. This question was approached by studying the ototoxic effects while they develop electrophysiologically and comparing these to earlier presented histological data (Van Ruijven et al., 2004). Guinea pigs were treated with intraperitoneal injections of cisplatin at a dose of 2 mg/kg/day for either 4, 6 or 8 consecutive days. This time sequence has not revealed any evidence of one ototoxic process triggering another. Therefore, we have to stay with the conclusion of Van Ruijven et al (2004) that both processes run in parallel.

1. Introduction

Cisplatin is a platinum-containing compound which is effectively and widely used in the treatment of epithelial tumors. However, this cytotoxic drug produces several adverse effects such as peripheral neuropathies, renal insufficiency, and ototoxicity. Cisplatin-induced ototoxicity in humans is generally manifested as sensorineural hearing loss, which may be accompanied by tinnitus. The hearing loss is dose-related, usually permanent, and is characterized by an initial high-frequency deficit in both ears (De Oliveira, 1989; Schweitzer, 1993).

Animal studies have demonstrated that the histological lesions in the cochlea after cisplatin administration are most prominent in the organ of Corti, and consist of degeneration and loss of the sensory cells. This loss typically starts at the first row of outer hair cells (OHCs) in the basal turn of the cochlea followed by the other OHC rows and, with increasing doses or prolonged administration, progresses towards the more apically located cochlear turns and eventually to the inner hair cells (IHCs) (Komune et al., 1981; Nakai et al., 1982; Konishi et al., 1983; De Oliveira, 1989; Schweitzer, 1993; Kaltenbach et al., 1997; De Groot et al., 1997; Kamimura et al., 1999; Cardinaal et al., 2000a; Van Ruijven et al., 2004). These findings correlate well with the permanent frequency-dependent elevation in the compound action potential (CAP) thresholds and the irreversible suppression of hair-cell related potentials and emissions, such as the cochlear microphonics (CM) and the distortion-product otoacoustic emissions (DPOAEs) (Komune et al., 1981; Konishi et al., 1983; Hamers et al, 1994; Sie and Norton, 1997; Stengs et al., 1998; Heijmen et al., 1999; Wang et al., 2003).

There is now overwhelming evidence that cisplatin also exerts a (reversible) effect upon the stria vascularis. Cisplatin-induced reduction of the endocochlear potential (EP) – which is generated and maintained by strial processes – has been described after application of single high-dose injections (Komune et al., 1981; Laurell and Engstrom, 1989; Komune et al., 1995; Ravi et al., 1995), following repeated systemic administration (Konishi et al., 1983; Klis et al., 2000, 2002; O'Leary and Klis, 2002; Hamers et al., 2003; Sluyter et al., 2003) and during perilymphatic administration (Ford et al., 1997; Tsukasaki et al., 2000; O'Leary et al., 2001; Wang et al., 2003). Histologically, damage to the stria vascularis consists of intermediate cell atrophy, swelling and blebbing of the marginal cells, and vacuolation of the latter's cytoplasm (Saito and Aran, 1994; Meech et al., 1998; Campbell et al., 1999; Cardinaal et al., 2000a; Sluijter et al., 2003).

Also, as cisplatin is known to affect peripheral sensory nerves and to induce peripheral neuropathies in patients (for a review, see Hamers et al., 1997), an

effect on neural elements in the cochlea might be expected. Cisplatin-induced neuronal death and degeneration of spiral ganglion cells (SGCs) is a common finding in organotypic explant cultures of the postnatal rodent cochlea and in dissociated spiral ganglia cultures (Zheng et al., 1995; Zheng and Gao, 1996; Gabaizadeh et al., 1997; Feghali et al., 2001; Bowers et al., 2002). Cardinaal et al. (2000a) observed cytoplasmic vacuolation in SGCs of adult guinea pigs treated with low doses (≤1.5 mg/kg/day) of cisplatin, which was later demonstrated to be due to mitochondrial swelling (Cardinaal et al., 2004). In addition, cisplatin may induce cell shrinkage (O'Leary et al., 2001) and nuclear shrinkage (Hamers et al., 2003) in SGCs of the adult guinea-pig cochlea. Moreover, Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in SGCs of mongolian gerbil cochleas after cisplatin administration, suggesting the involvement of apoptosis in cisplatin-induced SGC degeneration.

From these experimental findings it is evident that cisplatin ototoxicity has at least three major targets in the cochlea: the organ of Corti, the stria vascularis, and the spiral ganglion. There is no consensus of opinion, however, with regard to whether the drug's intracochlear effects develop in parallel or are mutually dependent. In an earlier paper (Van Ruijven et al., 2004), we investigated the time sequence of the histological degeneration pattern in the guinea pig cochlea during cisplatin administration, in order to gain more insight into this issue. In that study two main effects were described. First, cisplatin affects the organ of Corti, with OHC loss as the most prominent feature. The second effect was on the spiral ganglion and consisted of detachment of the myelin sheath enveloping the SGCs, which proved to be due to perikaryal shrinkage of the SGCs and simultaneous swelling of the myelin sheath. Apparently, cisplatin-induced OHC loss and myelin sheath detachment develop in parallel: they first occur between the fourth and sixth day of administration and progress after 8 days.

In order to differentiate between the individual contributions of the OHCs and SGCs in the deterioration of the cochlear response after cisplatin administration, we have compared the individual electrophysiological data (CAP, CM) with the most prominent histological parameters (OHC loss, detachment of the myelin sheath of SGCs).

2. Materials and methods

2.1. Experimental design

Healthy, female albino guinea pigs (strain Dunkin Hartley, weighing 250-350 g.) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the Animal Care Facility of the Central Laboratory Animal Institute of Utrecht University. Animals had free access to food and water and were kept under standard laboratory conditions. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC #89055). Animal care was under the supervision of the Central Laboratory Animal Institute of Utrecht University. Cisplatin (Platinol®, containing 1 mg cisplatin per ml injection fluid; Bristol-Myers Squibb, Woerden, the Netherlands) was diluted with sterile physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml. This cisplatin solution was stored in the dark at room temperature. During the entire experiment it was made sure that the shelf life did not exceed the maximum limit of 3 days, as recommended by the Hospital Pharmacist.

Thirty-five animals were assigned at random to either a non-treated group or one of the three experimental groups. Five animals received no cisplatin and served as controls. The remaining animals (n=30) were treated with cisplatin (2 mg/kg/day) by daily intraperitoneal injections. Animals were weighed every morning and the injection volume was adjusted accordingly for each individual animal. The first experimental group received cisplatin for 4 consecutive days (total dose: 8 mg/kg; n=10), the second group for 6 consecutive days (total dose: 12 mg/kg; n=10), and the third group for 8 consecutive days (total dose: 16 mg/kg; n=10). At the end of the experiment all animals had survived. These animals were also used in the histological study by Van Ruijven et al. (2004).

2.2. Electrocochleography

Electrocochleography of the right ears only was performed 24 h after the final injection (non-treated group: n=5 cochleas; three experimental groups: n=10 cochleas, each group). Techniques for electrocochleography were described previously (Stengs et al., 1997) and are only summarized here. Auditory evoked responses were recorded differentially with a silverball electrode at the apex of the cochlea and a reference electrode in the muscles of the neck. Trains of 8-ms tone bursts of 2, 4, 8, and 16 kHz were presented with alternating polarity. Averaged responses were stored for off-line analysis. The compound action potential (CAP) was obtained by addition of the responses to opposite polarity stimuli, the cochlear microphonics (CM) by subtraction. CAP amplitude was defined relative

to the summating potential. The CM was determined as the peak-to-peak value of the sinusoidal response. CM was not measured at 16 kHz, because it exceeds the upper cut-off frequency of our measurement system. Threshold was defined as the stimulus level that produced a 10 μV response. We determined the threshold by visual interpolation of the data of individual animals.

2.3. Histological examination and quantification

Immediately after electrocochleography, the left and right cochleas were fixed and processed for histological examination. OHCs counts and SGC analyses were performed in semithin (1 μm) midmodiolar sections of both the right and left cochleas from the non-treated group (n=10 cochleas) and the three experimental groups (n=20 cochleas, each group). OHC counts were expressed as the mean percentage of remaining OHCs per individual transection of the respective half turn (two transections each for the basal and middle turns; three transections for the apical turn; Fig. 1). SGC analyses consisted of determination of: (1) SGC densities; and (2) the percentage of type-I SGCs demonstrating detachment of their myelin sheaths. More detailed information concerning the histological procedures and the light-microscopic evaluation criteria can be found in the paper by Van Ruijven et al. (2004). An estimate was made of the characteristic frequency of locations along the basilar membrane at which the OHCs and SGCs were counted, using the place-frequency map for guinea pigs (Greenwood, 1990; cf., Fig 1). For the comparison of the individual electrophysiological data (CAP, CM) and the histological parameters (OHC loss, myelin sheath detachment), only the histological data of the right ears were used.

location	d (mm from apex)	c.f. (kHz)
b1	16.5	26.2
b2	12.5	10.4
m1	9.5	5.1
m2	7.5	2.7
a1	5.5	1.3
a2	3.5	0.7
a3	1.5	0.3

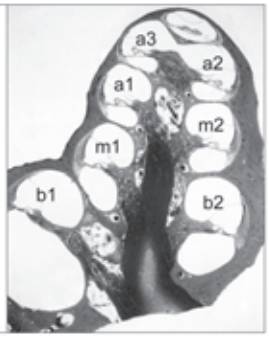


Figure 1.

Midmodiolar section (1 μm) of the cochlea. There are two transections each for the basal (b1 and b2) and middle (m1 and m2) turns, and three transections for the apical turn (a1, a2, a3). Distance (d) is the position along the basilar membrane in mm from the apex. The characteristic frequency (c.f.) was computed using the place-frequency map for guinea pigs (Greenwood, 1990).

We have chosen for the midmodiolar approach because it has the additional advantage that counting hair cells in midmodiolar sections is less time-consuming and that it not only gives a better impression of the gross morphology of the hair cells but also allows for morphological analyses of the other cochlear tissues (e.g. stria vascularis and spiral ganglion) in the corresponding cochlear turn(s). The latter feature of the method is essential in a correlation-oriented study like this.

3. Results

Figure 2 presents the averaged CAP and CM amplitude growth curves for the non-treated group and the three experimental groups, arranged according to stimulus frequency. The salient effect is that cisplatin administration causes a shift in the growth curves towards the higher stimulus levels, which is especially pronounced in the 8-day group, but clearly is already present in the 6-day group.

Two additional effects attract attention: (1) shifts in amplitude growth curves seem larger at the higher frequencies, especially for the CAP; and (2) shifts in the CM amplitude growth curve seem generally smaller than those for the CAP.

Analysis of variance (ANOVA) for repeated measurements was performed on separate datasets for both the CAP and CM. Logarithmic transformation was performed before analysis to improve homogeneity of variance. To avoid floor effects at lower levels, only the four highest stimulus levels were included in the analyses. Duration of treatment was a between-subjects factor, frequency and level of stimulation were within-subject factors.

For the CAP, we found significant main effects of duration of treatment ($F_{3,29}$=11.55, P<0.0001) as well as of (trivial) frequency and level of stimulation. All interactions were significant, including that between frequency and duration of treatment ($F_{9,87}$=4.95, P<0.0001). This indicates that at higher frequencies significantly larger shifts occur. Similar results were found for the CM, except for the interaction between frequency and duration of treatment ($F_{6,62}$=1.16, P=0.34), which indicates that the CM shift is independent of frequency.

To illustrate interanimal variability, we have plotted the individual CAP thresholds (10 μV iso-response levels) at 4-kHz stimulation, arranged per experimental group, in Fig. 3. It is apparent that the effect on threshold increases with duration of treatment. In the 6-day group, especially, a bimodal distribution appears to be present: some animals were not affected at all, while others showed threshold elevations of 50 dB or more.

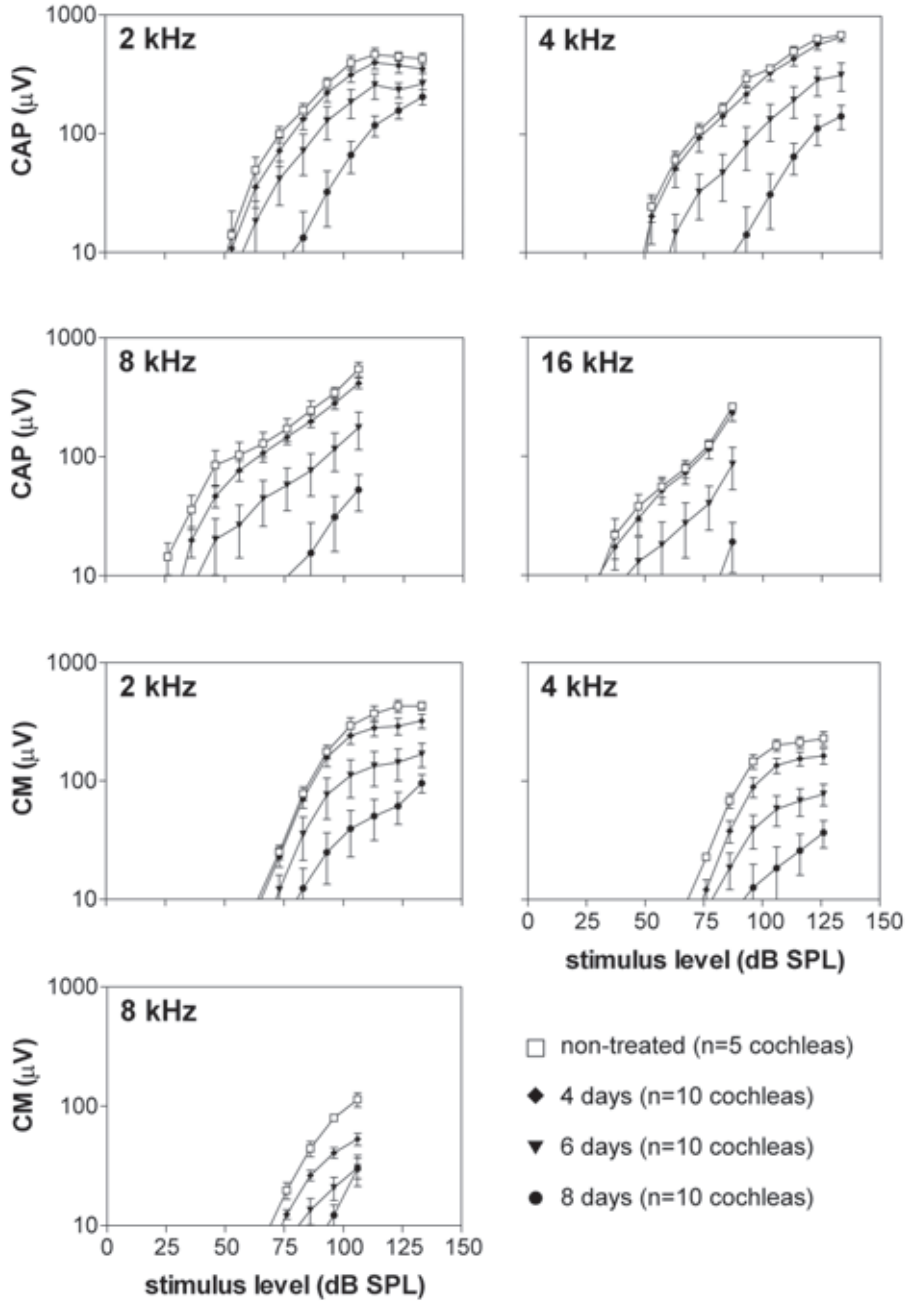


Figure 2.

Averaged CAP and CM amplitude (± standard error of the mean) growth curves for the non-treated group and the three experimental groups (4, 6, and 8 days at 2.0 mg/kg/day) arranged by stimulus frequency.

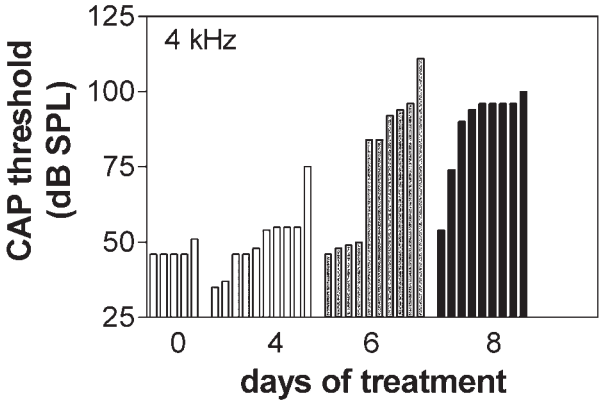


Figure 3.

Individual CAP thresholds (10 μV iso-response levels at 4-kHz stimulation) for the non-treated group and the experimental groups (4, 6, and 8 days at 2.0 mg/kg/day).

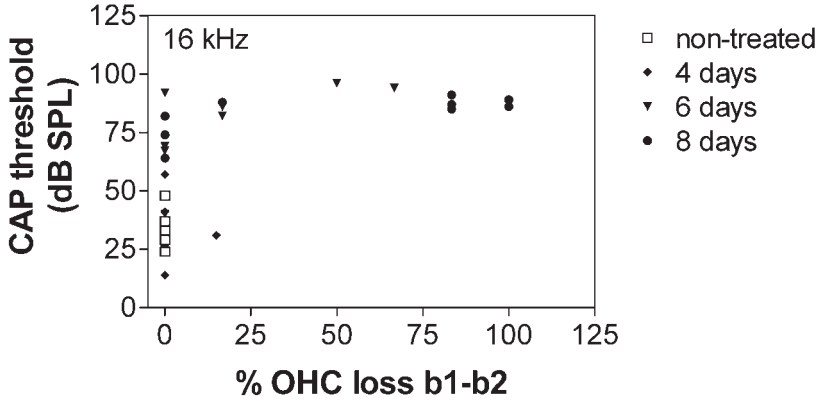


Figure 4.

CAP threshold at 16-kHz stimulation plotted against averaged OHC counts of the lower and upper basal turns (at locations b1 and b2; cf., Fig 1).

The correlation between individual electrophysiological data and histological data could be analyzed in view of high interanimal variability. OHCs were counted in midmodiolar sections in all animals. The averaged OHC counts of both basal locations (b1 and b2) were plotted against CAP threshold at 16-kHz stimulation (Fig. 4). Remarkably, about half of the animals with evidently raised thresholds (>50 dB SPL) did not have any OHC loss at all. However, the animals did show another histological feature which may account for this discrepancy. In some type-I SGCs the myelin sheath was detached from the perikaryon, leaving a void space between the cell body and the enveloping myelin sheath (Figs. 5A-B).

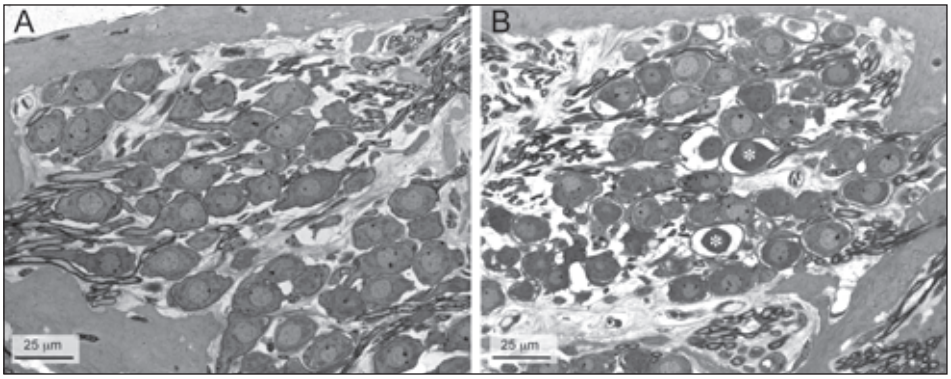


Figure 5.
Light micrographs of the spiral ganglion in basal turns of both a non-treated animal (A) and an animal treated with cisplatin for 8 days (B). Asterisks denote type-I SGCs demonstrating detachment of their myelin sheaths.

Previously, we have demonstrated that cisplatin-induced myelin sheath detachment is due to both perikaryal shrinkage and swelling of the myelin sheath (Van Ruijven et al., 2004). We counted the number of affected cells relative to the total number in midmodiolar sections of the basal turn (b1 and b2), and these were plotted against CAP threshold at the corresponding frequency of 16 kHz (Fig. 6). Clearly, two clusters of data points can be discerned: one with a lower CAP threshold (<60 dB SPL) and relatively few affected type-I SGCs (<25%), and a second cluster with a higher CAP threshold and more affected type-I SGCs. The animals in the second cluster all belong to the groups treated for 6 or 8 days. Cluster analysis (K-means clustering with 2 clusters) indeed assigned every animal to its expected cluster. Several animals in the second cluster (i.e., high CAP and high number of affected type-I SGCs) did not have any

associated OHC loss (Fig. 6: open symbols).
Within a treatment period of 5-11 days, administration of cisplatin at a daily dose of 2 mg/kg is known to result in a decrease in the EP (Klis et al., 2000, 2002). Such a decrease may be responsible for part of the threshold alterations. On the other hand, it also may cause histological alterations in the stria vascularis. However, in these animals significant morphological alterations in the stria vascularis were not observed (Van Ruijven et al., 2004). Therefore, histological data of the stria vascularis were not used for correlation analysis.

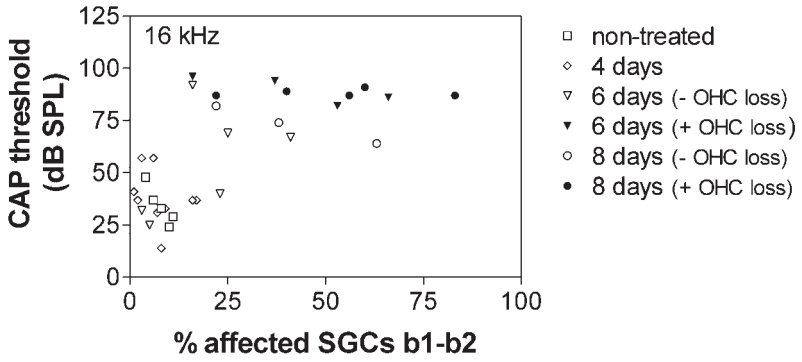


Figure 6.

The relative number of type-I SGCs showing myelin detachment is plotted against CAP threshold at 16-kHz stimulation for the non-treated group and the experimental groups (4, 6, and 8 days). Open symbols denote animals without OHC loss; closed symbols denote animals demonstrating (partial) OHC loss.

4. Discussion

CAP and CM data after cisplatin administration are in general agreement with previous studies from our group. Administration of cisplatin at a daily dose of 2 mg/kg results in a pronounced elevation of the CAP threshold after 8 days (Hamers et al., 1994; Stengs et al., 1998; Heijmen et al., 1999; Klis et al., 2002). A similar elevation has been reported already after 5 days of cisplatin administration (Wang et al., 2003), and is in line with the present data that some animals have a CAP threshold shift after 4-6 days.

In addition, CM thresholds increased after cisplatin administration. Comparable results were obtained by Konishi et al. (1983), Stengs et al. (1998), Heijmen et al. (1999) and Klis et al. (2002). In the 6-day group, some animals demonstrated a large threshold shift, whereas others hardly showed any effect at all (Fig. 3). This dichotomous distribution has been reported previously by Hamers et al. (1994) and Heijmen et al. (1999).

From Fig. 2 it is clear that the reduction in CAP amplitude is larger than the accompanying reduction in CM amplitude, implying that cisplatin, in addition, exerts an effect upon the SGCs or the auditory neurons (Stengs et al., 1997). This assumption is strengthened by our observation that several animals showed elevated CAP thresholds, but no obvious OHC loss (Fig. 4). SGCs, unlike OHCs, have received little attention in functional studies of cisplatin ototoxicity. Histologically, however, Cardinaal et al. (2000a, 2004) observed mitochondrial swelling in the SGCs of adult guinea pigs treated with low doses (≤1.5 mg/kg/day) of cisplatin, but the functional consequences, in terms of the CAP, were not investigated. Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in SGCs of Mongolian gerbils treated with cisplatin (4 mg/kg/day) for 5 days, suggesting that cisplatin interferes with the regulatory pathways involved in and leading to apoptotic cell death. However, we did not find any loss of SGCs after cisplatin administration, not even after 8 days of treatment (Van Ruijven et al., 2004). Hamers et al. (2003) reported that cisplatin may induce nuclear shrinkage in the SGCs of adult guinea pigs. O'Leary et al. (2001), on the other hand, reported shrinkage of the SGCs after intracochlear application of cisplatin with osmotic pumps, but they did not elaborate on the possible functional consequences of their observations.

The data presented in Fig. 6 suggest that detachment of the myelin sheath of the type-I SGCs may result in elevated CAP thresholds. However, concurrent effects of cisplatin on hair cell function and the EP may also have contributed to this increase. In addition, these effects will also interfere with the CM.

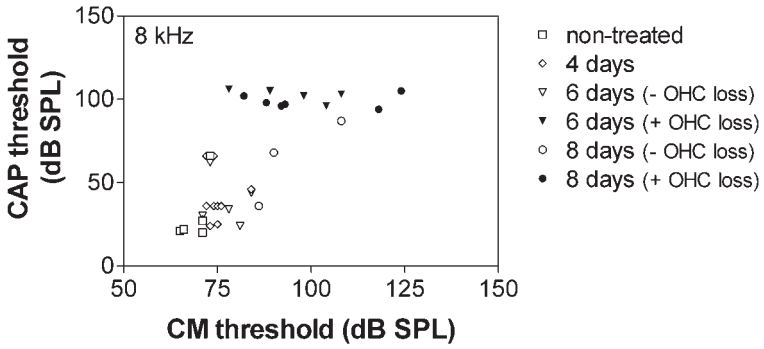


Figure 7.

CAP threshold as a function of CM threshold, distinguishing between the animals without and with OHC loss. Open symbols denote animals without OHC loss; closed symbols denote animals demonstrating (partial) OHC loss.

Figure 7 shows CAP threshold as a function of CM threshold, distinguishing between animals without OHC loss and those with (partial) loss. It is evident from this graph that animals with (partial) OHC loss have high CAP thresholds, even at low CM thresholds. The animals without OHC loss exhibit CAP thresholds that are proportional to the CM threshold. The latter simply reflects the fact that changes in CM, induced by changes in EP and/or by dysfunction of OHCs, will affect the CAP. However, the high CAP thresholds found at low CM thresholds for the animals with partial OHC loss, strongly suggest that a component other than hair cell function, or EP, has affected the CAP. Potentially, this component could be dysfunction of the SGCs. This inference is supported by further quantitative analysis. Arguably, the missing component would manifest itself in a relatively large difference between the CAP threshold and the CM threshold. We found that in the group with OHC loss, there is indeed a significant correlation between this difference ($threshold_{CAP}$ minus $threshold_{CM}$) and the percentage of affected SGCs ($r=0.72$; $P=0.01$). Thus, the present results suggest that once animals are severely affected by cisplatin administration (judging from the loss of OHCs), their CAP threshold shift cannot be explained completely by their CM threshold shift, but an additional component, apparently involving SGCs is active. However, we cannot exclude that concomitant damage to IHCs and possibly tectorial membrane malfunction also play a part. In conclusion, our results demonstrate that cisplatin at a daily dose of 2 mg/kg affects at least two sites in the cochlea: the organ of Corti (particularly the OHCs) and the SGCs. Furthermore, we know

from previous studies (Klis et al., 2000, 2002; Sluyter et al., 2003) that the EP is affected, although we could not correlate this with morphological alterations in the stria vascularis. Van Ruijven et al. (2004) showed that OHC loss and SGC degeneration occurred between 4 and 6 days of cisplatin administration. With the present study, we tried to differentiate between these two tissues using both electrophysiological and histological data. However, this time sequence has not revealed any evidence of one ototoxic process triggering another. Therefore, we have to stay with the conclusion of Van Ruijven et al (2004) that both processes run in parallel."

Acknowledgements
This study has been supported by grants from the Heinsius-Houbolt Foundation, the Netherlands. The authors wish to thank E.G.J. Hendriksen for his technical assistance, and N.L.M. Cappaert and F.L.C. Wolters for performing some of the electrophysiological measurements.
Marjolein W.M. van Ruijven is a Heinsius-Houbolt Foundation Research Fellow at the Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands.

References

Alam, S.A., Ikeda, K., Oshima, T., Suzuki, M., Kawase, T., Kikuchi, T., Takasaka, T., 2000. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. Hear. Res. 141, 28-38.

Bowers, W.J., Chen, X., Guo, H., Frisina, D.R., Federoff, H.J., Frisina, R.D., 2002. Neurotrophin-3 transduction attenuates cisplatin spiral ganglion neuron ototoxicity in the cochlea. Mol. Ther. 6, 12-18.

Campbell, K.C.M., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-methionine protects against cisplatin damage to the stria vascularis. Hear. Res. 138, 13-28.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000a. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000b. Histological effects of co-administration of an $ACTH_{(4-9)}$ analogue, ORG 2766, on cisplatin ototoxicity in the albino guinea pig. Hear. Res. 144, 157-167.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh.) 124, 144-154.

De Groot, J.C.M.J., Hamers, F.P.T., Gispen, W.H., Smoorenburg, G.F., 1997. Co-administration of the neurotropic $ACTH_{(4-9)}$ analogue, ORG 2766, may reduce the cochleotoxic effects of cisplatin. Hear. Res. 106, 9-19.

De Oliveira, J.A.A., 1989. Audiovestibular Toxicity of Drugs, Volume II. CRC Press, Boca Raton, Florida, pp. 181-198.

Feghali, J.G., Liu, W., Van De Water, T.R., 2001. L-*N*-acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. Laryngoscope 111, 1147-1155.

Ford, M.S., Zhongzhen, N., Whitworth, C., Rybak, L.P., Ramkumar, V., 1997. Up-regulation of adenosine receptors in the cochlea by cisplatin. Hear. Res. 111, 143-152.

Gaibaizadeh, R., Staecker, H., Liu, W., Kopke, R.D., Malgrange, B., Lefebvre, P.P., Van De Water, T., 1997. Protection of both auditory hair cells and auditory neurons from cisplatin induced damage. Acta Otolaryngol. (Stockh.) 117, 232-238.

Greenwood, D.D., 1990. A cochlear frequency-position function for several species. 29 years later. J. Acoust. Soc. Am. 87, 2592-2605.

Hamers, F.P.T., Klis, S.F.L., Gispen, W.H., Smoorenburg, G.F., 1994. Application of a neuroprotective $ACTH_{(4-9)}$ analog to affect cisplatin ototoxicity: An electrocochleographic study in guinea pigs. Eur. Arch. Otorhinolaryngol. 251, 23-29.

Hamers, F.P.T., Biessels, G.J., Van Dam, S., Gispen W.H., 1997. Neuroprotection in diabetic and toxic neuropathies. In: Bär, P.R., Flint Beal, M. (Eds.) Neuroprotection in CNS Diseases. Marcel Dekker, New York, Inc., pp. 513-554.

Hamers, F.P.T, Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by α-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

Heijmen, P. S., Klis, S.F.L., De Groot, J.C.M.J., Smoorenburg, G.F., 1999. Cisplatin ototoxicity and the possibly protective effect of alpha-melanocyte stimulating hormone. Hear. Res. 128, 27-39.

Kaltenbach, J., Church, M.W., Blakley, B.W., McCaslin, D.L., Burgio, D.L., 1997. Comparison of five agents in protecting the cochlea against the ototoxic effects of cisplatin in the hamster. Otolaryngol. Head Neck Surg. 117, 493-500.

Kamimura, T., Whitworth, C.A., Rybak, L.P., 1999. Effect of 4-methylthiobenzoic acid on cisplatin-induced ototoxicity in the rat. Hear. Res. 131, 117-127.

Klis, S.F.L., O'Leary, S.J., Hamers, F.P.T., De Groot, J.C.M.J., Smoorenburg, G.F., 2000. Reversible cisplatin ototoxicity in the albino guinea pig. NeuroReport 11, 623-626.

Klis, S.F.L., O'Leary, S.J., Wijbenga, J., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2002. Partial recovery of cisplatin-induced hearing loss in the albino guinea pig in relation to cisplatin dose. Hear. Res. 164, 138-146.

Komune, S., Asakuma, S., Snow, J.B.J., 1981. Pathophysiology of the ototoxicity of cis-diamminedichloroplatinum. Otolaryngol. Head Neck Surg. 89, 275-282.

Komune, S., Matsuda, K., Nakagawa, T., Kimitsuki, T., Hisashi, K., Inokuchi, A., Komiyama, S., Kobayashi, T., 1995. Disturbance of regulation of sodium by cis-diamminedichloroplatinum in perilymph of the guinea pig cochlea. Ann. Otol. Rhinol. Laryngol. 104, 149-154.

Konishi, T., Gupta, B.N., Prazma, J., 1983. Ototoxicity of cis-dichlorodiammine platinum (II) in guinea pigs. Am. J. Otolaryngol. 4, 18-26.

Laurell, G., Engström, B., 1989. The ototoxic effect of cisplatin on guinea pigs in relation to dosage. Hear. Res. 38, 27-34.

Meech, R.P., Campbell, K.C.M., Hughes, L.F., Rybak, L.P., 1998. A semiquantative analysis of the effects of cisplatin on the rat stria vascularis. Hear. Res. 124, 44-59.

Nakai, Y., Konishi, K., Chang, K.C., Ohashi, K., Morisaki, N., Minowa, Y., Morimoto, A., 1982. Ototoxicity of the anticancer drug cisplatin. An experimental study. Acta Otolaryngol. (Stockh.) 93, 227-232.

O'Leary, S.J., Klis, S.F.L., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2001. Perilymphatic application of cisplatin over several days in albino guinea pigs: Dose-dependency of electrophysiological and morphological effects. Hear. Res. 154, 135-145.

O'Leary, S.J., Klis, S.F., 2002. Recovery of hearing following cisplatin ototoxicity in the guinea pig. Anticancer Res. 22, 1525-1528.

Ravi, R., Somani, S.M., Rybak, L.P., 1995. Mechanism of cisplatin ototoxicity: Antioxidant system. Pharmacol. Toxicol. 76, 386-394.

Saito, T., Aran, J.M., 1994. Comparative ototoxicity of cisplatin during acute and chronic treatment. ORL 56, 315-320.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Sie, K.C.Y., Norton, S.J., 1997. Changes in otoacoustic emissions and auditory brain stem response after cis-platinum exposure in gerbils. Otolaryngol. Head Neck Surg. 116, 585-592.

Sluyter, S., Klis, S.F.L., De Groot, J.C.M.J., Smoorenburg, G.F., 2003. Alterations in the stria vascularis in relation to cisplatin ototoxicity and recovery. Hear. Res. 185, 49-56.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1997. Cisplatin-induced ototoxicity: Electrophysiological evidence of spontaneous recovery in the albino guinea pig, Hear. Res. 111, 103-113.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1998. Cisplatin ototoxicity. An electrophysiological dose-effect study in albino guinea pigs. Hear. Res. 124, 99-107.

Tsukasaki, N., Whitworth, C.A., Rybak, L.P., 2000. Acute changes in cochlear potentials due to cisplatin. Hear. Res. 149, 189-198.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Smoorenburg, G.F., 2004. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. Hear. Res. 197, 44-54.

Wang, J., Lloyd Faulconbridge, R.V., Fetoni, A., Guitton, M.J., Pujol, R., Puel, J.L., 2003. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacol. 45, 380-393.

Zheng, J.L., Stewart, R.R., Gao, W.Q., 1995. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. J. Neurosci. 15, 5079-5087.

Zheng, J.L., Gao, W.Q., 1996. Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur. J. Neurosci. 8, 1897-1905.

Immunohistochemical detection of platinated DNA in the cochlea of cisplatin-treated guinea pigs

Marjolein W.M. van Ruijven, John C.M.J. de Groot, Ferry Hendriksen, Guido F. Smoorenburg

Hearing Research 2005 (in press)

Chapter 4

Abstract

Cisplatin-induced ototoxicity is correlated with functional and morphological changes in the organ of Corti, the stria vascularis and the spiral ganglion. However, the cochlear sites of cisplatin uptake and accumulation have not been properly identified. Therefore, we have developed an immunohistochemical method to, indirectly, detect cisplatin in semithin cryosections of the guinea pig cochlea (basal turn) using an antiserum containing antibodies against cisplatin-DNA adducts. Platinated DNA was present in the nuclei of most cells in the organ of Corti and the lateral wall after cisplatin administration. Nuclear immunostaining was most pronounced in the outer hair cells, the marginal cells and the spiral ligament fibrocytes. This study is the first to demonstrate the presence of cisplatin in histological sections of the cochlea.

1. Introduction

Cisplatin-induced ototoxicity is characterized by an initial high-frequency hearing deficit, which is associated with a loss of the hair cells in the organ of Corti (for reviews, see De Oliveira, 1989; Schweitzer, 1993). In addition, functional and morphological changes have been observed in the stria vascularis, whereas there is also evidence that the spiral ganglion is affected after cisplatin administration (Schweitzer, 1993; Cardinaal et al., 2000; Hamers et al., 2003; Van Ruijven et al., 2004). However, despite several attempts, the cochlear sites of cisplatin uptake and accumulation have not been properly identified.

Numerous methods are available to demonstrate cisplatin in biological fluids or homogenized tissue samples (cf., Johnsson et al., 1995; Verschraagen et al., 2002). Using a radiographic method, Schweitzer (1993) detected [^{195m}Pt]-labelled cisplatin in homogenated samples of the organ of Corti and the stria vascularis. However, these techniques cannot be used to detect cisplatin in histological sections. Since the cisplatin molecule contains a central platinum (Pt) atom (Fig. 1), X-ray microanalytic techniques are the obvious choice for demonstrating the presence of cisplatin in ultrathin sections of tissues. Using X-ray microanalysis, Pt has been detected in cisplatin-treated kidney tissue (Berry et al., 1982; Makita et al., 1986; Saito and Aran, 1994a) and tumor cell lines (Khan and Sadler, 1978). In contrast, studies investigating the cochlear distribution of cisplatin using ultrastructural X-ray microanalysis have failed to detect Pt in the organ of Corti (Maruyama et al., 1993; Saito and Aran, 1994a; Welb, 1995), whereas data on the stria vascularis are conflicting. Although Maruyama et al. (1993) detected Pt in ultrathin sections of the stria vascularis after cisplatin administration by means of X-ray microanalysis, Welb (1995) could not corroborate this finding with sensitive techniques such as electron energy-loss spectrometry and laser microprobe mass spectrometry. The failure to detect cisplatin in ultrathin sections of the cochlea by means of X-ray microanalysis in the former studies may be explained by the fact that the intracellular concentration of Pt was below the detection limit (Saito and Aran, 1994a). On the other hand, cisplatin could be extracted from the cochlear tissues during chemical fixation and subsequent histological processing (Saito and Aran, 1994a).

Alternatively, (intra)cellular distribution of cisplatin can be assessed immunohistochemically. However, because of the aforementioned considerations as well as the fact that the production of antibodies against small molecules, such as cisplatin, is rather difficult and time-consuming, we decided to take another, indirect approach. It has been demonstrated that, in tumor cells, the

uncharged cisplatin molecule is transformed into several charged species that interact with nuclear DNA to form interstrand and intrastrand cross-links (Fig. 1; Reed et al., 1996; Fuertes et al., 2003). Antibodies against these cisplatin-DNA adducts react with cell nuclei in histological sections of tumor tissue (Bergström et al., 1997; Meijer et al., 2001). In addition, nuclear immunoreactivity for these adducts has been observed in, for instance, sections of cisplatin-treated kidney tissue (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999) and dorsal root ganglia (Terheggen et al., 1989; Meijer et al., 1999). These findings imply that cisplatin-DNA adducts are not only formed in tumor cells, but also in normal tissue. We have surmised that a similar molecular mechanism underlies the drug's ototoxic effect and that platination of DNA also may take place in cochlear tissues after cisplatin administration. Therefore, we have developed an immunohistochemical method to, indirectly, detect cisplatin in semithin cryosections of the guinea pig cochlea using an antiserum containing antibodies against cisplatin-DNA adducts.

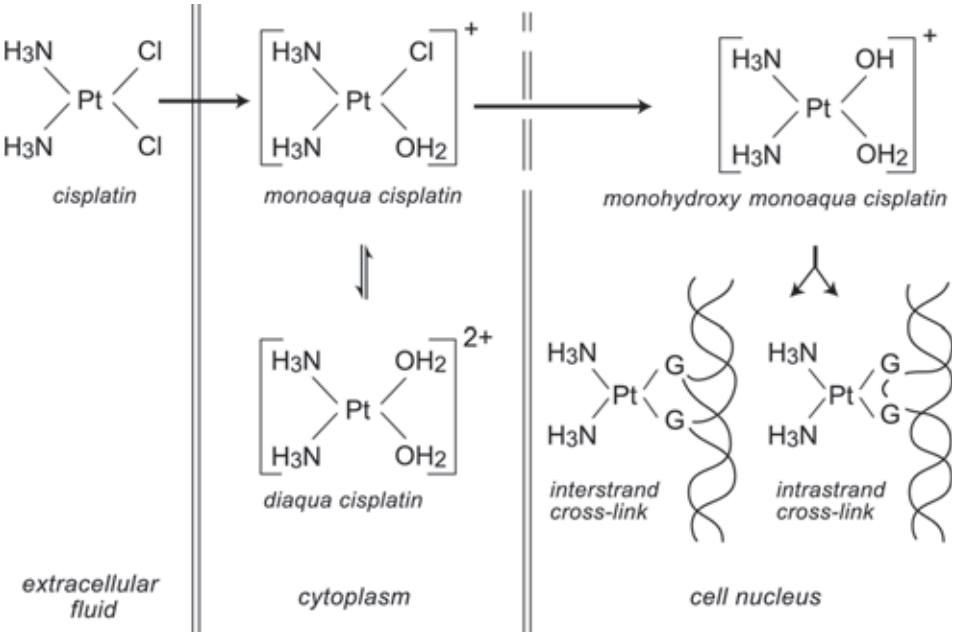


Figure 1.

The cisplatin molecule crosses the plasma membrane and is transformed within the cytoplasm into several charged species. Especially the monoaqua and monohydroxy monoaqua species are reactive and interact with nuclear DNA, resulting in the formation of interstrand and intrastrand cross-links (Adapted from Kartalou and Essigman, 2001).

2. Materials and methods

2.1. Animals

Albino, female Dunkin-Hartley guinea pigs (body weight 250-350 g.) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the Animal Care Facility of the Central Laboratory Animal Institute of Utrecht University. Animals had free access to both food and water and were kept under standard laboratory conditions. The experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC #89055). Animal care was under the supervision of the Central Laboratory Animal Institute of Utrecht University.

2.2. Drug administration

Platinol® injection fluid, containing 1 mg cisplatin per ml, was obtained from Bristol-Myers Squibb (Woerden, the Netherlands). This stock solution was diluted in sterile physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml, and stored in the dark at room temperature. During the entire experiment it was made sure that the shelf life did not exceed the maximum limit of 3 days, as recommended by the Hospital Pharmacist.

Two animals received intraperitoneal injections of cisplatin at a daily dose of 2 mg/kg. Animals were weighed prior to each application and the injection volume was adjusted accordingly for each individual animal. Cisplatin administration was started on Monday (day 1) and was continued until Friday (day 5), followed by two rest days (days 6-7). Administration was restarted on the next Monday (day 8) until the following Friday (day 12). After two additional rest days (days 13-14), the animals were anaesthetized (on day 15) with sodium pentobarbital (Nembutal®; Ceva Sante Animale, Maassluis, the Netherlands) and prepared for whole-body perfusion fixation. Another two animals did not receive cisplatin and were used as controls.

The rationale to treat animals with cisplatin (2 mg/kg/day) five times per week for a period of two weeks (cumulative dose: 20 mg/kg). is to allow cisplatin to accumulate in the cochlear and renal tissues, leading to intracellular cisplatin levels that are sufficiently high to be detected. In a previous study by Saito and Aran (1994a), cisplatin could be detected in renal cortex samples by means of ultrastructural X-ray microanalysis only after 14 days of administration (at a cisplatin dose of 1.5 mg/kg/day; cumulative dose: 21 mg/kg).

2.3. Preparation of cochleas

The method of processing cochleas for cryosectioning was modified from earlier procedures (Flock et al., 1981; Takagi et al., 1994). Immediately after anaesthesia, the animals were perfused intracardially with isotonic saline containing 50 IE/ml heparin for 10 min followed by perfusion with a mixture of 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After perfusion, the cochleas were removed from the bulla and immersed in the same fixative overnight at 4°C. Next, with the cochleas immersed in physiological saline, the bony capsule was removed using finely-pointed micro-hooks and the membranous parts were microdissected from the modiolus. The basal, middle and apical turns were separately collected and cut into smaller segments, with each segment containing both the basilar membrane with the organ of Corti and the lateral wall (i.e., the stria vascularis and spiral ligament). The subdivided segments were stored in the formaldehyde-glutaraldehyde fixative, followed by overnight decalcification in 10% EDTA.2Na (pH 7.4) at 4°C. Next, the segments were infiltrated with molten gelatin through a graded series (1%, 2%, 5%, and 10% in phosphate-buffered saline (PBS), pH 7.4, at 37°C) followed by final embedding in molten gelatin (10% in PBS, pH 7.4) at 37°C. The segments were placed in an embedding mould and oriented in such a way that the organ of Corti is cut in a transverse plane during cryosectioning. After solidification at 4°C, the gelatin slabs containing the cochlear segments were trimmed to an appropriate size and the resulting blocks were infused overnight with a cryoprotectant solution consisting of 1.6 M sucrose and 20% PVP in 44 mM Na_2CO_3 at pH 7.4 (Tokuyasu, 1989). Next, the blocks were mounted on specimen stubs, and frozen and stored in liquid nitrogen. Serial, semithin (0.5 μm) cryosections were cut with a glass knife in a Leica FCS ultracryotome. During cryosectioning, the temperatures of the knife, sample and cryochamber were all set at -100°C. Sections were collected on a drop of 2.3 M sucrose with a stainless-steel wire loop and mounted on organosilane-coated glass slides. Slides were stored overnight in a humid incubation chamber at 4°C.

2.4. Preparation of renal cortex samples

Renal cortex samples were used as positive controls, since cisplatin-DNA adducts are known to be formed in kidneys after cisplatin administration (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999). Following intracardial perfusion, kidneys were removed and small (1 mm^3) cubes of renal cortex were collected and stored overnight in the formaldehyde-glutaraldehyde fixative at 4°C. The samples were processed as described for the cochleas, except for the

gelatin infiltration and embedding steps, which can be omitted when processing solid tissues for cryosectioning.

2.5. NKI-A59 antiserum

NKI-A59 antiserum is a polyclonal rabbit antiserum containing antibodies that recognize cisplatin-DNA adducts (Terheggen et al., 1987, 1991). The NKI-A59 antiserum was a kind gift of Drs. A. Begg and B. Floot (Netherlands Cancer Institute, Amsterdam, the Netherlands). During initial analyses, it was observed that a weak but conspicuous immunostaining was present in nearly all cell nuclei of the renal cortex samples from non-treated animals. This indicates that the NKI-A59 antiserum also contains antibodies that react with nuclear antigens other than cisplatin-DNA adducts. In order to eliminate this non-specific staining in our experiment, the NKI-A59 antiserum is routinely pre-absorbed with a homogenate of non-treated guinea pig kidneys prior to each immunostaining.

2.6. Pre-absorption with kidney homogenate

Kidneys of non-treated guinea pigs were chopped and homogenized in PBS (pH 7.4) on ice with an ultrasonic homogenizer and diluted in PBS to a concentration of 26.5 mg (dry weight) per ml and stored at -20°C. Immediately before use, the NKI-A59 antiserum was diluted 1:100 in PBS and added to eight equal volumes of the kidney homogenate. After constant rotation for 60 min, an equal volume of PBS containing 1% PEG 6000 and 2% normal goat serum (NGS) was added and rotation was continued for 30 min. Next, the suspension was centrifuged at 15,000*g* for 10 min at 4°C. Unless otherwise specified, all steps were carried out at room temperature.

The supernatant containing the antibodies against cisplatin-DNA adducts is used as the primary antiserum during the immunohistochemical procedure. Final dilution of the NKI-A59 antiserum is 1:1,800.

2.7. Immunohistochemical procedure

Immunostaining was performed using either an indirect peroxidase-labelled extravidin-biotin method or an indirect avidin-biotin-peroxidase complex (ABC) method. Semithin cryosections of cochlear segments from the basal turn from either a non-treated or a cisplatin-treated animal were mounted on glass slides. In addition, each slide supported semithin cryosections of renal cortex samples from both non-treated and cisplatin-treated animals, which served as negative and positive controls for immunostaining. Unless otherwise specified, all incubation steps were carried out at room temperature. After each incubation

step, the slides were washed two times in PBS (pH 7.4), 4 min each.
Prior to immunostaining, sections were treated with 0.6% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidases and boiled in an antigen-demasking solution (10 mM sodium citrate, 1.5 mM citric acid; pH 6.0) for 10 min. After cooling in the same buffer until room temperature, sections were digested with proteinase K (0.01 mg/ml in PBS; Merck, Darmstadt, Germany) for 3 min at a temperature of 37°C to increase DNA accessibility.
For immunostaining, the sections were pre-incubated with 1% NGS in PBS (NGS/PBS) for 30 min to prevent non-specific staining. The sections were incubated with the primary antiserum (pre-absorbed NKI-A59 antiserum (see Section 2.6.) at a final dilution of 1:1,800) for 60 min. Two staining controls were performed. In the first control, incubation with the pre-absorbed antiserum was omitted and replaced with an additional incubation step with 1% NGS in PBS. For the second staining control, sections were treated using the same protocol for pre-absorption, except that the NKI-A59 antiserum was replaced by PBS.
Next, the sections were incubated with biotinylated goat anti-rabbit IgG (1:300 in 1% NGS/PBS; Vector Laboratories, Burlingame, USA) for 60 min, followed by incubation in ExtrAvidin®-peroxidase conjugate (1:50 in 1% NGS/PBS; Sigma-Aldrich, St. Louis, USA) or avidin-biotin-peroxidase complex (PK 6100 Vectastain®; Vector Laboratories, Burlingame, USA) for 30 min. Tissue-bound peroxidase was visualized by incubation with a 0.06% solution of DAB containing 0.03% H_2O_2 for 60 min, followed by several rinses in PBS and distilled water. The sections were counterstained with 1% methylene blue and 1% azur II in 1% sodium tetraborate, rinsed in distilled water and subsequently air-dried, cleared in xylene and mounted in Entellan® mounting medium. Sections were examined and photographed with a Leica DM RA microscope equipped with a digital videocamera.

3. Results and discussion

3.1. General findings

Immunoreactivity for platinated DNA was observed in both renal cortex samples (Fig. 2) and basal turns of microdissected cochleas (Figs. 3-6) from cisplatin-treated guinea pigs. This finding indicates that cisplatin is internalized in cochlear tissues and that cisplatin-DNA adducts are also formed in the cochlea, similar to tumor cells and other tissues (Terheggen et al., 1987). Staining controls were negative and did not demonstrate background staining in kidney tissue (Fig. 2b), nor in cochlear tissues (data not shown).

3.2. Renal cortex

Since cisplatin-DNA adducts are known to be formed in cisplatin-treated kidneys (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999), renal cortex samples were used as positive controls. Weak but conspicuous immunostaining with the original NKI-A59 antiserum was present in nearly all cell nuclei in the renal cortex samples from non-treated animals (Fig. 2c). A similar staining was reported by Terheggen et al. (1987). This non-specific staining could be due to the presence of antibodies against nuclear antigens other than cisplatin-DNA adducts (i.a., histones). Terheggen et al. (1987) therefore used NKI-A59 antiserum pre-absorbed with calf thymus DNA, but non-specific staining was not completely abolished in their sections. As cross-reactivity of rabbit antibodies from the NKI-

Figure 2.

Immunohistochemical detection (indirect ABC method) of cisplatin-DNA adducts in renal cortex samples from cisplatin-treated guinea pigs (A, B, E) as compared to non-treated animals (C, D, F).

(A, B): Specificity of the NKI-A59 antiserum. (A): nuclear immunostaining (brown; arrows) is present after incubation with pre-absorbed antiserum. (B): immunostaining is completely absent after incubation with normal goat serum.

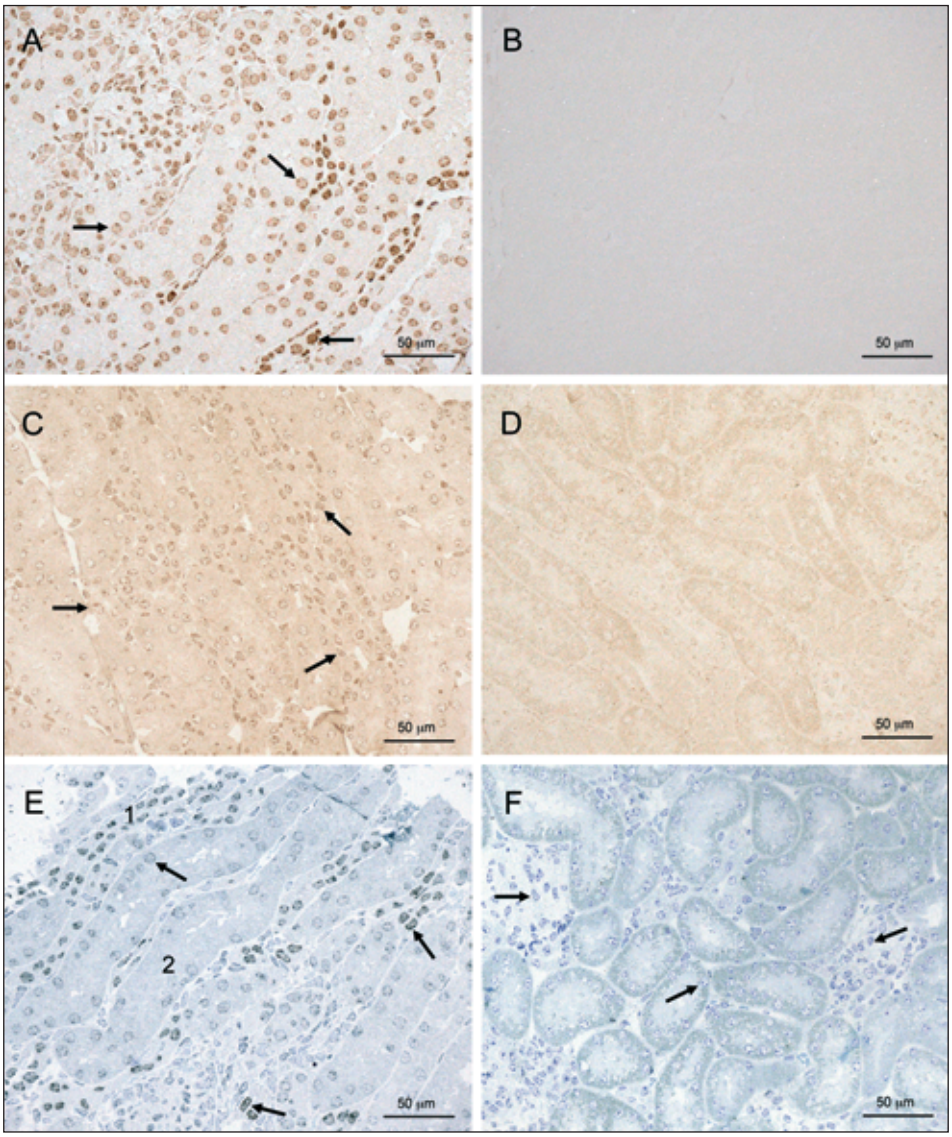
(C, D): Prevention of non-specific staining. (C): in non-treated animals, nuclear immunostaining (brown; arrows) and cytoplasmic background staining are present after incubation with the original NKI-A59 antiserum. (D): non-specific staining in the nuclei is eliminated by incubation with pre-absorbed antiserum, but cytoplasmic background staining remains present (non-treated animal).

(E, F): Incubation with pre-absorbed NKI-A59 antiserum. (E): nuclei in renal tubules from cisplatin-treated animals demonstrate distinct immunostaining (black, arrows). Immunostaining within tubules is equally present in the nuclei of all individual cells, whereas it is present in varying degrees between the different types of tubules. Compare tubule '1' and tubule '2'. (F): nuclei from non-treated animals lack immunostaining and only demonstrate counterstaining (blue, arrows).

[A-D: no counterstaining; E-F: counterstaining with methylene blue and azur II].

A59 antiserum with guinea-pig tissue antigens may account for the non-specific staining, we pre-absorbed the antiserum with a homogenate of non-treated guinea pig kidneys. Cell nuclei in renal cortex samples from non-treated animals did not react with pre-absorbed antiserum (Fig. 2d).

In sections without counterstaining, it is difficult to differentiate between the different tubules and between individual cells within the tubules. Therefore, sections were counterstained with methylene blue and azur II (Figs. 2e-f). In renal cortex samples from cisplatin-treated animals, immunostaining was present in nearly all nuclei of the glomerular cells, proximal tubule cells, and



distal tubule cells as well as in the collecting tubules of the medullary rays (Fig. 2e). Immunostaining within tubules was equally present in the nuclei of all individual cells, whereas it was present in varying degrees between the different types of tubules. These results correspond with those obtained by other authors (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999).

In the corresponding controls (i.e., non-treated animals), immunostaining was absent in all nuclei (Fig. 2f); the nuclei only demonstrated counterstaining. Remarkably, all proximal tubule cells in these controls demonstrated some cytoplasmic background staining in their basal regions (Figs 2c, d, f), but it is absent in cisplatin-treated renal tissue (Figs 2a, e). This background staining does not disappear when sections are incubated with higher dilutions of the primary antibody (pre-absorbed NKI-A59 antiserum) or the secondary antibody (biotinylated goat anti-rabbit IgG). At present, we do not have an explanation for the cytoplasmic background staining in the proximal tubule cells in non-treated animals. Remarkably, this staining seems to be restricted to the basolateral infoldings, which are rich in mitochondria. It should be noted that this background staining is not present in cisplatin-treated animals, suggesting that the antigen(s) involved in the background staining may get lost during cisplatin treatment. We have not undertaken further steps to reduce this background staining, especially since it does not occur in the cochlear tissues of non-treated animals.

Short decalcification of the microdissected cochlear turns is necessary since bony fragments may remain after microdissection, resulting in damage during cryosectioning. The possible effect of decalcification with EDTA on antigenicity was tested in renal cortex samples. Half of the samples were treated overnight with 10% EDTA.2Na, similar to the microdissected cochlear segments. The degree and intensity of immunostaining in EDTA-treated samples was not different from that in the non-treated samples, implying that EDTA does not interfere with the epitopes of the cisplatin-DNA adducts (data not shown).

3.3. Organ of Corti

Semithin (0.5 μm) cryosections of cochlear tissues are very fragile, which may be due to the less-than-optimal-fixation. They are occasionally damaged by the boiling step during the antigen demasking procedure. As the organ of Corti is not a solid tissue, its structure may become distorted during collection and mounting of the sections after cryosectioning (e.g., Fig. 3b). Due to this fragility and distortion, IHCs cannot always be discerned from the surrounding supporting cells. Immunoreactivity for the pre-absorbed NKI-A59 antiserum, visualized with the ABC method, is present in the organ of Corti of cisplatin-treated animals (Fig.

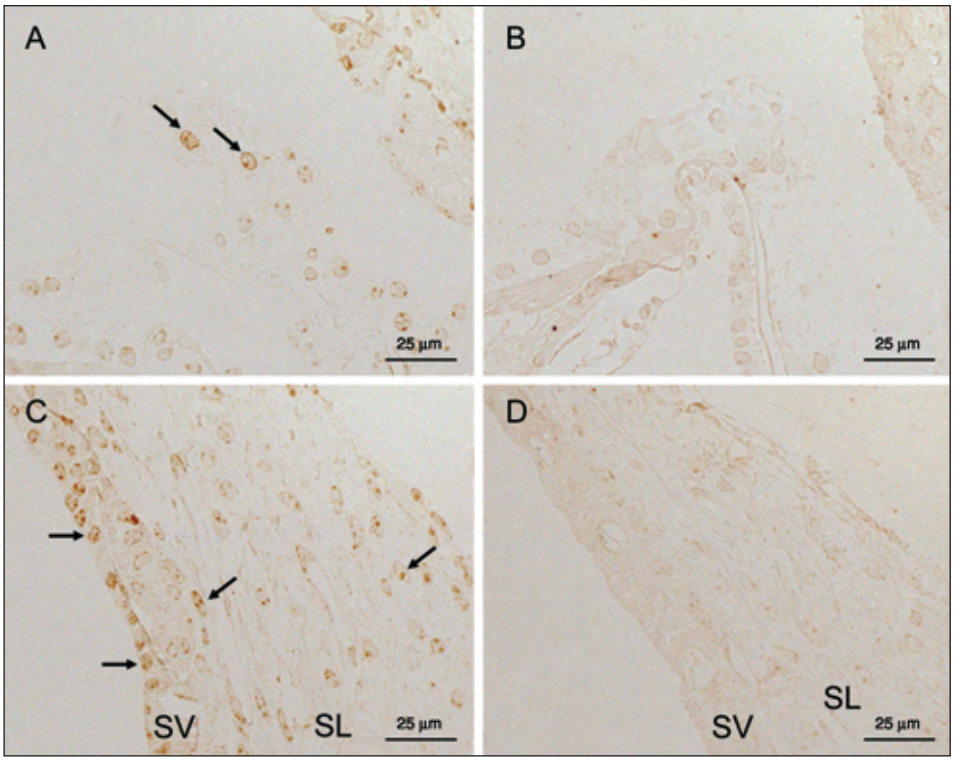


Figure 3.

Immunohistochemical detection (indirect ABC method) of cisplatin-DNA adducts in basal-turn segments containing the organ of Corti (A, B) and lateral wall (C, D). Compare to Figs. 4-6.

(A): nuclear immunostaining (brown; arrows) is seen within the organ of Corti of cisplatin-treated animals. (B): nuclei in the organ of Corti from non-treated animals lack immunostaining. (C): nuclear immunostaining (brown, arrows) is also present in the stria vascularis (SV) and the spiral ligament (SL). (D): nuclei in the lateral wall from non-treated animals lack immunostaining.

[No counterstaining].

3a). The nuclei of the outer hair cells demonstrate prominent immunostaining, whereas the nuclei of all other (supporting) cells are only weakly stained. In the corresponding sections of non-treated cochleas, immunostaining was not observed (Fig. 3b).

Although immunostaining is clearly discernible in Fig. 3a, it is difficult to differentiate accurately between the individual cells. Therefore, sections were counterstained with methylene blue and azur II (Fig. 4) and this confirmed that the nuclei of the outer hair cells were most reactive with the NKI-A59 antiserum.

After visualisation with extravidin-biotin and counterstaining, intense

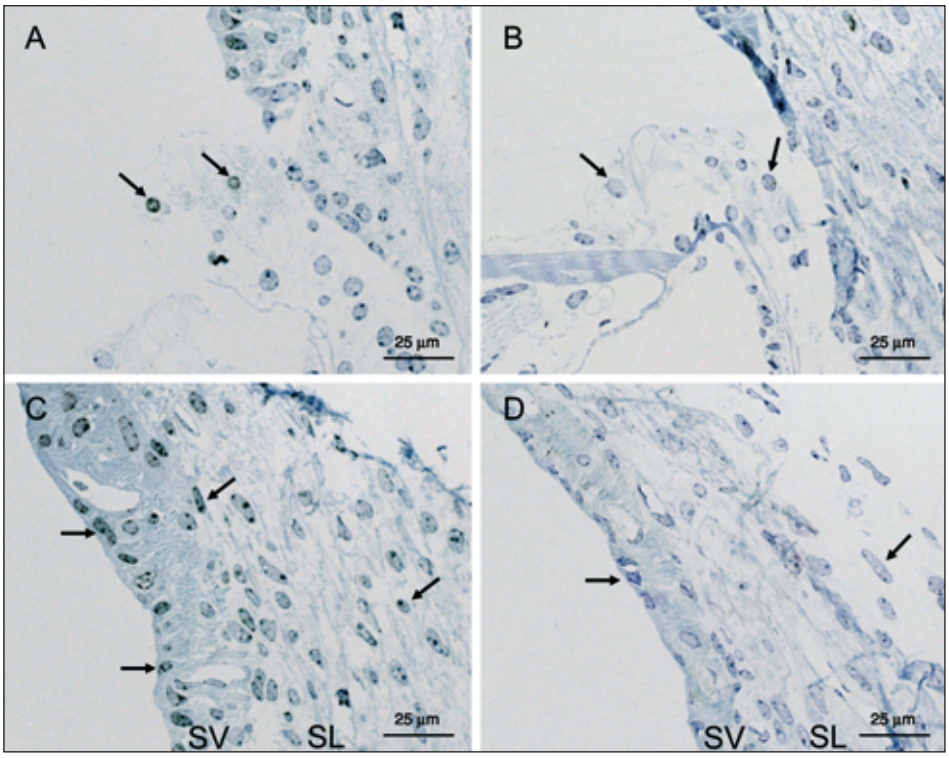


Figure 4.

Immunohistochemical detection (indirect ABC method) of cisplatin-DNA adducts in basal-turn segments containing the organ of Corti (A, B) and lateral wall (C, D), counterstained with methylene blue and azur II. Compare to Fig. 3. (A): nuclear immunostaining (black; arrows) is seen within the organ of Corti of cisplatin-treated animals. (B): nuclei (blue, arrows) in the organ of Corti from non-treated animals lack immunostaining. (C): nuclear immunostaining (black, arrows) is also present in the stria vascularis (SV) and the spiral ligament (SL). (D): nuclei (blue, arrows) in the lateral wall from non-treated animals lack immunostaining.

immunostaining was seen in the nuclei of the outer hair cells, but not so intense in those of the supporting cells (Fig. 5a). Cells in the medial aspect of the organ of Corti occasionally demonstrated nuclear immunostaining, but it was not possible to discern whether these nuclei belong to the inner hair cell or the inner phalangeal cells (Fig. 5a; open arrow). The counterstaining with methylene blue and azur II completely masks immunoreactivity in nuclei that are weakly stained. However, it should be noted that without counterstaining nearly all nuclei are reactive with the NKI-A59 antiserum (Fig. 3a). This means that, with this treatment protocol, nearly all cells in the organ of Corti contain cisplatin-DNA adducts. The presence

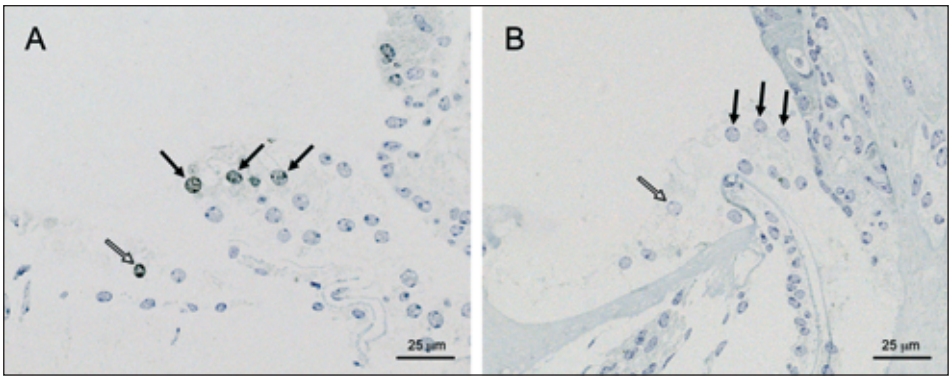


Figure 5.

Immunohistochemical detection (indirect extravidin-biotin method) of cisplatin-DNA adducts in basal-turn segments containing the organ of Corti, counterstained with methylene blue and azur II. Compare to Figs. 4A-B.

(A): immunostaining (black, closed arrows) is primarily present in the nuclei of the outer hair cells. Immunostaining (black, open arrow) is also obvious in a nucleus in the medial aspect of the organ of Corti, but it is not certain whether it belongs to the inner hair cell or an inner phalangeal cell. (B): in non-treated animals, nuclei of the outer hair cells (blue, closed arrows) and the inner hair cell (blue, open arrows) lack immunostaining.

of cisplatin-DNA adducts is in line with the results obtained by Schweitzer (1993), who detected [^{195m}Pt]-labelled cisplatin in homogenated samples of the organ of Corti. Sections from non-treated animals were devoid of immunostaining (Fig. 5b).

The finding that cisplatin-DNA adducts are also present in the nuclei of the supporting cells in the organ of Corti, substantiates the morphological and molecular evidence that cisplatin also affects these cells (Estrem et al., 1981; Anniko and Sobin, 1986; Endo et al., 2002; Cardinaal et al., 2004; Ramírez-Camacho et al., 2004). However, the major effect was seen in the nuclei of the OHCs.

3.4. Lateral wall

Immunoreactivity for cisplatin-DNA adducts, after visualization with the ABC method, is present in the lateral wall in cisplatin-treated animals. Immunostaining in the stria vascularis is present in the nuclei of most cell types, but is most prominent in those of the marginal cells (Fig. 3c). In addition, nearly all nuclei of the fibrocytes in the spiral ligament demonstrate immunostaining (Fig. 3c). In the corresponding sections of non-treated cochleas, immunostaining was not observed (Fig. 3d).

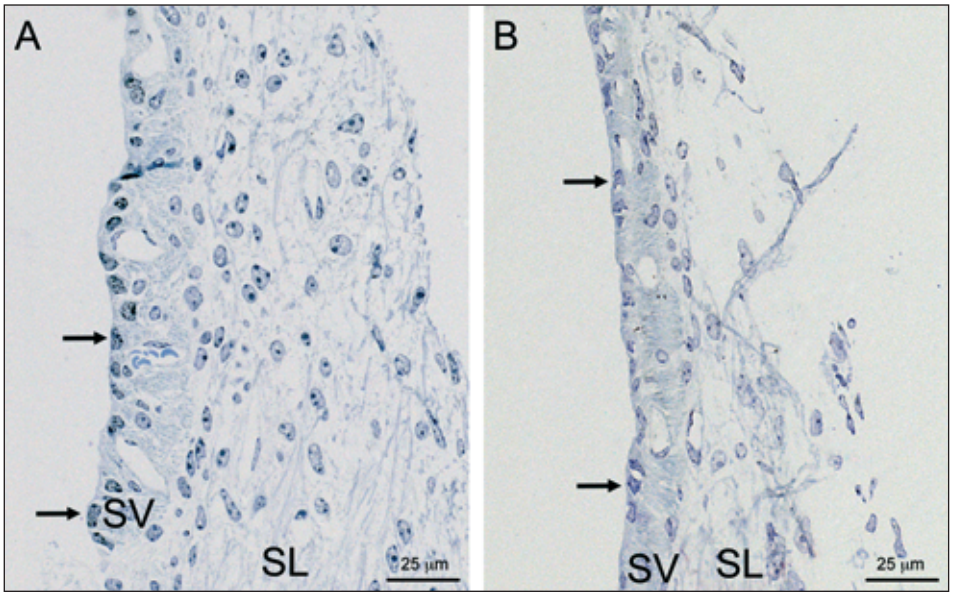


Figure 6.

Immunohistochemical detection (indirect extravidin-biotin method) of cisplatin-DNA adducts in basal-turn segments containing the lateral wall, counterstained with methylene blue and azur II. Compare to Figs. 4C-D.

(A): intense immunostaining (black, arrows) is primarily present in the nuclei of the marginal cells of the stria vascularis (SV). (B): in the non-treated animals, immunostaining of the nuclei (blue, arrows) in the lateral wall is not present.

Although immunostaining is clearly discernible in Fig. 3c, it is difficult to differentiate accurately between the individual cells. Therefore, sections were counterstained with methylene blue and azur II (Fig. 4), and this confirmed that the nuclei of the marginal cells as well as the spiral ligament fibrocytes were most reactive with the NKI-A59 antiserum.

After visualisation with extravidin-biotin and counterstaining, immunostaining is present in the lateral wall. In the stria vascularis, intense immunoreactivity is present in the nuclei of the marginal cells, whereas weak staining is present in the intermediate cells, basal cells, and strial capillaries (Fig. 6a). Literature reports that morphological changes in the stria vascularis primarily occur in the marginal cells (Tange and Vuzevski, 1984; Kohn et al., 1988; Laurell and Bagger-Sjöbäck, 1991; Saito and Aran, 1994b; Meech et al., 1998; Campbell et al., 1999; Wang, 2003). In the spiral ligament, immunostaining was present in the nuclei of the fibrocytes (Fig. 6a). These findings are in agreement with studies demonstrating the presence

of cisplatin in homogenated samples of the stria vascularis (Schweitzer, 1993) and in ultrathin sections of the stria vascularis (Maruyama et al., 1993). Sections from non-treated animals lacked immunostaining (Fig. 6b).

3.5. Comparison of the visualization methods

There is a marked difference in nuclear staining between the two visualization methods tested. With the ABC method, most nuclei in the cochlear tissues demonstrated immunoreactivity for the antiserum, but nuclear staining is diffuse and of low intensity (Figs. 4a-c). In contrast, nuclear staining after visualisation with the extravidin-biotin method is more intense (Figs. 5a, 6a). This indicates that extravidin-biotin method is the method of choice for visualisation of the NKI-A59 antiserum in the cochlea. In renal tissue, there are no obvious differences between the ABC and extravidin-biotin methods. This difference in staining density may be explained by differences in sensitivity between the two methods. Such differences in sensitivity become obvious, especially, in tissues in which the amount of antigen(s) are similar to the (lower) detection limit of the method. It is likely that the amount of cisplatin in the cochlear tissues are lower that those in the renal tubule cells, and therefore sections of kidney tissue do not demonstrate any differences in nuclear immunostaining with either method.

3.6. Concluding remarks

This study is the first to demonstrate the presence of cisplatin in histological sections of the basal cochlear turn. Cisplatin is internalized in cochlear tissues and interacts with DNA to form cisplatin-DNA adducts. Platinated DNA was immunohistochemically detected in the nuclei of nearly all cells in the organ of Corti and the lateral wall of guinea pigs treated with cisplatin at a daily dose of 2 mg/kg, five times per week for a period of two weeks. Immunostaining was most pronounced in the hair cells, the marginal cells of the stria vascularis and the spiral ligament fibrocytes. The formation of cisplatin-DNA adducts may not completely account for the ototoxic effect of cisplatin. It cannot be excluded that cisplatin interacts with other cellular macromolecules (e.g., glutathione, metallothionein) and organelles (e.g., mitochondria, endoplasmic reticulum), resulting in cell degeneration. This method might be a tool to answer the important question whether or not the changes in the organ of Corti, the stria vascularis and the spiral ganglion run in parallel. Time-related distribution of cisplatin in the cochlea will be reported in a future study. Moreover, immunohistochemical detection of cisplatin-DNA adducts could be a useful approach to study the effects of otoprotective agents on cisplatin ototoxicity.

Acknowledgements
This study has been supported by grants from the Heinsius-Houbolt Foundation, the Netherlands. The purchase of a Leica DM RA light microscope was made possible by a generous donation of Mr. L.G.M. van Gansewinkel. The authors wish to thank Drs. A. Begg and B. Floot (Netherlands Cancer Institute, Amsterdam, the Netherlands) for their kind gift of the NKI-A59 antiserum. We are also grateful to Mr. C. Guikers for his invaluable histotechnical advice.
Marjolein W.M. van Ruijven is a Heinsius-Houbolt Research Fellow at the Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands.

References

Anniko, M., Sobin, A., 1986. Cisplatin: Evaluation of its ototoxic potential. Am. J. Otolaryngol. 7, 276-293.

Bergström, P., Johnsson, A., Cavallin-Stahl, E., Bergenheim, T., Henriksson, R., 1997. Effects of cisplatin and amphotericin B on DNA adduct formation and toxicity in malignant glioma and normal tissues in rat. Eur. J. Cancer 33, 153-159.

Berry, J.P., Brille, P., LeRoy, A.F., Gouveia, Y., Ribaud, P., Galle, P., Mathe, G., 1982. Experimental ultrastructural and X-ray microanalysis study of cisplatin in the rat: Intracellular localization of platinum. Cancer Treat. Rep. 66, 1529-1533.

Campbell, K.C.M., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear. Res. 138, 13-28.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh) 124, 144-154.

De Oliveira, J.A.A., 1989. Audiovestibular Toxicity of Drugs, Volume II. CRC Press, Boca Raton, Florida, pp. 181-198.

Endo, T., Nakagawa, T., Lee, J.E., Dong, Y., Kim, T.S., Iguchi, F., Taniguchi, Z., Naito, Y., Ito, J., 2002. Alteration in expression of p27 in auditory epithelia and neurons of mice during degeneration. Neurosci. Lett. 334, 173-176.

Estrem, S.A., Babin, R.W., Ryu, J.H., Moore, K.C., 1981. Cis-diamminedichloroplatinum (II) ototoxicity in the guinea pig. Otolaryngol. Head Neck Surg. 89, 638-645.

Flock, A., Hoppe, Y., Wei, X., 1981. Immunofluorescence localization of proteins in semithin 0.2-1 μm frozen sections of the ear. A report of improved techniques including gelatin encapsulation and cryoultramicrotomy. Arch. Otorhinolaryngol. 233, 55-66.

Fuertes, M.A., Castilla, J., Alonso, C., Perez, J.M., 2003. Cisplatin biochemical mechanism of action: From cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. Curr. Med. Chem. 10, 257-266.

Hamers, F.P.T., Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by alpha-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

Johnsson, A., Olsson, C., Nygren, O., Nilsson, M., Seiving, B., Cavallin-Stahl, E., 1995. Pharmacokinetics and tissue distribution of cisplatin in nude mice: Platinum levels and cisplatin-DNA adducts. Cancer Chemother. Pharmacol. 37, 23-31.

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

Khan, M.J., Sadler, P.J., 1978. Distribution of a platinum anti-tumour drug in HeLa cells by analytical electron microscopy. Chem. Biol. Interact. 21, 227-232.

Kohn, S., Fradis, M., Pratt, H., Zidan, J., Podoshin, L., Robinson, E., Nir, I., 1988. Cisplatin ototoxicity in guinea pigs with special reference to toxic effects in the stria vascularis. Laryngoscope 98, 865-871.

Laurell, G., Bagger-Sjöbäck, D., 1991. Degeneration of the organ of Corti following intravenous administration of cisplatin. Acta Otolaryngol. (Stockh.) 111, 891-898.

Makita, T., Hakoi, K., Ohokawa, T., 1986. X-ray microanalysis and electron microscopy of platinum complex in the epithelium of proximal renal tubules of the cisplatin administered rabbits. Cell Biol. Int. Rep. 10, 447-454.

Maruyama, K., Furuya, N., Daimon, T., 1993. Distribution of platinum in the inner ear of guinea pig after treatment with cisplatin. J. Otolaryngol. (Jpn) 96, 1758-1759.

Meech, R.P., Campbell, K.C.M., Hughes, L.F., Rybak, L.P., 1998. A semiquantative analysis of the effects of cisplatin on the rat stria vascularis. Hear. Res. 124, 44-59.

Meijer, C., De Vries, E.G.E., Marmiroli, P., Tredici, G., Frattola, L., Cavaletti, G., 1999. Cisplatin-induced DNA-platination in experimental dorsal root ganglia neuropathy. Neurotoxicol. 20, 883-888.

Meijer, C., Van Luyn, M.J.A., Nienhuis, E.F., Blom, N., Mulder, N.H., Vries, E.G.E., 2001. Ultrastructural morphology and localisation of cisplatin-induced platinum-DNA adducts in a cisplatin-sensitive and -resistant human small cell lung cancer cell line using electron microscopy. Biochem. Pharmacol. 61, 573-578.

Ramírez-Camacho, R., García-Berrocal, J.R., Buján, J., Martín-Marero, A., Trinidad, A., 2004. Supporting cells as a target of cisplatin-induced inner ear damage: Therapeutic implications. Laryngoscope 114, 533-537.

Reed, E., Dabholkar, M., Chabner, B.A., 1996. Platinum analogs. In: Chabner, B.A., Longo, D.L. (Eds.) Cancer Chemotherapy and Biotherapy, 2nd Edition. Lippincott-Raven Publishers, Philadelphia, pp. 357-378.

Saito, T., Aran, J.M., 1994a. X-ray microanalysis and ion microscopy of guinea pig cochlea and kidney after cisplatin treatment. ORL 56, 310-314.

Saito, T., Aran, J.M., 1994b. Comparative ototoxicity of cisplatin during acute and chronic treatment. ORL 56, 315-320.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Takagi, I., Hendriksen, E.G.J., De Groot, J.C.M.J., Veldman, J.E., 1994. A new, high-resolution cryotechnique in inner ear immunohistochemistry: A more precise localization of intermediate filament proteins in the guinea pig inner ear. In: Mogi, G., Veldman, J.E., Kawauchi, H. (Eds.) Immunobiology in Otorhinolaryngology. Progress of a Decade. Kugler Publications, Amsterdam/New York, pp. 297-301.

Tange, R.A., Vuzevski, V.D., 1984. Changes in the stria vascularis of the guinea pig due to cis-platinum. Arch. Otorhinolaryngol. 239, 41-47.

Terheggen, P.M., Gerritsen Van Der Hoop, R., Floot, B.G., Gispen, W.H., 1989. Cellular distribution of cis-diamminedichloroplatinum(II)-DNA binding in rat dorsal root spinal ganglia: Effect of the neuroprotecting peptide ORG 2766. Toxicol. Appl. Pharmacol. 99, 334-343.

Terheggen, P.M., Floot, B.G., Lempers, E.L., Van Tellingen, O., Begg, A.C., Den Engelse, L., 1991. Antibodies against cisplatin-modified DNA and cisplatin-modified (di)nucleotides. Cancer Chemother. Pharmacol. 28, 185-191.

Terheggen, P.M.A.B., Floot, B.G.J., Scherer, E., Begg, A.C., Fichtinger-Schepman, A.M.J., Den Engelse, L., 1987. Immunocytochemical detection of interaction products of cis- diam minedichloroplatinum(II) and cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in rodent tissue sections. Cancer Res. 47, 6719-6725.

Tokuyasu, K.T., 1989. Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. Histochem. J. 21, 163-171.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Smoorenburg, G.F., 2004. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. Hear. Res. 197, 44-54.

Verschraagen, M., Van Der Born, K., Zwiers, T.H., Van Der Vijgh, W.J., 2002. Simultaneous determination of intact cisplatin and its metabolite monohydrated cisplatin in human plasma. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 772, 273-281.

Wang, J., Lloyd Faulconbridge, R.V., Fetoni, A., Guitton, M.J., Pujol, R., Puel, J.L., 2003. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacol. 45, 380-393.

Welb, R., 1995. Experimentelle Studie über die Wirkung von Cisplatin auf das Innenohr. PhD Thesis, Medizinischen Fakultät der Heinrich-Heine-Universität, Düsseldorf, Germany.

Time-dependent formation of platinated DNA in the cochlear targets of cisplatin

Marjolein W.M. van Ruijven, Ferry Hendriksen,
John C.M.J. de Groot, Guido F. Smoorenburg

Chapter 5

Abstract

Using an immunohistochemical method, the time-related distribution of cisplatin in cochlear tissues was investigated, in order to answer the question of whether or not there is a primary target of cisplatin in the cochlea. Albino guinea pigs were treated with intraperitoneal injections of cisplatin (2 mg/kg/day) for 2, 4, 6, 8, 10, 12, 14 or 16 consecutive days. Twenty-four hours after the final injection, cochlear function was measured by means of electrocochleography. After fixation, the cochleas were microdissected and cochlear segments containing the organ of Corti, spiral ganglion and the lateral wall were processed for cryosectioning. Semithin cryosections were incubated with the NKI-A59 antiserum containing antibodies against cisplatin-DNA adducts. Cisplatin-DNA adducts were first obvious in the organ of Corti after 6 days. In the basal turn, immunostaining was present in the nuclei of all three rows of outer hair cells (OHCs) and, to a lesser degree, in the nuclei of the Deiters' cells. Immunostaining demonstrated a longitudinal gradient, starting in the basal turn and progressing to the middle and apical turns. Immunostaining in the lateral wall (i.e., stria vascularis and spiral ligament) started in the basal turn after 8 days, and had progressed to the middle and apical turns after 12 days. Spiral ganglion cells from the basal turn did not show immunostaining until after 14 days of continuous administration. This study demonstrates that cisplatin-DNA adducts are present in OHCs and Deiters' cells at an earlier moment than in strial cells and spiral ganglion cells, suggesting that the organ of Corti is the primary target of cisplatin. Thresholds of the compound action potential (CAP) and cochlear microphonics (CM) at 8 kHz were increased at the same time that cisplatin-DNA adducts were present in the basal turn. At 4 and 2 kHz, however, increased CAP and CM thresholds preceded the occurrence of cisplatin-DNA adducts in the middle and apical turns, resepctively. This suggests that the presence of cisplatin-DNA adducts in OHCs is not directly related with the threshold shifts in CAP and CM.

1. Introduction

It is thought that the anti-tumor activity of cisplatin is related to its binding capacity to nuclear DNA and the formation of so-called cisplatin-DNA adducts. The exact mechanism, however, that leads to tumor cell death following treatment with cisplatin is not known, but several models have been proposed to explain the role of adduct-binding proteins in mediating cisplatin cytotoxicity (Zamble, 1995; Reed et al., 1996; Kartalou and Essigmann, 2001; Gonzalez et al., 2001).Antibodies against cisplatin-DNA adducts can be used to indirectly localize cisplatin in histological sections. Nuclear immunostaining of these adducts has been observed in sections of, for instance, cisplatin-treated tumor tissue (Bergström et al., 1997; Meijer et al., 2001), kidney tissue (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999) and dorsal root ganglia (Terheggen et al., 1989; Meijer et al., 1999). Recently, Van Ruijven et al. (2005) demonstrated that, in guinea pig cochleas treated with cisplatin for 2x5 days, immunostaining of cisplatin-DNA adducts was mainly present in the nuclei of the outer hair cells (OHCs), the marginal cells of the stria vascularis and the spiral ligament fibrocytes.Animal studies have demonstrated that cisplatin administration results in morphological and functional changes in the organ of Corti, the stria vascularis, and the spiral ganglion (Schweitzer, 1993; Cardinaal et al., 2000; Klis et al., 2000, 2002; Hamers, et al., 2003). However, the question of whether or not there is a primary target of cisplatin in the cochlea remains unanswered. This is mainly due to the fact that the cellular sites of cisplatin uptake and distribution in cochlear tissues have not been properly identified. Furthermore, there is a lack of data concerning the time sequence of the degeneration pattern in the cochlea. A recent time-sequence study (Van Ruijven et al., 2004) demonstrated that both OHC loss and myelin sheath detachment in the type-I spiral ganglion cells (SGCs) are present after 6 days of cisplatin administration, whereas histological changes in the stria vascularis were not obvious, not even after 8 days. It appears from this study that the changes in the OHCs and type-I SGCs occur simultaneously and develop in parallel. Identification of the first site(s) of cisplatin uptake may answer the question of whether the organ of Corti, the spiral ganglion or the stria vascularis is the primary target of cisplatin in the cochlea. Therefore, we have investigated the intracochlear distribution of cisplatin using a time-sequence study. For this purpose, guinea pigs were treated with cisplatin at a daily dose of 2 mg/kg for either 2, 4, 6, 8, 10, 12, 14 or 16 days. Cisplatin was indirectly detected in semithin cryosections of the guinea pig cochlea, with a polyclonal rabbit antiserum that contains antibodies against cisplatin-DNA adducts (Van Ruijven et al., 2005).

2. Materials and methods

2.1. Animals

Healthy, female albino guinea pigs (strain: Dunkin Hartley, weighing 250-350 g.) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the Animal Care Facility of the Central Laboratory Animal Institute of Utrecht University. Animals had free access to both food and water and were kept under standard laboratory conditions. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC #89055). Animal care was under the supervision of the Central Laboratory Animal Institute of Utrecht University.

2.2. Drug administration

Platinol® injection fluid, containing 1 mg cisplatin per ml, was obtained from Bristol-Myers Squibb (Woerden, the Netherlands). This stock solution was diluted in sterile physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml, and stored in the dark at room temperature. During the entire experiment it was made sure that the shelf life did not exceed the maximum limit of 3 days, as recommended by the Hospital Pharmacist.

Guinea pigs were randomly assigned to either the non-treated group (n=2) or one of the eight experimental groups. The animals in the experimental groups were treated with cisplatin (2 mg/kg/day) by daily intraperitoneal injections for either 2, 4, 6, 8, 10, 12, 14 or 16 days (n=2 animals, each group). Animals were weighed every morning and the injection volume was adjusted accordingly for each individual animal.

Two animals, both from the 14-day group, died during cisplatin treatment and were substituted with new animals, which survived throughout the experiment. Four animals (one from the 12-day group, one from the 14-day group, and two from the 16-day group) demonstrated weight losses of ≥10%, as compared to the preceding day. Cisplatin administration in these animals was interrupted for the next 24 h. As cisplatin is not completely eliminated from the serum within 24 h, the animals remained in their allotted group throughout the entire experiment.

2.3. Electrocochleography

Electrocochleography of the right ears only was performed 24 h after the final injection. Techniques for electrocochleography were described previously (Stengs et al., 1997) and are only summarized here. Auditory evoked responses were recorded differentially with a silverball electrode at the apex of the cochlea

and a reference electrode in the muscles of the neck. Trains of 8-ms tone bursts of 2, 4, 8, and 16 kHz were presented with alternating polarity. Averaged responses were stored for off-line analysis. The compound action potential (CAP) was obtained by addition of the responses to opposite polarity stimuli, the cochlear microphonics (CM) by subtraction. CAP amplitude was defined relative to the summating potential. The CM was determined as the peak-to-peak value of the sinusoidal response. CM was not measured at 16 kHz, because it exceeds the upper cut-off frequency of our measurement system. Threshold was defined as the stimulus level that produced a 10 μV response. We determined the threshold by visual interpolation of the data of individual animals.

2.4. Preparation of cochleas

The method for processing cochleas for ultracryomicrotomy was modified from earlier procedures (Flock et al., 1981; Takagi et al., 1994). Immediately after anaesthesia, the animals were perfused intracardially with isotonic saline containing 50 IE/ml heparin for 10 min followed by perfusion with a mixture of 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After perfusion, the cochleas were removed from the bulla and immersed in the same fixative overnight at 4°C. Next, with the cochleas immersed in physiological saline, the bony capsule was removed using finely-pointed micro-hooks and the membranous parts were microdissected from the modiolus. The basal, middle and apical turns were separately collected and cut into smaller segments, with each segment containing the basilar membrane with the organ of Corti, lateral wall (i.e., the stria vascularis and spiral ligament) and the spiral ganglion. The subdivided segments were stored in the formaldehyde-glutaraldehyde fixative, followed by overnight decalcification in 10% EDTA.2Na (pH 7.4) at 4°C. Next, the segments were infiltrated with molten gelatin through a graded series (1%, 2%, 5%, and 10% in phosphate-buffered saline (PBS), pH 7.4, at 37°C) followed by final embedding in molten gelatin (10% in PBS, pH 7.4) at 37°C. The segments were placed in an embedding mould and oriented in such a way that the organ of Corti is cut in a transverse plane during cryosectioning. After solidification at 4°C, the gelatin slabs containing the cochlear segments were trimmed to an appropriate size and the resulting blocks were infused overnight with a cryoprotectant solution consisting of 1.6 M sucrose and 20% PVP in 44 mM Na_2CO_3 at pH 7.4 (Tokuyasu, 1989). Next, the blocks were mounted on specimen stubs, and frozen and stored in liquid nitrogen. Serial, semithin (0.5 μm) cryosections were cut with a glass knife in a Leica FCS ultracryotome. During cryosectioning, the temperatures of the knife, sample and cryochamber were all

set at -100°C. Sections were collected on a drop of 2.3 M sucrose with a stainless-steel wire loop and mounted on organosilane-coated glass slides. Slides were stored overnight in a humid incubation chamber at 4°C.

2.5. Preparation of renal cortex samples

Renal cortex samples were used as positive controls, since cisplatin-DNA adducts are known to be formed in kidneys after cisplatin administration (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999). Following intracardial perfusion, kidneys were removed and small (1 mm^3) cubes of renal cortex were collected and stored overnight in the formaldehyde-glutaraldehyde fixative at 4°C. The samples were processed as described for the cochleas, except for the gelatin infiltration and embedding steps, which can be omitted when processing solid tissues for cryosectioning.

2.6. Immunohistochemical procedure

Immunostaining was performed using an indirect peroxidase-labelled extravidin-biotin method (Van Ruijven et al., 2005). Several serial semithin cryosections of cochlear segments of each animal were mounted on glass slides. In addition, each slide contained semithin cryosections of renal cortex samples from both non-treated and cisplatin-treated animals, which served as negative and positive controls for the immunostaining. Unless otherwise specified, all incubation steps were carried out at room temperature. After each incubation step, the slides were washed two times in PBS (pH 7.4), 4 min each.

Prior to immunostaining, sections were treated with 0.6% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidases and boiled in an antigen-demasking solution (10 mM sodium citrate, 1.5 mM citric acid; pH 6.0) for 10 min. After cooling in the same buffer until room temperature, sections were digested with proteinase K (0.01 mg/ml in PBS; Merck, Darmstadt, Germany) for 3 min at a temperature of 37°C to increase DNA accessibility.

For immunostaining, the sections were pre-incubated with 1% normal goat serum (NGS) in PBS (NGS/PBS) for 30 min to prevent non-specific staining. The sections were incubated with the NKI-A59 antiserum at a final dilution of 1:1,800 for 60 min. The NKI-A59 antiserum was pre-absorbed with a homogenate of non-treated guinea pig kidneys prior to each immunostaining (Van Ruijven et al., 2005). Control sections were incubated with 1% NGS/PBS.

Next, the sections were incubated with biotinylated goat anti-rabbit IgG (1:300 in 1% NGS/PBS; Vector Laboratories, Burlingame, USA) for 60 min, followed by incubation in ExtrAvidin®-peroxidase (1:50 in 1% NGS/PBS; Sigma-Aldrich,

St. Louis, USA) for 30 min. Tissue-bound peroxidase was visualized by incubation with a 0.06% solution of 3,3'-diaminobenzidine.4HCl (DAB) containing 0.03% H_2O_2 for 60 min, followed by several rinses in PBS and distilled water. The section were subsequently air-dried, cleared in xylene and mounted in Entellan® mounting medium. To prevent masking of immunostaining by counterstaining solutions (Van Ruijven et al., 2005), sections were not counterstained. The sections were examined and photographed with a Leica DM RA microscope equipped with a digital videocamera.

3. Results

3.1. Detection of cisplatin-DNA adducts in the renal cortex

Since cisplatin-DNA adducts are known to be formed in cisplatin-treated kidneys (Terheggen et al., 1987; Bergström et al., 1997; Meijer et al., 1999), renal cortex samples were used as positive controls. Immunostaining with the NKI-A59 antiserum was not present in the non-treated group (Table 1; 0 days). Immunostaining was present in the nuclei of both the proximal and distal tubule cells in all experimental groups, but not before 4 days of cisplatin administration (Table 1).

3.2. Detection of cisplatin-DNA adducts in the cochlea

Semithin (0.5 μm) cryosections are very fragile, which may be due to the less-than-optimal-fixation. They are occasionally damaged by the boiling step during the antigen demasking procedure. As the organ of Corti is not a solid tissue, its structure may become distorted during collection and mounting of the sections after cryosectioning (Fig. 2A). Due to this fragility and distortion, IHCs cannot always be discerned from the surrounding supporting cells.

Immunostaining with the NKI-A59 antiserum is visible as black staining in the cell nuclei. During the first days of cisplatin administration, staining was not present. After prolonged treatment, the staining became apparent and eventually, the staining was prominently present. The degree of immunostaining was scored as: absent (-), moderate (+), and prominent (++). Immunostaining for the different cell types, cochlear turns and experimental groups are summarized in Table 1. Scores are averaged over 2 animals (=4 cochleas) per experimental group.

3.2.1 Detection of cisplatin-DNA adducts in the organ of Corti

None of the animals from the non-treated group demonstrated immunostaining with the NKI-A59 antiserum in the organ of Corti of the basal, middle and apical turns (Figs. 1A, D, G).

Immunostaining in the organ of Corti was first observed in the basal turn after 6 days of cisplatin administration (Fig. 1B). Staining was most prominent in the nuclei of all three rows of OHCs, and to a lesser degree, in the nuclei of the Deiters' cells. Immunostaining was not obvious in the IHCs and other supporting cells. The organ of Corti in the middle and apical turns did not demonstrate immunostaining.

In the 8-day group, immunostaining was present in OHCs, but it was not observed in the IHCs and supporting cells.

Cisplatin	kidney	cochlear	organ of Corti			stria vascularis				SL	SG
Days		turn	OHC	IHC	SC	MC	IC	BC	CAP	fibrocytes	SGC
0	–	b	–	–	–	–	–	–	–	–	–
		m	–	–	–	–	–	–	–	–	–
		a	–	–	–	–	–	–	–	–	–
2	–	b	–	–	–	–	–	–	–	–	–
		m	–	–	–	–	–	–	–	–	–
		a	–	–	–	–	–	–	–	–	–
4	++	b	–	–	–	–	–	–	–	–	–
		m	–	–	–	–	–	–	–	–	–
		a	–	–	–	–	–	–	–	–	–
6	++	b	++	–	+	–	–	–	–	–	–
		m	–	n.d.	–	–	–	–	–	–	–
		a	–	–	–	–	–	–	–	–	–
8	++	b	++	–	–	+	+	+	+	+	–
		m	–	n.d.	–	–	–	–	–	–	–
		a	–	–	–	–	–	–	–	–	–
10	++	b	++	n.d.	+	+	–	–	–	+	–
		m	+	+	–	–	–	–	–	–	–
		a	+	–	–	–	–	–	–	–	–
12	++	b	++	n.d.	+	++	–	–	–	++	–
		m	++	++	+	++	+	+	+	++	–
		a	++	++	+	–	–	–	–	+	–
14	++	b	++	++	++	++	++	++	+	++	+
		m	++	+	++	++	+	+	+	++	–
		a	++	–	++	++	–	–	–	++	–
16	++	b	++	n.d.	++	++	++	++	++	++	++
		m	++	++	++	++	++	++	++	++	++
		a	++	+	++	++	+	+	+	++	+

Table 1.

Summary of immunostaining data in kidneys and cochleas of guinea pigs treated with cisplatin at a dose of 2.0 mg/kg/day. First column indicates the number of days of cisplatin administration ("0": non-treated). Second column gives data for kidney tissue as a positive control. Data for the cochlea are specified for the basal (b), middle (m) and apical (a) turns. Cochlear data are divided into four

groups: organ of Corti, stria vascularis, spiral ligament (SL), and spiral ganglion (SG). OHC: outer hair cells; IHC: inner hair cells; SC: supporting cells; MC: marginal cells; IC: intermediate cells; BC: basal cells; CAP: capillaries; SGC: spiral ganglion cells (immunostaining: –: absent; +: moderate; ++: prominent). IHCs could not always be discerned from the surrounding supporting cells (n.d.: non-discernable).

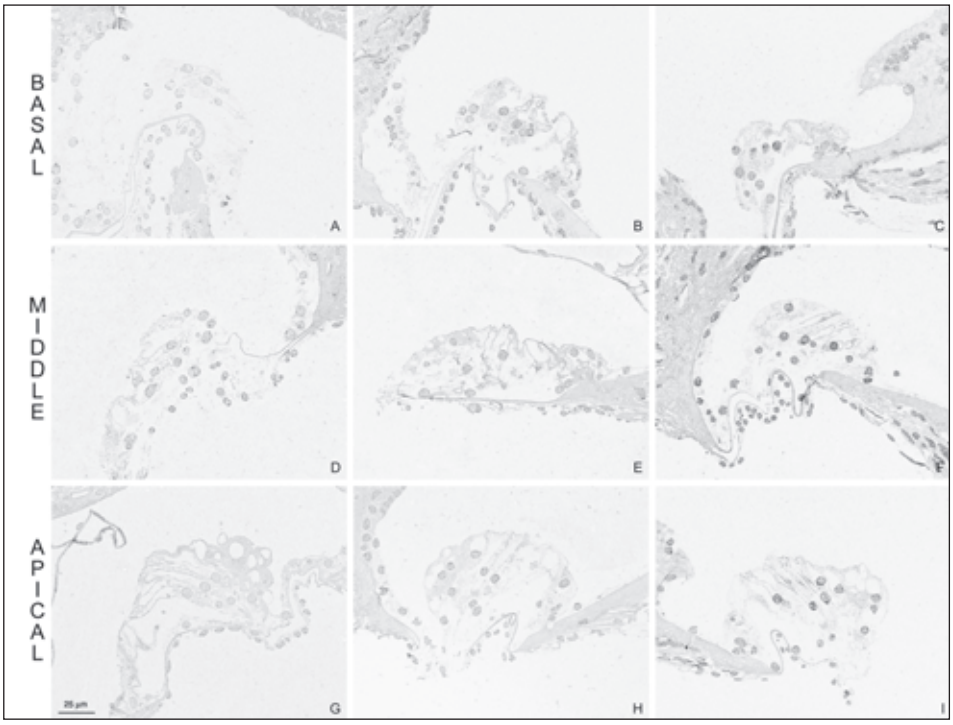


Figure 1.

Immunohistochemical detection of cisplatin-DNA adducts in the organ of Corti. In the non-treated cochleas, immunostaining was absent in the basal (A), middle (D) and apical (G) turns. The light micrographs in the middle column represent the points of time at which immunostaining was first obvious in the different cochlear turns: In the basal turn after 6 days (B), in the middle (E) and apical (H) turns after days. The light micrographs in the right column are from cochleas treated for 16 days: Immunostaining was present in the basal (C), middle (F) and apical (I) turns.

After 10 days of cisplatin administration, prominent immunostaining was observed in the OHCs and, to a lesser degree, the Deiters' cells of the basal turn. IHCs could not be discerned in sections of the basal turn. Immunostaining in the IHCs was first observed in the middle turn. In the middle and apical turns, the nuclei of the OHCs were immunostained, but less pronounced as compared to the

basal turn (Figs. 1E, H). Supporting cells did not demonstrate immunostaining in the middle and apical turns.
At 12 days, the nuclei of the OHCs in the basal, middle and apical turns were intensely stained with the NKI-A59 antiserum. In the basal turn, IHCs could not be discerned from the surrounding supporting cells. In the middle and apical turns, however, the IHCs demonstrated immunostaining comparable to that of the OHCs. In this group, nuclear immunostaining was manifest in all supporting cells in the organ of Corti.
After 14 days of cisplatin administration, OHC loss was observed. In the remaining OHCs as well as in the supporting cells immunostaining was present in all turns. Immunostaining in the IHCs was prominent in the basal turn, moderate in the middle turn and absent in the apical turn.
In the 16-day group, immunostaining for the OHCs and supporting cells was similar to that seen after 14 days (Figs. 1C, F, I). IHCs in the basal turn could not be discerned. Immunostaining in the IHCs was prominently present in the middle turn and, to a lesser degree, in the apical turn.

3.2.2. Detection of cisplatin-DNA adducts in the lateral wall

Animals from the non-treated group did not demonstrate immunostaining with the NKI-A59 antiserum in the lateral wall (i.e., stria vascularis and spiral ligament) of the basal, middle and apical turns (Figs. 2A, D, G).
Immunostaining in the lateral wall was first observed, in both animals, in the basal turn after 8 days of cisplatin administration (Fig. 2B). In the stria vascularis, all cell types (i.e., marginal cells, intermediate cells and basal cells) and the strial capillaries demonstrated moderate immunostaining. In the spiral ligament, the nuclei of the fibrocytes were stained.
After 10 days, moderate immunostaining was only present in the marginal cells and the spiral ligament fibrocytes of the basal turns. In contrast to the 8-day group, immunostaining for the intermediate and basal cells as well as the strial capillaries was completely absent, in both animals.
Prominent immunostaining was present after 12 days only in the nuclei of the marginal cells and the spiral ligament fibrocytes, both in the basal and middle turn (Figs. 2E). Immunostaining was moderate in the nuclei of the intermediate and basal cells as well as the strial capillaries of the middle turn, but remarkably these cell types were not immunostained in the basal turn. In the apical turn, the stria vascularis was devoid of labelling, but the spiral ligament fibrocytes were moderately immunostained.
After 14 days, prominent immunostaining was present in all cell types of the stria

vascularis in the basal turn, but the strial capillaries were only moderately stained. The spiral ligament fibrocytes demonstrated prominent labelling. In the middle turn, immunostaining was comparable to that of the 12-day group. In the apical turn, only the marginal cells and the spiral ligament fibrocytes demonstrated prominent staining (Fig. 2H).

In the 16-day group, nuclear immunostaining was present in all cell types of the stria vascularis as well as the spiral ligament fibrocytes, both in the basal and middle turns (Figs. 2C, F). In the apical turn, the nuclei of the marginal cells stained prominently, whereas the other cell types of the stria vascularis demonstrated only moderate staining. Prominent staining was present in the spiral ligament fibrocytes (Fig 2I).

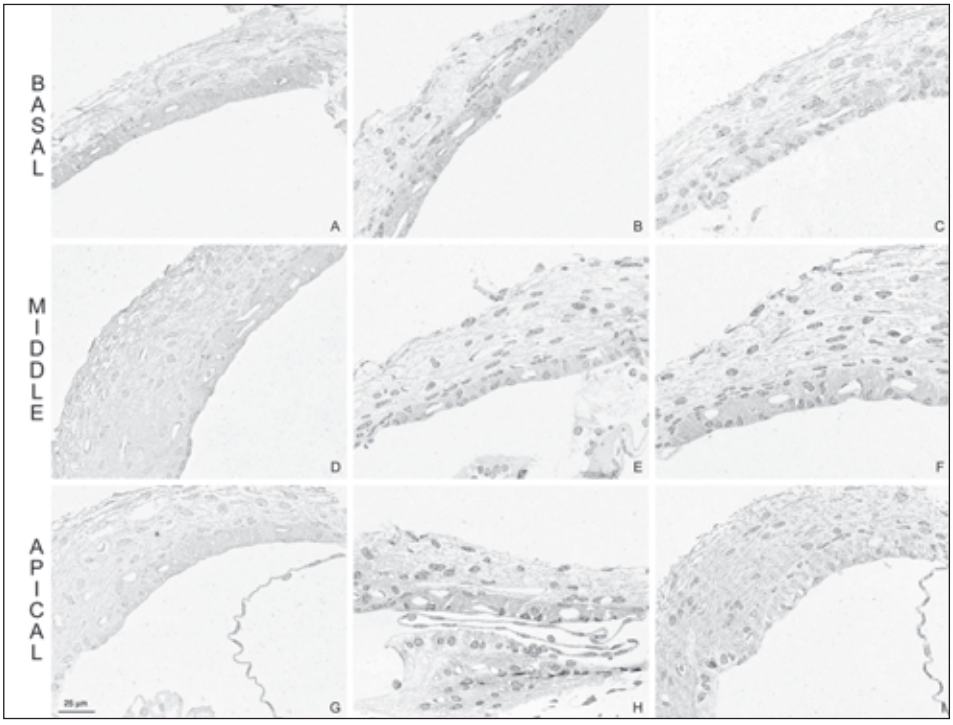


Figure 2.

Immunohistochemical detection of cisplatin-DNA adducts in the lateral wall (stria vascularis and spiral ligament). In the non-treated cochleas, immunostaining was absent in the basal (A), middle (D) and apical (G) turns. The light micrographs in the middle column represent the points of time at which immunostaining was first obvious in the different cochlear turns: In the basal turn after 8 days (B), in the middle turn after 12 days (E) and in the apical turn after 14 days (H). The light micrographs in the right column are from cochleas treated for 16 days: Immunostaining was present in the basal (C), middle (F) and apical (I) turns.

3.2.3 Detection of cisplatin-DNA adducts in the spiral ganglion

None of the animals from the non-treated group demonstrated immunostaining with the NKI-A59 antiserum in the spiral ganglion of the basal, middle and apical turns (Figs. 3A, D, G). Immunostaining in the SGCs was first observed in the basal turn after 14 days of cisplatin administration (Fig. 3B). The middle and apical turns were devoid of labelling (Figs. 3E, H). After 16 days of cisplatin administration, immunostaining was prominent in the nuclei of the SGCs in the basal and middle turns (Figs. 3C, F) and less prominent in the apical turn (Fig. 3I). In none of the groups did the Schwann cells demonstrate immunostaining.

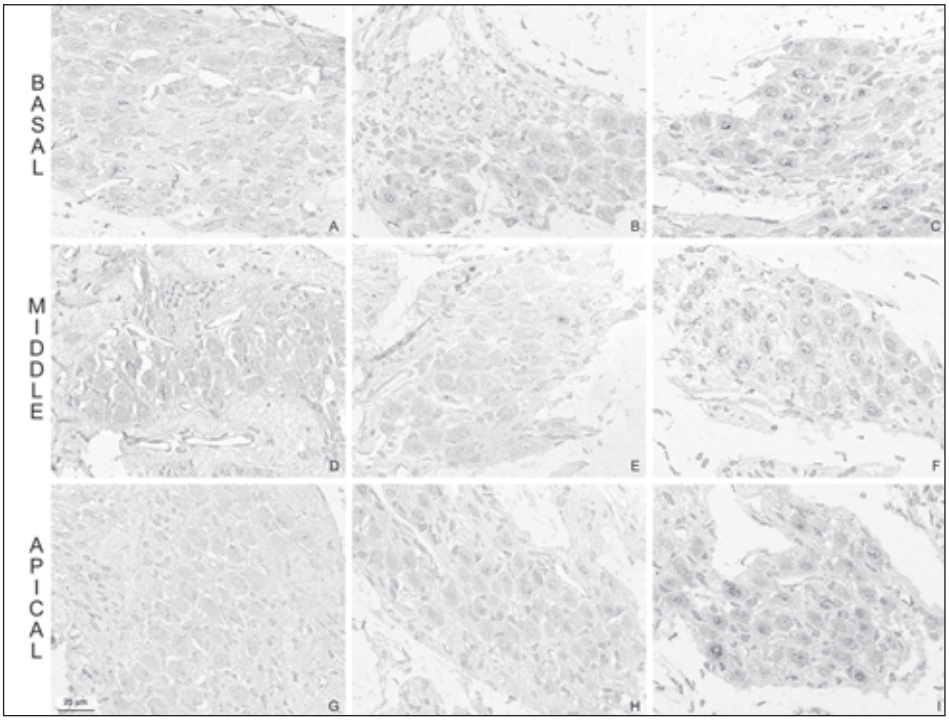


Figure 3.

Immunohistochemical detection of cisplatin-DNA adducts in the spiral ganglion. In the non-treated cochleas, immunostaining was absent in the basal (A), middle (D) and apical (G) turns. Immunostaining in the spiral ganglion was first obvious after 14 days in the basal (B) turn. The middle (E) and apical turns (H) were devoid of labelling at this point of time. After 16 days, immunostaining was present in the basal (C), middle (F) and apical (I) turns.

3.3. Electrocochleography

Electrocochleography was performed to determine whether the presence of cisplatin-DNA adducts is related to functional loss. Shifts in the amplitude growth curves of both CAP and CM were first seen after 8 days of cisplatin administration, wheras cisplatin-DNA adducts were first detected after 6 days.

Figure 4 shows the time-dependent relation between the presence of cisplatin-DNA adducts and the CAP and CM thresholds. Presence of cisplatin-DNA adducts is represented as the averaged degree of immunostaining (4 cochleas per experimental group). Subjective scores were given a numerical notation, allowing averaging: absent (-) = 0; moderate (+) =1; and prominent (++) = 2. Immunostaining was scored in the basal, middle and apical turns. Therefore, we compared immunostaining in the basal turn with the CAP and CM thresholds at 8 kHz, in the middle turn with the thresholds at 4 kHz, and in the apical turn with thresholds at 2 kHz. Thresholds of the CAP and CM at 8 kHz were increased at the same time that cisplatin-DNA adducts were present in the basal turn. At 4 and 2 kHz, however, increased CAP and CM thresholds preceded the occurrence of cisplatin-DNA adducts in the middle and apical turns, respectively. This suggests that the presence of cisplatin-DNA adducts in OHCs is not directly related with the threshold shifts in CAP and CM.

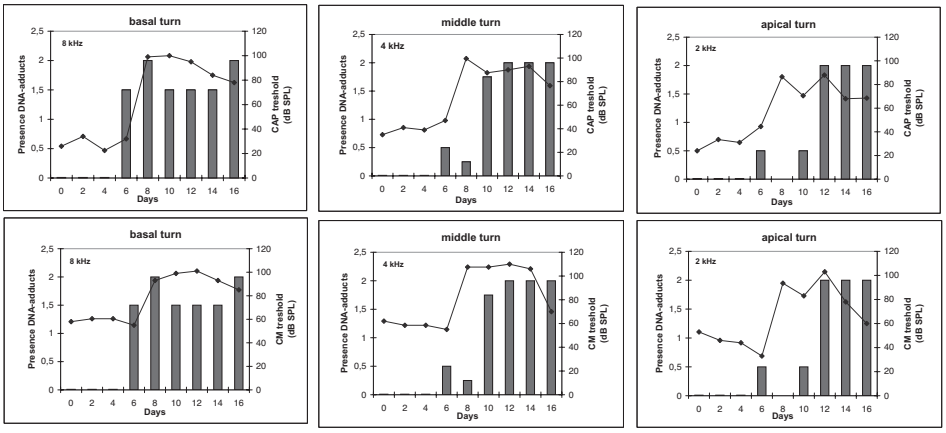


Figure 4.

The relation between the functional changes and the presence of cisplatin-DNA adducts in OHCs. The upper three panels depict the CAP threshold (dB SPL; lines) and the presence of cisplatin-DNA adducts (columns) during cisplatin administration. The lower three panels depict the CM threshold (dB SPL; lines) and the presence of cisplatin-DNA adducts (columns) during cisplatin administration. The presence of DNA-adducts is scored on a scale where "0" corresponds to absence of immunostaining and "2" to prominent immunostaining (- and ++, respectively, in Table 1).

4. Discussion

The results from the present study confirm the earlier conception that cisplatin interferes with cochlear function and morphology at three main levels, i.e., the organ of Corti, the stria vascularis and the spiral ganglion (Schweitzer, 1993; Cardinaal et al., 2000; Klis et al., 2000, 2002; Hamers, et al., 2003; Van Ruijven et al., 2004). In this study, we have tried to answer the question of whether or not one of these tissues is the primary target of cisplatin in the cochlea. For this, we have used an antiserum containing antibodies against cisplatin-DNA adducts to indirectly localize cisplatin in cryosections of the cochlea.

From this time-sequence study, it is apparent that the tissue in which cisplatin-DNA adducts first occur is the organ of Corti. After 6 days, immunostaining of cisplatin-DNA adducts was obvious in the OHCs of the basal turn. This is in line with a morphological time-sequence study using the same dose of cisplatin: Van Ruijven et al. (2004) demonstrated that OHC loss was present after 6 days.

Similar to the morphological changes seen after cisplatin administration, the presence of cisplatin-DNA adducts follows a longitudinal gradient in the cochlea i.e. cisplatin-DNA adducts are present in the basal turn at an earlier stage than in the middle and apical turns (Fig. 4). Remarkably, there was no difference in degree of immunostaining with the NKI-A59 antiserum between the different rows of OHCs. Morphological studies, however, have demonstrated that cisplatin-induced OHC loss follows a radial gradient, in that the first row of OHCs disappears at an earlier stage than the second and third rows (e.g., Van Ruijven et al., 2004). Apparently, the radial gradient in OHC loss is determined by differences in intrinsic vulnerability (e.g., oxidative stress, DNA repair) rather than the formation of cisplatin-DNA adducts.

Unfortunately, immunostaining in the IHCs was not always obvious in the cryosections. This is mainly due to the fact that it was not always possible to discern between the IHCs and the surrounding cells because of the distortion of the organ of Corti. Therefore, the limited data does not allow us to draw specific conclusions about the precise point of time, at which cisplatin-DNA adducts are formed in the nuclei of IHCs. However, although IHCs could not be observed in the basal turn after 10 days, they were present in the middle turn and demonstrated immunostaining.

In addition to the OHCs, immunostaining with the NKI-A59 antiserum is present in the Deiters' cells, although generally in a lesser degree. The finding that cisplatin-DNA adducts are also present in the nuclei of the supporting cells in the organ of Corti, substantiates the morphological and molecular evidence

that cisplatin also affects these cells (Estrem et al., 1981; Anniko and Sobin, 1986; Laurell and Bagger-Sjöbäck, 1991; Endo et al., 2002; Cardinaal et al., 2004; Ramírez-Camacho et al., 2004).

In this study, we also measured cochlear function by means of electrocochleography in order to study the relation of the presence of cisplatin-DNA adducts in OHCs to functional changes. Shifts in the thresholds of both CAP and CM were first seen after 8 days of cisplatin administration. In the basal turn, functional changes at 8 kHz occurred after the formation of cisplatin-DNA adducts in the nuclei of the OHCs (6 days). In contrast, in the middle and apical turns the threshold shifts at lower frequencies precede the presence of relatively large amounts of adducts by several days. This suggest that the presence of cisplation-DNA adducts in OHCs is not directly related with the threshold shift in CAP and CM.

Immunostaining in the lateral wall is first seen after 8 days in all cell types of the stria vascularis and the spiral ligament fibrocytes. In contrast to the 8-day group, immunostaining of cisplatin-DNA adducts was completely absent from the intermediate cells, basal cells and the strial capillaries in the basal turn after 10 and 12 days. Remarkably, after 14 days, all strial cells in the basal turn again demonstrated immunostaining. The lack of immunostaining in the intermediate cells and basal cells as well as the strial capillaries in the basal turn at 10 and 12 days cannot be explained at present. From 12 days onwards, immunostaining of cisplatin-DNA adducts in the stria vascularis and the spiral ligament fibrocytes progresses to the more apically located turns.

Since morphological changes were not found in the stria vascularis after 8 days using the same treatment protocol (Van Ruijven et al., 2004), it may be surmised that formation of cisplatin-DNA adducts does not immediately result in morphological changes in the stria vascularis.

Formation of cisplatin-DNA adducts in SGCs is apparently a late event, since immunostaining of cisplatin-DNA adducts is first seen after 14 days. This late appearance of adducts may be explained by a natural barrier: The Schwann cell with its myelin sheath, surrounding the perikaryon of the SGC, prevents cisplatin uptake by the latter. A recent morphological study (Van Ruijven et al., 2004) has demonstrated that after 6 days of cisplatin administration the myelin sheaths detach from the perikarya of the SGCs. This detachment may enable cisplatin to enter the perikaryon, which eventually results in the formation of cisplatin-DNA adducts.

Schweitzer (1993) detected radiolabelled cisplatin in homogenated samples of the organ of Corti and the stria vascularis within 1 h after a single injection. In homogenated samples, cisplatin is detected not only in the cellular fraction of the

tissue but also in the extracellular fraction. In our study, cisplatin is detected after it has entered the cell nucleus and interacted with nuclear DNA. As illustrated by immunostaining of cisplatin-DNA adducts in renal cortex samples, it takes some days (4 days) for the cisplatin-DNA adducts to reach a concentration exceeding the detection limit of the method. A delay between uptake and detection of cisplatin is also observed in the cochlear tissues (organ of Corti: 6 days; lateral wall: 8 days; spiral ganglion: 14 days).

A recent time-sequence study (Van Ruijven et al., 2004) demonstrated that OHC loss and myelin sheath detachment in the type-I SGCs are present after 6 days of cisplatin administration, whereas histological changes in the stria vascularis were not obvious, not even after 8 days. It appears from that study that the changes in the OHCs and type-I SGCs occur simultaneously and develop in parallel. This observation cannot be explained by the formation and presence of cisplatin-DNA adducts, since DNA adducts are only formed in the spiral ganglion after 14 days. The formation of cisplatin-DNA adducts is probably not the only cause of cisplatin ototoxicity. Cisplatin is also known to interact with other cellular macromolecules (e.g., glutathione, metallothionein) and organelles (e.g., mitochondria, endoplasmic reticulum), and these interactions may also result in cell degeneration.

From the present study we can conclude that cisplatin is internalized in the organ of Corti (6 days), the lateral wall (8 days) and the spiral ganglion (14 days), that it interacts with DNA to form cisplatin-DNA adducts. The observation that cisplatin-DNA adducts are first observed in the organ of Corti, several days before staining is obvious in the lateral wall, suggests that the toxic effect on the OHCs is not secondary to an effect of cisplatin upon the lateral wall tissues.

Acknowledgements

This study has been supported by grants from the Heinsius-Houbolt Foundation, the Netherlands. The purchase of a Leica DM RA light microscope was made possible by a generous donation of Mr. L.G.M. van Gansewinkel. The authors wish to thank Drs. A. Begg and B. Floot (Netherlands Cancer Institute, Amsterdam, the Netherlands) for their kind gift of the NKI-A59 antiserum. Dr. S.F.L. Klis is kindly acknowledged for performing the electrophysiological measurements.

Marjolein W.M. van Ruijven is a Heinsius-Houbolt Research Fellow at the Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands.

References

Anniko, M., Sobin, A., 1986. Cisplatin: Evaluation of its ototoxic potential. Am. J. Otolaryngol. 7, 276-293.

Bergström, P., Johnsson, A., Cavallin-Stahl, E., Bergenheim, T., Henriksson, R., 1997. Effects of cisplatin and amphotericin B on DNA adduct formation and toxicity in malignant glioma and normal tissues in rat. Eur. J. Cancer 33, 153-159.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh) 124, 144-154.

Endo, T., Nakagawa, T., Lee, J.E., Dong, Y., Kim, T.S., Iguchi, F., Taniguchi, Z., Naito, Y., Ito, J., 2002. Alteration in expression of p27 in auditory epithelia and neurons of mice during degeneration. Neurosci. Lett. 334, 173-176.

Estrem, S.A., Babin, R.W., Ryu, J.H., Moore, K.C., 1981. Cis-diamminedichloroplatinum (II) ototoxicity in the guinea pig. Otolaryngol. Head Neck Surg. 89, 638-645.

Flock, A., Hoppe, Y., Wei, X., 1981. Immunofluorescence localization of proteins in semithin 0.2-1 μm frozen sections of the ear. A report of improved techniques including gelatin encapsulation and cryoultramicrotomy. Arch. Otorhinolaryngol. 233, 55-66.

Gonzalez, V.M., Fuertes, M.A., Alonso, C., Perez, J.M., 2001. Is cisplatin-induced cell death always produced by apoptosis? Mol. Pharmacol. 59, 657-663.

Hamers, F.P.T, Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by α-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

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

Klis, S.F.L., O'Leary, S.J., Hamers, F.P.T., De Groot, J.C.M.J., Smoorenburg, G.F., 2000. Reversible cisplatin ototoxicity in the albino guinea pig. NeuroReport 11, 623-626.

Klis, S.F.L., O'Leary, S.J., Wijbenga, J., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2002. Partial recovery of cisplatin-induced hearing loss in the albino guinea pig in relation to cisplatin dose. Hear. Res. 164, 138-146.

Laurell, G., Bagger-Sjoback, D., 1991. Degeneration of the organ of Corti following intravenous administration of cisplatin. Acta Otolaryngol. (Stockh.) 111, 891-898.

Meijer, C., De Vries, E.G.E., Marmiroli, P., Tredici, G., Frattola, L., Cavaletti, G., 1999. Cisplatin-induced DNA-platination in experimental dorsal root ganglia neuropathy. Neurotoxicol. 20, 883-888.

Meijer, C., Van Luyn, M.J.A., Nienhuis, E.F., Blom, N., Mulder, N.H., Vries, E.G.E., 2001. Ultrastructural morphology and localisation of cisplatin-induced platinum-DNA adducts in a cisplatin-sensitive and -resistant human small cell lung cancer cell line using electron microscopy. Biochem. Pharmacol. 61, 573-578.

O'Leary, S.J., Klis, S.F., 2002. Recovery of hearing following cisplatin ototoxicity in the guinea pig. Anticancer Res. 22, 1525-1528.

Ramírez-Camacho, R., García-Berrocal, J.R., Buján, J., Martín-Marero, A., Trinidad, A., 2004. Supporting cells as a target of cisplatin-induced inner ear damage: Therapeutic implications. Laryngoscope 114, 533-537.

Reed, E., Dabholkar, M., Chabner, B.A., 1996. Platinum analogs. In: Chabner, B.A., Longo, D.L. (Eds.) Cancer Chemotherapy and Biotherapy, 2nd Edition. Lippincott-Raven Publishers, Philadelphia, pp. 357-378.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Takagi, I., Hendriksen, E.G.J., De Groot, J.C.M.J., Veldman, J.E., 1994. A new, high-resolution cryotechnique in inner ear immunohistochemistry: A more precise localization of intermediate filament proteins in the guinea pig inner ear. In: Mogi, G., Veldman, J.E., Kawauchi, H. (Eds.) Immunobiology in Otorhinolaryngology. Progress of a Decade. Kugler Publications, Amsterdam/New York, pp. 297-301.

Tokuyasu, K.T., 1989. Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. Histochem. J. 21, 163-171.

Terheggen, P.M.A.B., Floot, B.G.J., Scherer, E., Begg, A.C., Fichtinger-Schepman, A.M.J., Den Engelse, L., 1987. Immunocytochemical detection of interaction products of cis- diam minedichloroplatinum(II) and cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in rodent tissue sections. Cancer Res. 47, 6719-6725.

Terheggen, P.M., Gerritsen Van Der Hoop, R., Floot, B.G., Gispen, W.H., 1989. Cellular distribution of cis-diamminedichloroplatinum(II)-DNA binding in rat dorsal root spinal ganglia: Effect of the neuroprotecting peptide ORG 2766. Toxicol. Appl. Pharmacol. 99, 334-343.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Hendriksen, F., Smoorenburg, G.F., 2004. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. Hear. Res. 197, 44-54.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Hendriksen, F., Smoorenburg, G.F., 2005. Immunohistochemical detection of platinated DNA in the cochlea of cisplatin-treated guinea pigs. Hear. Res. (in revision).

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1997. Cisplatin-induced ototoxicity: Electrophysiological evidence of spontaneous recovery in the albino guinea pig. Hear. Res. 111, 103-113.

Zamble, D.B., Lippard, S.J., 1995. Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem. Sci. 20, 435-439.

Chapter 6

General discussion and summary

Cisplatin is used extensively in cancer treatment. Therefore, its ototoxic side effects are of great concern. Numerous studies have already shown that cisplatin ototoxicity involves functional and morphological changes in the organ of Corti, the stria vascularis, and the spiral ganglion. However, it remains unknown whether or not these changes are mutually dependent. In other words: Is there a primary target of cisplatin in the cochlea or do these changes develop in parallel? Determination of the time sequence of the degeneration patterns in cochlear tissues, as well as the precise localization of the cellular site(s) of uptake and accumulation of cisplatin in the cochlea may shed more light onto this issue.

Time sequence of cisplatin ototoxicity

In Chapter 2, we investigated the key tissues that are implicated in cisplatin ototoxicity within the time window during which degeneration starts. This study demonstrates that cisplatin-induced loss of OHCs starts at the basal turn between 4 and 6 days. After 8 days, it has progressed to the middle turn of the cochlea. These data demonstrate that the degeneration pattern in the organ of Corti follows a longitudinal gradient, with OHC loss starting in the basal turn and progressively extending to the more apically located turns. At this point of time, loss of IHCs is not apparent in midmodiolar sections. From the surface preparations of cochleas from 8-days treated animals, it is evident that OHC loss also demonstrates a radial gradient. In the 8-day group, OHC loss in the first row is more extensive than in the second and third row. Also, signs of IHC loss are not obvious.

After 6 days, a significant amount of myelinated (type-I) SGCs in the basal turn demonstrated detachment of their myelin sheath. After 8 days, type-I SGCs in the the middle turn were also affected. SGC loss may succeed myelin sheath detachment. However, SGC densities did not decrease after 6 and 8 days of cisplatin administration. This indicates that, although histological changes developed between 4 and 6 days, loss of SGCs did not occur up to 8 days. This new finding of myelin sheath detachment is discussed in more detail in the next section.

In order to determine to what extent the OHCs and the SGCs contribute to the deterioration of the cochlear response after cisplatin administration, the individual electrophysiological data (CAP, CM) were compared with the degree of OHC loss and the amount of type-I SGCs demonstrating myelin sheath detachment (Chapter 3). Analysis of these data did not provide evidence that the

observed changes in the type-I SGCs are secondary to loss of OHCs, or vice versa. Therefore, we infer that the effects in the organ of Corti and the spiral ganglion start simultaneously between the fourth and sixth day of cisplatin administration and that they appear to develop in parallel.

Morphological changes were not observed in the stria vascularis, not even after 8 days. This finding is in line with previous studies applying this treatment protocol (De Groot et al., 1997, Cardinaal et al., 2000a). It should be noted that the development of histological changes in the stria vascularis depends on the total dose of cisplatin (Cardinaal 2000a, b), the treatment protocol and time after cessation of treatment. An increase in strial volume immediately after a single high-dose was reported by Meech et al. (1998) and Campbell et al. (1999), whereas Sluyter et al. (2003) observed a decrease in strial cross-sectional area only after post-treatment survival of ≥4 weeks following treatment with cisplatin (1.5 mg/kg/day). In addition, endolymphatic hydrops – which may be regarded as a histological indication of strial dysfunction – was not found. Although morphological changes could not be discerned in the stria vascularis within this time window (4-8 days) at this dose (2 mg/kg/day), it cannot be excluded that cisplatin acts at a molecular level. Klis et al. (2000, 2002) measured changes in the EP using a similar treatment protocol.

The spiral ganglion

The effects of cisplatin on the organ of Corti and the stria vascularis are well established (for reviews, see De Oliveira 1989; Schweitzer, 1993). On the other hand, the finding that cisplatin has an effect on the SGCs in adult cochleas is relatively new. In addition, studies that describe the effect of cisplatin on the spiral ganglion in adult cochleas are rather limited, and a variety of effects have been reported. Alam et al. (2000) reported an increase in the bax/bcl-2 ratio in SGCs of mongolian gerbil cochleas after cisplatin administration, suggesting the involvement of apoptosis in cisplatin-induced SGC degeneration. Cardinaal et al. (2004) observed mitochondrial swelling in SGCs of cisplatin-treated guinea pigs. In addition, cisplatin may induce cell shrinkage (O'Leary et al., 2001) and nuclear shrinkage (Hamers et al., 2003) in SGCs of the adult guinea-pig cochlea.

In the first study (Chapter 2) we observed that, during systemic administration, in many of the myelinated (type-I) SGCs, the myelin sheaths were detached from the perikarya, leaving a void space between the perikarya and the enveloping myelin sheaths. The number of SGCs showing this effect progressed after 6 and 8 days of treatment. Morphometrical analysis demonstrated that detachment of the

myelin sheath was due to both perikaryal shrinkage and swelling of the myelin sheath. It was not possible, however, to determine whether cisplatin initially affects the surrounding Schwann cell with its myelin sheath or the auditory neuron itself. It can be surmised that cisplatin first affects the Schwann cells resulting in swelling of the myelin sheath, which may lead to osmotic changes in the SGCs and, eventually, to perikaryal shrinkage. The effect of cisplatin on Schwann cells may be similar to its effect on the satellite cells in the dorsal root ganglia. These "glial" cells are more affected by cisplatin than the neuronal cells themselves (*cf.*, Ter Laak et al., 2000).

It is generally assumed that loss of SGCs is secondary to degeneration of the organ of Corti. Ylikoski et al. (1974) and Spoendlin (1979) found that retrograde degeneration of SGCs occurs only after IHCs have degenerated, and that it is a slow and progressive process. The morphological time-sequence study (Chapter 2) indicates that detachment of the myelin sheath of type-I SGCs is not due to retrograde degeneration, especially since IHC loss was not present, not even after 8 days of cisplatin administration.

SGCs, unlike OHCs, have received little attention in functional studies of cisplatin ototoxicity. In the second study (Chapter 3), we observed that high CAP thresholds, but low CM thresholds, are present in animals showing partial OHC loss. This strongly suggests that a component other than hair cell function and the EP has affected the CAP. This component could be dysfunction of the SGCs. When comparing the histological and electrophysiological data, we found a correlation between the extent of morphological damage to the type-I SGCs and the differences in the CM and CAP data. This study demonstrates that once animals are severely affected by cisplatin administration (judging from the loss of OHCs), their CAP threshold shift cannot be explained by the CM threshold shift alone, but an additional component, probably involving the SGCs, is active. In conclusion, the data presented in Chapters 2 and 3 definitely prove that the spiral ganglion, also, is a cochlear target of cisplatin.

Cochlear distribution of cisplatin

Since attempts to identify the cellular sites of cisplatin uptake and accumulation in histological sections of the cochlea have failed (Saito and Aran, 1994; Welb, 1995), we developed an immunohistochemical method to indirectly localize cisplatin in semithin cryosections of cochlear tissues (Chapter 4). Cisplatin was detected in microdissected segments from basal cochlear turns of guinea pigs treated with cisplatin for 2x5 days, using a polyclonal rabbit antiserum containing antibodies against cisplatin-DNA adducts. Immunostaining was observed in all cell nuclei

of the organ of Corti and the lateral wall, but it was most prominent present in the OHCs, the marginal cells of the stria vascularis and the spiral ligament fibrocytes. This is the first study to demonstrate the presence of cisplatin-DNA adducts – hence, the presence of cisplatin – in histological sections of cochlear tissues.

Using this immunohistochemical method, the time-related distribution of cisplatin in cochlear tissues was investigated in order to determine the drug's primary target (Chapter 5). Cisplatin-DNA adducts were first obvious in the organ of Corti in the basal turn: After 6 days, immunostaining was present in the nuclei of all three rows of OHCs and, to a lesser degree, in the nuclei of the Deiters' cells. Immunostaining in the organ of Corti demonstrated a longitudinal gradient, starting in the basal turn and continuing to progress to the more apically located turns. From day 10 onwards, immunostaining was also obvious in the middle and apical turns.

Immunostaining in the lateral wall also demonstrated a longitudinal gradient, starting in the basal turn after 8 days of cisplatin administration. All strial cell types (i.e., marginal cells, intermediate cells and basal cells) and the strial capillaries demonstrated moderate immunostaining. At this point of time, the nuclei of the spiral ligament fibrocytes were also immunostained. After prolonged administration (12 days or more), immunostaining had extended to the middle and apical turns. In view of our previous studies, the presence of cisplatin-DNA adducts in spiral ligament fibrocytes was not anticipated. It may be related to cisplatin-induced changes in solute transport in the cochlea (Suzuki and Kaga, 1996, 1997; Laurell et al., 1997), especially since the different types of spiral ligament fibrocytes are involved in potassium-recycling pathways between the perilymphatic and endolymphatic compartments (Weber et al., 2001). In addition, cisplatin-induced changes in the spiral ligament fibrocytes have been described. In adult guinea pig cochleas, immunostaining for caspase-activated deoxyribonuclease, caspase 3, and fragments of single-stranded DNA has been observed in the spiral ligament after cisplatin administration (Watanabe et al., 2002, 2003). Furthermore, Touliatos et al. (2000) observed strong positive immunostaining for glutathione-*S*-transferase in cisplatin-treated adult rats, with the most intense staining found in the spiral ligament. Taken together, these results indicate that the spiral ligament fibrocytes are also affected by cisplatin.

The third cochlear tissue in which immunostaining for cisplatin-DNA adducts was observed is the spiral ganglion. SGCs from the basal and middle turns showed immunostaining for cisplatin-DNA adducts not before 14 days of continuous administration. The surrounding Schwann cells were devoid of

immunostaining, even after 16 days. However, detachment of the myelin sheath was already observed after 6 days of cisplatin administration.

Although we have demonstrated that cisplatin enters cochlear cells and reacts with nuclear DNA to form cisplatin-DNA adducts, the formation of cisplatin-DNA adducts per se cannot account for the morphological and/or functional damage in the cochlea. It should be noted that the toxic effect of cisplatin in tumor cells arises from the drug's ability to form cisplatin-DNA adducts, which eventually results in mitotic arrest. However, it is unlikely that, for example, hair cell loss is mediated by a similar mechanism since hair cells are terminally differentiated (post-mitotic) cells that do not replicate. In addition, in the immunohistochemical time-sequence study, it is evident that all three rows of OHCs stain in an equal degree with the antibodies against cisplatin-DNA adducts, and do not demonstrate a radial gradient. Apparently, the radial gradient in OHC loss (Chapter 2) is determined by differences in intrinsic vulnerability (e.g., glutathione depletion, oxidative stress, DNA repair), rather than the formation of cisplatin-DNA adducts (Sha et al., 2001). In conclusion, despite the fact that we have demonstrated that cisplatin-DNA adducts are formed in cochlear cells, actual cell degeneration may be induced by the reaction of cisplatin with other cellular macromolecules (e.g., glutathione, metallothionein) and organelles (e.g., mitochondria, endoplasmic reticulum). This is strengthened by numerous publications which report that cisplatin administration causes depletion of intracellular glutathione levels as well as decreased activities of antioxidant enzymes in the cochlea, resulting in the accumulation of reactive oxygen species and peroxidation of membrane phospholipids (Schweitzer, 1993; Ravi et al., 1995; Rybak et al., 1995; Kamimura et al., 1999).

The cochlear targets of cisplatin and future research

Taken together, the morphological and immunohistochemical (time-sequence) studies seem to indicate that the organ of Corti, and in particular the OHCs, is the main cochlear target of cisplatin. With regard to the organ of Corti, there is a high concordance of the morphological and immunohistochemical data. Both OHC loss and the first occurrence of cisplatin-DNA adducts are a relatively early event (between 4 and 6 days). However, it cannot be concluded that the organ of Corti is the only target to be first affected by cisplatin, since histological changes in the spiral ganglion do occur in the same time window during which OHC loss first develops.

In the spiral ganglion, cisplatin administration results in detachment of the myelin sheath in type-I SGCs. This detachment is due to perikaryal shrinkage

of the SGC itself and swelling of the myelin sheath of the surrounding Schwann cell. It seems unlikely that these morphological changes are the result of the formation of cisplatin-DNA adducts, since immunostaining was only observed in the SGCs after 14 days of cisplatin administration whereas in Schwann cells immunostaining was not observed at all. Studies using a more narrow time-window may be helpful to better define the starting points of the effects in the organ of Corti and spiral ganglion.

Morphological changes in the stria vascularis were not observed in our study, not even after 8 days of cisplatin administration. However, immunostaining for cisplatin-DNA adducts was already observed after 8 days. The latter finding indicates that cisplatin is internalized by the strial cells. It is likely that, in the stria vascularis, cisplatin acts at a molecular level and may interfere with strial processes which may lead to a depression in the EP (Klis et al., 2000, 2002). Future studies should focus on both the ultrastructural and molecular changes in strial cells during cisplatin administration. Also, it would be interesting to investigate why cisplatin at daily doses <2 mg/kg does induce morphological changes that are obvious in light microscopy (Cardinaal et al., 2000).

The spiral ligament fibrocytes showed immunostaining for cisplatin-DNA adducts. The effect of cisplatin on these cells is not well known. However, several studies have shown that cisplatin does affect the spiral ligament fibrocytes (Suzuki and Kaga 1996, 1997; Laurell et al., 1997; Touliatos et al., 2000; Watanabe et al., 2002, 2003). Thus, future studies should not only investigate the effects of cisplatin on the organ of Corti, the stria vascularis and the spiral ganglion, but also on the spiral ligament fibrocytes.

Some studies suggest that alterations in cochlear function and morphology may spontaneously recover after cessation of cisplatin administration (Stengs et al., 1997; Cardinaal et al., 2000b, 2004; Klis et al., 2002; O'Leary and Klis, 2002). Therefore, it would be very interesting to study the presence of cisplatin-DNA adducts during and after cessation of cisplatin administration. Will there be DNA repair in cochlear cells during and after recovery?

More research should be done on the identification of the subcellular target(s) of cisplatin, such as mitochondria, lysosomes and endoplasmic reticulum. Future studies may include detection of cisplatin-DNA adducts at the ultrastructural level (using immuno-electron microscopy), in order to determine whether these adducts are also present in mitochondria.

A disadvantage of our immunohistochemical method is that it can only be used to localize platinated DNA. Furthermore, only a small fraction of internalized cisplatin will react with nuclear DNA (Bose, 2002; Fuertes et al., 2003) and,

therefore, this method can only localize a small part of the total amount of internalized cisplatin. Alternatively, X-ray microanalytic techniques could be used to detect elemental platinum in ultrathin sections of tissues. However, all attempts to detect cisplatin in ultrathin sections of the cochlea using this approach have failed (Maruyama et al., 1993; Saito and Aran, 1994a; Welb, 1995). This failure may be explained by the fact that the intracellular concentrations of platinum were below the detection limit. Also, cisplatin could have been extracted from the cochlear tissues during chemical fixation and subsequent histological processing. The extraction of cisplatin during tissue processing may be prevented by precipitation of the platinum followed by autometallographic amplification (Guthrie and Balaban, 2004).

Research directed at the identification of the exact mechanisms by which cisplatin acts upon the different cochlear tissues should be continued. It may be helpful in the development and implementation of more sophisticated treatment protocols to prevent the ototoxic side effects of this drug.

Acknowledgements
This study has been supported by grants from the Heinsius-Houbolt Foundation, the Netherlands. The purchase of a Leica DM RA light microscope was made possible by a generous donation of Mr. L.G.M. van Gansewinkel. The authors wish to thank Drs. A. Begg and B. Floot (Netherlands Cancer Institute, Amsterdam, the Netherlands) for their kind gift of the NKI-A59 antiserum. Dr. S.F.L. Klis is kindly acknowledged for performing the electrophysiological measurements.
Marjolein W.M. van Ruijven is a Heinsius-Houbolt Research Fellow at the Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands.

References

Alam, S.A., Ikeda, K., Oshima, T., Suzuki, M., Kawase, T., Kikuchi, T., Takasaka, T., 2000. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. Hear. Res. 141, 28-38.

Bose, R.N., 2002. Biomolecular targets for platinum antitumor drugs. Mini Rev. Med. Chem. 2, 103-111.

Campbell, K.C.M., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear. Res. 138, 13-28.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000a. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. Hear. Res. 144, 135-146.

Cardinaal, R.M., De Groot, J.C.M.J., Huizing, E.H., Veldman, J.E., Smoorenburg, G.F., 2000b. Cisplatin-induced ototoxicity: Morphological evidence of spontaneous outer hair cell recovery in albino guinea pigs? Hear. Res. 144, 147-156.

Cardinaal, R.M., De Groot, J.C.M.J, Huizing, E.H., Smoorenburg, G.F., Veldman, J.E., 2004. Ultrastructural changes in the albino guinea pig cochlea at different survival times following cessation of 8-day cisplatin administration. Acta Otolaryngol. (Stockh.) 124, 144-154.

De Groot, J.C.M.J., Hamers, F.P.T., Gispen, W.H., Smoorenburg, G.F., 1997. Co-administration of the neurotropic $ACTH_{(4-9)}$ analogue, ORG 2766, may reduce the cochleotoxic effects of cisplatin. Hear. Res. 106, 9-19.

De Oliveira, J.A.A., 1989. Audiovestibular Toxicity of Drugs, Volume II. CRC Press, Boca Raton, FL, pp. 181-198.

Fuertes, M.A., Castilla, J., Alonso, C., Perez, J.M., 2003. Cisplatin biochemical mechanism of action: From cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. Curr. Med. Chem. 10, 257-266.

Guthrie, O.W., Balaban, C., 2004. Autometallographical amplification of intracellular anti-cancer platinum molecules. Abstractbook 27th Midwinter Meeting of the ARO, Daytona Beach, p. 137.

Hamers, F.P.T, Wijbenga, J., Wolters, F.L.C., Klis, S.F.L., Sluyter, S., Smoorenburg, G.F., 2003. Cisplatin ototoxicity involves organ of Corti, stria vascularis and spiral ganglion: Modulation by α-MSH and ORG 2766. Audiol. Neurootol. 8, 305-315.

Kamimura, T., Whitworth, C.A., Rybak, L.P., 1999. Effect of 4-methylthiobenzoic acid on cisplatin-induced ototoxicity in the rat. Hear. Res. 131, 117-127.

Klis, S.F.L., O'Leary, S.J., Hamers, F.P.T., De Groot, J.C.M.J., Smoorenburg, G.F., 2000. Reversible cisplatin ototoxicity in the albino guinea pig. NeuroReport 11, 623-626.

Klis, S.F.L., O'Leary, S.J., Wijbenga, J., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2002. Partial recovery of cisplatin-induced hearing loss in the albino guinea pig in relation to cisplatin dose. Hear. Res. 164, 138-146.

Laurell, G., Teixeira, M., Sterkers, O., Ferrary, E., 1997. Paracellular transport properties of inner ear barriers do not account for cisplatin toxicity in the rat. Hear. Res. 110, 135-140.

Maruyama, K., Furuya, N., Daimon, T., 1993. Distribution of platinum in the inner ear of guinea pig after treatment with cisplatin. J. Otolaryngol. (Jpn) 96, 1758-1759.

Meech, R.P., Campbell, K.C.M., Hughes, L.F., Rybak, L.P., 1998. A semiquantative analysis of the effects of cisplatin on the rat stria vascularis. Hear. Res. 124, 44-59.

O'Leary, S.J., Klis, S.F.L., De Groot, J.C.M.J., Hamers, F.P.T., Smoorenburg, G.F., 2001. Perilymphatic application of cisplatin over several days in albino guinea pigs: Dose-dependency of electrophysiological and morphological effects. Hear. Res. 154, 135-145.

O'Leary, S.J., Klis, S.F., 2002. Recovery of hearing following cisplatin ototoxicity in the guinea pig. Anticancer Res. 22, 1525-1528.

Ravi, R., Somani, S.M., Rybak, L.P., 1995. Mechanism of cisplatin ototoxicity: Antioxidant system. Pharmacol. Toxicol. 76, 386-394.

Rybak, L.P., Ravi, R., Somani, S.M., 1995. Mechanism of protection by diethyldithiocarbamate against cisplatin ototoxicity: Antioxidant system. Fundam. Appl. Toxicol. 26, 293-300.

Sha, S.H., Taylor, R., Forge, A., Schacht, J., 2001. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear. Res. 155, 1-8.

Saito, T., Aran, J.M., 1994. X-ray microanalysis and ion microscopy of guinea pig cochlea and kidney after cisplatin treatment. ORL 56, 310-314.

Schweitzer, V.G., 1993. Cisplatin-induced ototoxicity: The effect of pigmentation and inhibitory agents. Laryngoscope 103, Supplement 59.

Sluyter, S., Klis, S.F.L, De Groot, J.C.M.J., Smoorenburg, G.F., 2003. Alterations in the stria vascularis in relation to cisplatin ototoxicity and recovery. Hear. Res. 185, 49-56.

Spoendlin, H., 1979. Anatomischen-pathologische Aspekte der Elektrostimulation des ertaubten Innenohres. Arch. Otorhinolaryngol. 223, 1-75.

Stengs, C.H.M., Klis, S.F.L., Huizing, E.H., Smoorenburg, G.F., 1997. Cisplatin-induced ototoxicity: Electrophysiological evidence of spontaneous recovery in the albino guinea pig. Hear. Res. 111, 103-113.

Suzuki, M., Kaga, K., 1996. Effect of cisplatin on the negative barrier in strial vessels of the guinea pig. A transmission electron microscopy study using polyethyleneimine molecules. Eur. Arch. Otorhinolaryngol. 253, 351-355.

Suzuki, M., Kaga, K., 1997. Effect of cisplatin on the basement membrane anionic sites in the ampulla, macula, and stria vascularis of guinea pigs. Ann. Otol. Rhinol. Laryngol. 106, 971-975.

Ter Laak, M.P., Lankhorst, A.J., Schrama, L.H., Gispen, W.H., 2000. Neuropathies. Neurosc. Res. Commun. 26, 227-239.

Touliatos, J.S., Neitzel, L., Whitworth, C., Rybak, L.P., Malafa, M., 2000. Effect of cisplatin on the expression of glutathione-S-transferase in the cochlea of the rat. Eur. Arch. Otorhinolaryngol. 257, 6-9.

Watanabe, K.C., Jinnouchi, K., Hess, A., Michel, O., Baba, S., Yagi, T., 2002. Carboplatin induces less apoptosis in the cochlea of guinea pigs than cisplatin. Chemotherapy 48, 82-87.

Watanabe, K., Inai, S., Jinnouchi, K., Baba, S., Yagi, T. 2003. Expression of caspase-activated deoxyribonuclease (CAD) and caspase 3 (CPP32) in the cochlea of cisplatin (CDDP)-treated guinea pigs. Auris Nasus Larynx 30, 219-225.

Welb, R., 1995. Experimentelle Studie über die Wirkung von Cisplatin auf das Innenohr. PhD Thesis, Medizinischen Fakultät der Heinrich-Heine-Universität, Düsseldorf, Germany.

Ylikoski, J., Wersäll, J. Björkroth, B., 1974. Correlative studies on the cochlear Pathology and hearing loss in guinea pigs after intoxication with ototoxic antibiotics. Acta Otolaryngol. (Stockh.) Supplement 326, 23-41.

Nederlandse samenvatting
De aangrijpingspunten van cisplatine in de cochlea

Cisplatine is een cytostaticum dat gebruikt wordt bij de behandeling van kwaadaardige hoofd-halstumoren, zaadbal- en eierstoktumoren. Behandeling met cisplatine kan leiden tot ernstige bijwerkingen zoals een sterk verminderde nierfunctie (nefrotoxiciteit), uitval van de zenuwfunctie in de ledematen (perifere neuropathie) en verslechtering van het gehoor (ototoxiciteit). Patiënten die worden behandeld met cisplatine kunnen een gehoorsverlies ontwikkelen dat gekarakteriseerd wordt door oorsuizen en/of tweezijdig, meestal permanent verlies in de hogere frequenties. Op termijn kunnen ook de lagere frequenties, die betrokken zijn bij het spraakverstaan, aangedaan worden. Proefdieronderzoek heeft aangetoond dat behandeling met cisplatine leidt tot schade in verschillende weefsels van het binnenoor (*cochlea*, het slakkenhuis). Eén van deze weefsels is het orgaan van Corti, waarin de binnenste en buitenste haarcellen liggen. Deze zintuigcellen zijn betrokken bij de omzetting van geluidssignalen in zenuwimpulsen. Tijdens behandeling met cisplatine raken in eerste instantie de buitenste haarcellen in de basale winding (waar de hoge tonen waargenomen worden) beschadigd. Ze sterven vervolgens af. Dit verlies van buitenste haarcellen breidt zich, na langdurige behandeling of toediening van hogere doseringen, uit naar de middelste en apicale windingen (waar lagere tonen waargenomen worden). Op termijn sterven ook de binnenste haarcellen af. Zonder haarcellen is er geen overdracht van geluidssignalen naar elektrische zenuwimpulsen meer mogelijk, hetgeen resulteert in gehoorsverlies. Naast het orgaan van Corti veroorzaakt cisplatine ook functionele en morfologische veranderingen in de stria vascularis. Deze structuur speelt een regulerende rol bij de elektrolytensamenstelling van de endolymfe en is betrokken bij de generatie van de endocochleaire potentiaal. Recent onderzoek lijkt erop te duiden dat cisplatine ook een schadelijk effect heeft op het spirale ganglion, waarin de zenuwcellen liggen. Deze spirale ganglioncellen zorgen voor de geleiding van de zenuwimpulsen naar de hersenen.

Hoewel bekend is dat deze drie structuren de aangrijpingspunten van cisplatine in de cochlea zijn, bestaat er vooralsnog geen duidelijkheid over de vraag of één van deze structuren het primaire aangrijpingspunt is – gevolgd door effecten op de andere structuren – of dat de effecten parallel aan elkaar lopen en de structuren dus onafhankelijk van elkaar beschadigd worden. Deze onduidelijkheid komt voort uit het feit dat nog niet bekend is hoe de degeneratie van de structuren verloopt in de tijd. Daarnaast is het niet bekend waar cisplatine in de cochlea

wordt opgenomen en opgeslagen. Het doel van de in dit proefschrift beschreven studies is het vinden van het primaire aangrijpingspunt van cisplatine in de cochlea.

In de **eerste studie** (Hoofdstuk 2) werd door middel van een tijdreeks onderzocht welke weefsels in de cochlea als eerste morfologische afwijkingen vertonen. Albino cavia's werden dagelijks behandeld met intraperitoneale cisplatine injecties (2 mg/kg/dag) gedurende 4, 6 of 8 achtereenvolgende dagen. Onbehandelde dieren dienden als controle groep. Eén dag na de laatste injectie werd de functionele gehoorschade gemeten d.m.v. elektrocochleografie (EcochG), waarna de cochlea's opgewerkt werden voor histologische bestudering. In coupes die over de lengteas van de cochlea zijn gesneden, zgn. midmodiolaire coupes (1 µm), werden kwantitatieve metingen verricht naar haarcelverlies, oppervlakteveranderingen in de stria vascularis en de mate van bolling van het membraan van Reissner. Uitbolling van het membraan van Reissner (endolymfatische hydrops) is een indicatie voor dysfunctie van de stria vascularis. In het spirale ganglion werd het totale aantal ganglioncellen geteld alsmede het aantal cellen die morfologische veranderingen vertoonden. Tenslotte werd geverifieerd of haarceltellingen in midmodiolaire coupes een goede indruk geven van het haarcelverlies. Daarvoor werden deze tellingen vergeleken met tellingen gebaseerd op oppervlaktepreparaten.
Na 4 dagen vertoonden het orgaan van Corti, de stria vascularis en het spirale ganglion geen histologische afwijkingen. Verlies van buitenste haarcellen trad op na 6 dagen en breidde zich uit behandeling gedurende 8 dagen. Verlies van binnenste haarcellen werd in geen van de groepen waargenomen. De haarceltellingen in midmodiolaire coupes kwamen overeen met die van de oppervlaktepreparaten. Het uitvoeren van haarceltellingen in midmodiolaire coupes is eenvoudiger en minder tijdsrovend. Tevens geeft een midmodiolaire coupe een beter inzicht in de veranderingen van de celstructuur. Door de overeenkomst tussen de tellingen in midmodiolaire coupes en oppervlaktepreparaten kunnen in de toekomst haarceltellingen in midmodiolaire coupes volstaan. Een bijkomend voordeel van midmodiolaire coupes is dat andere structuren in de cochlea, zoals de stria vascularis en het spirale ganglion, ook onderzocht kunnen worden.
In de stria vascularis werden geen histologische veranderingen waargenomen, na 4, 6 of 8 dagen. In tegenstelling tot ander studies werden geen aanwijzingen voor het optreden van een endolymfatische hydrops gevonden, zelfs niet na cisplatine behandeling gedurende 8 dagen. Ondanks dat er geen histologische veranderingen plaatsvinden in de stria vascularis, kunnen we niet uitsluiten dat cisplatine in deze

periode een effect heeft gehad op fysiologisch of biochemisch niveau. Eerdere studies hebben aangetoond dat behandeling met cisplatine een effect heeft op de endocochleaire potentiaal. Maar hier is geen (licht-)microscopisch correlaat voor. Verlies van spirale ganglioncellen werd niet waargenomen. Vanaf 6 dagen vertoonde een relatief groot aantal ganglioncellen krimp. Tevens trad zwelling van de myelineschede op. Na 8 dagen vertoonden ook ganglioncellen in de middelste winding deze afwijkingen. Deze studie levert het histologisch bewijs dat cisplatine ook schade toebrengt aan de ganglioncellen. Deze histologische afwijkingen lijken te ontstaan in hetzelfde tijdsbestek waarin verlies van buitenste haarcellen zich voor het eerst manifesteert, nl. na 6 dagen. Uit deze resultaten kunnen we concluderen dat de effecten op het orgaan van Corti en op het spirale ganglion gelijktijdig ontstaan en zich parallel aan elkaar ontwikkelen.

In de **tweede studie** (Hoofdstuk 3) werd onderzocht of de effecten op het orgaan van Corti inderdaad parallel lopen aan de veranderingen in de spirale ganglioncellen of dat er een causaal verband tussen beide effecten bestaat. Tevens werd onderzocht of de histologische veranderingen in de spirale ganglioncellen effect hebben op het functioneren van de cochlea. Daarvoor werden de individuele data van de ECochG metingen (CAP, CM) van de dieren uit de eerste studie vergeleken met het haarcelverlies en het percentage afwijkende spirale ganglioncellen. Na 6 dagen werden verhoogde drempels gemeten, zowel voor de Samengestelde Actie Potentiaal (CAP) als voor de Cochleaire Microfonie (CM). De bevinding dat de drempel van de CAP een grotere stijging vertoont dan die van de CM, impliceert dat cisplatine ook een effect heeft op een neurale component in de cochlea. Dit wordt bevestigd door de waarneming dat in sommige dieren wél de drempel van de CAP verhoogd was, maar nog géén verlies van buitenste haarcellen aangetoond kon worden. Het verschil tussen de veranderingen in de CAP en de CM correleert met het percentage van spirale ganglioncellen die krimp vertonen. Uit deze resultaten trekken we de conclusie dat de histologische veranderingen in de spirale ganglioncellen ook een effect hebben op het functioneren van de cochlea. Echter, een causaal verband tussen het effect van cisplatine op het orgaan van Corti en het optreden van histologische veranderingen in het spirale ganglion kon niet aangetoond worden.

Aangezien het niet mogelijk bleek onze vraagstelling met behulp van morfologische en electrofysiologische methoden volledig te beantwoorden, werd besloten voor een andere, immunohistochemische benadering. Van cisplatine is bekend dat het na opname in tumorcellen reageert met DNA in de celkern en daar

cisplatine-DNA adducten vormt.
In de **derde studie** (Hoofdstuk 4) hebben we onderzocht of cisplatine-DNA adducten ook in de cochlea gevormd worden en of deze herkend worden door het antiserum NKI-A59. Dit antiserum bevat antilichamen tegen cisplatine-DNA adducten en werd ter beschikking gesteld door Prof. A. Begg en B. Floot (Nederlands Kanker Instituut, Amsterdam).
Albino cavia's werden dagelijks behandeld met intraperitoneale injecties cisplatine (2 mg/kg/dag) gedurende 2x5 dagen. Vervolgens werden de cochlea's gefixeerd en werden segmenten van het orgaan van Corti en de laterale wand (met de stria vascularis en het spirale ligament) uit het benige kapsel geprepareerd en ingevroren. Van het ingevroren weefsel werden vriescoupes (0,5 µm) gesneden. Als positieve controle werd nierweefsel gebruikt, omdat eerdere studies hebben aangetoond dat cisplatine-DNA adducten ook in nierweefsel gevormd worden.
Er bleek non-specifieke aankleuring te zijn in nierweefsel van onbehandelde cavia's. Dit gaf aanleiding tot een voorbewerking van het NKI-A59 antiserum om non-specifieke antilichamen uit het antiserum te halen. Deze voorbewerking was succesvol, waarna de immunokleuringen werden verricht met voorbehandeld antiserum.
Een positieve reactie met het NKI-A59 antiserum, duidend op de aanwezigheid van cisplatine-DNA adducten, werd waargenomen in bijna alle cellen in het orgaan van Corti en in de laterale wand, maar in de buitenste haarcellen in het orgaan van Corti, de marginale cellen in de stria vascularis en de fibrocyten in het spirale ligament was de sterkste reactie te zien. Dit is de eerste studie waarin de aanwezigheid van cisplatine-DNA adducten in histologische coupes van de cochlea is aangetoond. De aanwezigheid van cisplatine-DNA adducten in de celkernen van nagenoeg alle cellen in de cochlea impliceert dat cisplatine wordt opgenomen door deze cochleaire cellen. Met behulp van deze methode kan de vraag of er een primair aangrijpingspunt van cisplatine in de cochlea is, opnieuw bekeken worden.

In de **vierde studie** (Hoofdstuk 5) werd de distributie van cisplatine, met behulp van de in Hoofdstuk 4 beschreven immunohistochemische methode, in verschillende cochleaire weefsels en nierweefsel onderzocht middels een tijdreeks. In deze studie werd de aanwezigheid van cisplatine-DNA adducten gebruikt als indicator voor de aanwezigheid van cisplatine in de cochleaire cellen. Albino cavia's werden dagelijks behandeld met intraperitoneale injecties cisplatine (2 mg/kg/dag) gedurende 2, 4, 6, 8, 10, 12, 14 of 16 achtereenvolgende dagen. Onbehandelde dieren vormden de controlegroep. Vriescoupes werden gesneden

van uitgeprepareerde segmenten van het orgaan van Corti, het spirale ganglion en de laterale wand, alsmede van nierweefsel. De immunokleuring werd uitgevoerd met het voorbewerkte NKI-A59 antiserum.
Immunoreactiviteit voor cisplatine-DNA adducten was als eerste waarneembaar in nierweefsel na 4 dagen en was aanwezig in alle celkernen van de proximale en distale tubuli. In de cochlea was na 6 dagen een positieve reactie waarneembaar in het orgaan van Corti in de basale winding. De celkernen van de buitenste haarcellen vertoonden een sterke reactie, terwijl de immunoreactiviteit in de Deiterse cellen minder prominent was. Na 10 dagen was immunoreactiviteit ook aanwezig in de celkernen van de buitenste haarcellen in de middelste en apicale windingen, maar niet zo sterk als in de basale winding. Na 12 dagen vertoonden de buitenste haarcellen in alle windingen een sterke reactie met het antiserum. Aangezien in de fragiele vriescoupes de binnenste haarcellen moeilijk te onderscheiden zijn van de omringende cellen, was het tijdstip waarop deze cellen positief reageren moeilijk vast te stellen.
De cellen in de stria vascularis en de fibrocyten in het spirale ligament vertoonden pas na 8 dagen in de basale winding reactie met het antiserum. Na 16 dagen vertoonden alle cellen in de stria vascularis en de fibrocyten in het spirale ligament in alle windingen een duidelijke reactie.
In de spirale ganglioncellen was pas na 14 dagen immunoreactiviteit aantoonbaar in de basale winding. Na 16 dagen vertoonden de ganglioncellen in alle windingen immunoreactiviteit.
Immunoreactiviteit voor cisplatine-DNA adducten wordt dus duidelijker zichtbaar naarmate de cavia's langer behandeld zijn met cisplatine. Daarnaast is er een longitudinale gradiënt zichtbaar in de cochlea: de cellen in de basale winding vertonen eerder immunoreactiviteit, waarna de cellen in de middelste winding en tenslotte de cellen in de apicale winding volgen.

In Hoofdstuk 6 worden de resultaten van de verschillende studies vergeleken, bediscussieerd en worden er suggesties gedaan voor vervolgonderzoek. Uit de eerste studie blijkt dat er in de tijdreeks van 4, 6 en 8 dagen cisplatine behandeling histologische veranderingen te zien zijn in zowel het orgaan van Corti als in het spirale ganglion. Degeneratie van de buitenste haarcellen en van de spirale ganglioncellen vindt plaats vanaf 6 dagen. Histologische veranderingen werden niet waargenomen in de stria vascularis. De tweede studie gaf met behulp van de elektrofysiologische data geen aanleiding te veronderstellen dat het effect op het orgaan van Corti causaal gerelateerd is aan de veranderingen in het spirale ganglion, of vice versa.

Reeds lange tijd is bekend dat cisplatine in de cochlea een effect heeft op het orgaan van Corti en de stria vascularis. De bevinding dat cisplatine ook een effect heeft op het spirale ganglion is relatief nieuw en niet uitgebreid beschreven. Het wordt algemeen aangenomen dat celverlies in het spirale ganglion volgt op verlies van binnenste haarcellen. In deze studie is dat niet het geval, aangezien er geen binnenste haarcelverlies, maar wel een effect op de ganglioncellen, werd waargenomen.

Uit de eerste studie wordt duidelijk dat cisplatine een effect heeft op zowel de spirale ganglioncellen zelf als op de Schwann cellen waarvan de myelineschede een onderdeel is. Krimp van de ganglioncellen werd waargenomen, alsmede zwelling van de myelineschede. Dit is nog niet eerder beschreven. Schwann cellen lijken op satellietcellen in de dorsale streng ganglia. Van deze satellietcellen is bekend dat deze gevoeliger voor cisplatine zijn dan de ganglioncellen zelf.

Uit de tweede studie wordt duidelijk dat de histologische veranderingen in de ganglioncellen ook een effect hebben op de functie van de cochlea. Deze hoofdstukken leveren bewijs dat ook het spirale ganglion een rol speelt bij cisplatine ototoxiciteit.

Aangezien verscheidene studies hebben gefaald om cisplatine aan te tonen in histologische coupes van de cochlea, hebben we een immunohistochemische methode ontwikkeld waarmee cisplatine, indirect, kan worden gelocaliseerd in semidunne vriescoupes van cochleaire weefsels (Hoofdstuk 4). Cisplatine werd gelocaliseerd middels antilichamen tegen cisplatine-DNA adducten. Deze methode werd vervolgens gebruikt om de distributie van cisplatine in diverse cochleaire weefsels te onderzoeken middels een tijdreeks (Hoofdstuk 5). Immunoreactiviteit voor cisplatine-DNA adducten was als eerste waarneembaar in het orgaan van Corti. Daarna was immunoreactiviteit aanwezig in de stria vascularis en de fibrocyten in het spirale ligament. Immunoreactiviteit in de ganglioncellen werd als laatste waargenomen. De aanwezigheid van cisplatine-DNA adducten in de celkernen van nagenoeg alle cellen in de cochlea (na 16 dagen cisplatine behandeling) impliceert dat cisplatine wordt opgenomen door alle cochleaire cellen. Echter, deze bevinding toont niet aan dat vorming van cisplatine-DNA adducten in de celkernen ook het enige werkingsmechanisme van cisplatine in de cochlea is. We kunnen niet uitsluiten dat cisplatine reageert met andere organellen en/of macromoleculen en langs deze wegen celdood bewerkstelligt.

Met de resultaten van dit promotieonderzoek hebben we meer inzicht verkregen in het verloop van de degeneratie van diverse cochleaire weefsels na cisplatine behandeling. Bovendien hebben we voor het eerst cisplatine aangetoond in coupes van cochleair weefsel. Door deze methode is een nieuwe ingang

gevonden om cisplatine ototoxiciteit te bestuderen. Met deze methode kan het exacte mechanisme dat leidt tot celdood in de verschillende cochleaire weefsels ontrafeld worden. Uiteindelijk zal deze kennis bijdragen aan de verfijning van de behandelingsmethoden teneinde de ototoxische bijwerkingen van cisplatine tegen te gaan.

Curriculum Vitae

Marjolein van Ruijven werd op 16 september 1977 geboren te Delft. In 1993 behaalde zij het diploma aan de Thomas van Aquino MAVO te Poeldijk, gevolgd door het HAVO diploma in 1995 aan het Westland College te Naaldwijk. Na het behalen van het propedeutisch diploma Levensmiddelentechnologie aan de Hogeschool Delft in 1996, startte ze de opleiding Biologie aan de Universiteit Leiden. Tijdens haar doctoraalstudie werd de hoofdstage gelopen bij de afdeling Endocrinologie en Stofwisselingziekten van het Leids Universitair Medisch Centrum onder begeleiding van Dr. G. van der Pluijm en Ing. B. Boers-Sijmons. Een tweede stage werd gelopen bij de afdeling Bioactieve Componenten van Numico Research te Wageningen onder begeleiding van Dr. L. van der Zee, Drs. C. Rouws en Ing. F. Dijk. In augustus 2000 werd het diploma biologie met als specialisatie medische biologie gehaald. In september van dat jaar werd de auteur aangesteld als Heinsius Houbolt Research Fellow bij de Hearing Research Laboratories, afdeling Keel-, Neus- en Oorheelkunde, Universitair Medisch Centrum Utrecht. Het promotieonderzoek werd verricht onder begeleiding van Prof. Dr. G.F. Smoorenburg en Dr. J.C.M.J. de Groot.

Publications

Van Ruijven, M.W.M., De Groot, J.C.M.J., Smoorenburg, G.F., 2002. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. Acta Otorhinolaryngol. Belg. 56, 270 (abstract).

Van Ruijven, M.W.M., De Groot, J.C.M.J., Smoorenburg, G.F., 2004. Time sequence of degeneration pattern in the guinea pig cochlea during cisplatin administration. A quantitative histological study. Hear. Res. 197, 44-54.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Klis, S.F.L., Smoorenburg, G.F. The cochlear targets of cisplatin. An electrophysiological and morphological time-sequence study. Hear. Res., in revision.

Van Ruijven, M.W.M., De Groot, J.C.M.J., Hendriksen, F., Smoorenburg, G.F. Immunohistochemical detection of platinated DNA in the cochlea of cisplatin-treated guinea pigs. Hearing Research 2005 (in press).

Van Ruijven, M.W.M., F. Hendriksen, De Groot, J.C.M.J., Smoorenburg, G.F. Time-dependent formation of platinated DNA in the cochlear targets of cisplatin. In preparation.

Dankwoord

Hora Est! De tijd is verstreken! De vier jaar zijn alweer voorbij, maar wel met resultaat: het proefschrift is af. In deze periode heb ik niet alleen wetenschappelijk onderzoek gedaan, maar ook zeer waardevolle contacten gelegd. Van deze contacten wil ik een aantal persoonlijk bedanken die hebben geholpen bij de totstandkoming van dit proefschrift.

Prof. G.F. Smoorenburg: bedankt voor de laatste en vaak essentiële aanpassingen in mijn manuscripten. Ik wens u alvast een fijne tijd in Frankrijk toe.

John de Groot: het is echt ongelooflijk hoe jij elk verkeerd puntje, komma of haakje in de manuscripten weet op te sporen. Ook kwamen mijn gecorrigeerde manuscripten vaak rood bij mij terug, maar na wat discussie en een kopje koffie zijn we er toch altijd weer uitgekomen. Bedankt voor de gezellige jaren, ik heb veel geleerd op jouw lab. Ik wens je heel veel succes toe met je grote passie: het oude Egypte.

Ferry Hendriksen: zonder jou was het boekje niet zo snel afgekomen. Bedankt ook voor de gezelligheid op het lab en tijdens de lunches en dat je altijd klaar stond als ik weer iets 'blonds' had gedaan.

Sjaak Klis: bedankt voor alle hulp bij de uitwerking van de elektrofysiologische experimenten. Bedankt ook voor de gezellige tijd in en rond Daytona Beach: tijdens zowel het congres, mij leren krab eten en het uit eten gaan.

Frits Meeuwsen: bedankt voor de gezelligheid in het lab, tijdens de lunch en alle goede adviezen.

Prof. Begg en Ben Floot: bedankt voor het gebruik van het antilichaam NKI-A59. Ook in de cochlea toont dit antilichaam cisplatine-DNA adducten aan. Prof. Begg, ik stel het zeer op prijs dat u zitting neemt in de promotiecommissie.

Kees Guikers: bedankt voor de goede en waardevolle ideeën tijdens het opzetten van de immunokleuring. En de aanvullende gezelligheid op het lab.

Rolph Houben: Je bent een ontzettend leuke collega en ook nog eens goed in o.a. statistiek. Wat fijn dat ik bij je terecht kon als ik weer eens vast zat met mijn toetsen en andere zaken. Het geeft me een goed gevoel dat je op 18 februari achter me staat.

Petra Boddé: jou wil ik graag bedanken voor het verwerken van alle foto's en de daarbij horende kopjes koffie. Ook bedankt voor al je hulp bij m'n idee voor de kaft.

Ciska van Doesum: het was leuk en leerzaam dat ik je paranimph was in 2003. Nu ben ik zelf aan de beurt.

Martijn Agterberg: je was een verfrissende nieuwe kamergenoot: veel succes met je eigen promotie.

Huib Versnel, Frank Hamers en Natalie Cappaert: jullie ook bedankt voor de gezelligheid en interesse, o.a. bij de wekelijkse koffie.

Rob Speijk: jij bedankt dat je me al die tijd op de weg gehouden hebt. Ik heb met het ritje Wassenaar-Utrecht en vice versa 3 auto's versleten, maar gelukkig sta jij altijd klaar als ik een lekke band heb.

Mijn allerliefste Peter, geen woorden kunnen uitdrukken wat jij voor mij betekent. Je bent mijn steun en toeverlaat en staat altijd achter me als ik weer iets nieuws wil ondernemen. Nog even en dan kunnen we in ons eigen, nieuwe huis in Wassenaar. Is het niet allemaal geweldig?!