THE PROTECTIVE EFFECT OF MELANOCORTINS ON CISPLATIN-INDUCED HEARING LOSS

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THE PROTECTIVE EFFECT OF MELANOCORTINS ON CISPLATIN-INDUCED HEARING LOSS

Het beschermend effect van melanocortinen op door cisplatine veroorzaakte gehoorschade

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Contents

Chapter 1

General introduction

Hearing is very important in everyday life. Humans depend on their hearing in a number of respects, such as for communication, socializing, learning, listening and to be warned for approaching danger. So when hearing is lost, it can have a disabling effect on a person's life. It has been estimated that in the Netherlands > 1 million people have an average hearing loss of at least 35 dB at the frequencies involved in speech perception (1, 2, and 4 kHz). In general, two types of hearing loss can be distinguished: conductive and sensorineural hearing loss. Conductive hearing loss is due to a blockage of the anatomical cascade that conducts the sound waves from the outer to the inner ear. Examples are middle ear infections, perforation of the eardrum, and otosclerosis, a disorder in which the stapes may become immobile because of excessive growth of the bone. The other type of hearing loss is called "sensorineural" and refers to damage of the cochlea and/or auditory nerve. Sensorineural hearing loss can be induced by aging (presbycusis), loud music or noise, viral or bacterial infections and drugs (such as aminoglycoside antibiotics or the anti-cancer drug cisplatin).
In this thesis will be investigated whether the side effects of cisplatin upon the auditory system can be reduced or even prevented.

The peripheral auditory system

The peripheral auditory system can be subdivided into three parts: the outer, middle, and inner ear. The outer ear consists of the auricle and the external auditory canal and plays a role in sound localization, partly by frequency-selective modification of the sound wave, while it is transferred to the tympanic membrane (eardrum). Subsequently, these acoustic vibrations progress along the tympanic cavity (= middle ear) via the cascade of three tiny ossicles: the malleus (hammer), the incus (anvil) and the stapes (stirrup). Since there will be an energy loss when sound is transferred directly from air to the fluid in the cochlea, the ossicles amplify the sound and transfer the sound-induced vibrations via the stapes to the oval window, efficiently converting the sound waves into vibrations of the cochlear fluids.
The cochlea, which together with the vestibular apparatus comprises the inner ear (Fig. 1A) is responsible for the transduction of the sound-induced vibrations into electrochemical impulses in the auditory nerve. The cochlea consists of a fluid-filled spiraling tube that progressively diminishes in diameter towards the apex. In a cross section of the cochlea (Fig. 1B) the tube seems to consist of three scalae: the scala vestibuli, the scala tympani, and the scala media.

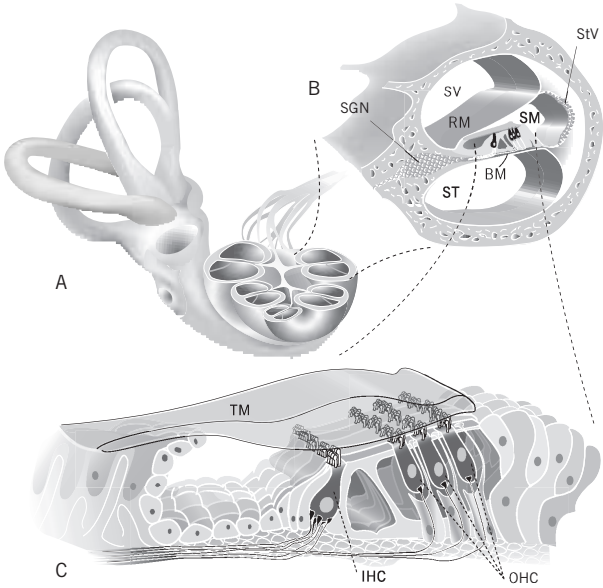

Figure 1: A. The structure of the human inner ear, containing the vestibular apparatus and the cochlea. B. Cross section of the cochlea that shows the arrangement of the three scalae: the scala tympani (ST), scala vestibuli (SV) and scala media (SM). The stria vascularis (StV) is situated on he lateral wall of the scala media. SGN: Spiral ganglion cells; BM: basilar membrane; RM Reissner's membrane. C. Detailed structure of the organ of Corti, which contains the receptor cells: outer hair cells (OHCs) and inner hair cells (IHCs). TM: tectorial membrane.

At its base, the scala vestibuli is sealed off by the oval window membrane, to which the stapes is connected. The scala tympani is closed at its base by another thin elastic membrane: the round window membrane. The scala tympani and the scala vestibuli are in open connection at the apex of the cochlea by an opening known as the helicotrema. In between these two compartments lies the scala media, which is separated from the scala vestibuli by Reissner's membrane and from the scala tympani by the basilar membrane. On top of the latter is situated the organ of Corti, which contains ±16000 receptor cells (hair cells).

The scala tympani and scala vestibuli contain perilymph, which is like normal extracellular fluid in composition and is at or near ground potential. The scala media contains endolymph, which is more like intracellular fluid with high levels of K^+ and low levels of Na^+. K^+ provides the major charge carrier for sensory transduction in the hair cells (Wangemann, 2002). The electrolyte composition and potential of the endolymph (+ 80 mV) is regulated by an energy-consuming mechanism involving multiple ion transport processes in

the stria vascularis located in the lateral wall of the cochlea. The stria vascularis is a complex, multilayered structure, containing three layers of different cell types. Facing the endolymphatic space is a luminal layer of marginal cells, which are characterized by the presence of numerous basolateral membranes that are rich in mitochondria. The middle layer of the stria vascularis is composed of the capillaries and intermediate cells. Facing the spiral ligament there is a layer of multiple flat, basal cells.
The sensory epithelium of the inner ear, the organ of Corti, (Fig. 1C) is positioned on top of the basilar membrane. It contains two types of hair cells: the outer hair cells (OHCs) and the inner hair cells (IHCs). The OHCs are arranged in three rows and the IHCs in a single row. Both types contain a bundle of hair-like structures, the so-called stereocilia, on the surface facing the scala media. The tops of the stereocilia of the OHCs are inserted into holes in the tectorial membrane. When a sound-induced vibration reaches the cochlea, the basilar membrane moves up and down because of differences in the fluid pressure between the scala vestibuli and the scala tympani. This movement of the basilar membrane is accompanied by a shearing motion between the organ of Corti and the tectorial membrane, causing the stereocilia to bend. In response to these movements the hair cells generate a stream of electrical signals that code the frequency, intensity and duration of the sound. The electrical signals are generated in both IHCs and OHCs, but the neural information predominantly originates from the IHCs, which receive 90-95% of the afferent nerve fibers (Spoendlin, 1972). The signals are transported through the eighth cranial nerve (vestibulocochlear nerve) to the brain. The OHCs are responsible for the sensitivity and frequency selectivity of the cochlea. It is thought that OHCs can generate forces, by actively contracting and relaxing, enhancing the basilar membrane motion (Brownell et al., 1985).
Several factors, such as noise, bacteria, viruses, aging, drugs and other chemical agents, may cause hearing loss (ototoxicity). Some of the clinically applied ototoxic agents, such as aminoglycosides and cisplatin, have such a critical role in the treatment of serious, life-threatening diseases that the ototoxic risk can considered to be of less importance.

Cisplatin

The biological activity of *cis*-diamminedichloroplatinum (II) or cisplatin (Fig. 2) was discovered in 1965 by Rosenberg and co-workers during their studies to the effects of an electric current on bacterial growth. They noticed that an electrical field caused inhibition of *Escherichia coli* cell division (Rosenberg et

al., 1965). Further investigation indicated that the active agents responsible for this effect were platinum salts, which were produced at the electrode during electrolysis (Rosenberg et al., 1967). Several platinum complexes were tested for their biological activity and some of them, including cisplatin, suppressed cell division and induced filamentous growth of bacteria (Howle and Gale, 1970), which was known to be an indicator of DNA damage.
Therefore, it was plausible to assume that cisplatin would also interfere with cell division in eukaryotes and subsequent studies revealed that cisplatin treatment indeed results in arrested growth of tumors. Surprisingly, the *trans*-isomer of cisplatin had no effect on tumor growth (Rosenberg et al., 1969).

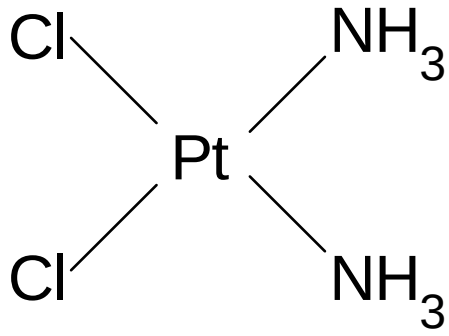

Figure 2: chemical structure of cisplatin

The first clinical trials with cisplatin started in the 1970s. Nowadays, cisplatin is a widely used antineoplastic agent. Cisplatin-based combination chemotherapy displays significant efficacy in the treatment of testis tumors, ovarian carcinoma, squamous cell carcinoma of the head and neck, and non-small-cell carcinoma of the lung. This anti-tumor effect is due to a covalent binding between the platinum atom and genomic or mitochondrial DNA. Once cisplatin enters the cell the chlorine atoms are replaced by water, resulting in the formation of a positively charged aquated species that can react easily with nitrogen or sulphur atoms in intracellular macromolecules to form protein-, RNA-, or DNA-adducts. If there is another potentially reactive site nearby, cisplatin can react further to form intra- and inter-strand crosslinks (Kartalou and Essigmann, 2001), eventually leading to apoptotic (programmed) cell death of tumor cells. The clinical use of cisplatin, however, is limited by dose-dependent side effects, such as renal dysfunction, peripheral neuropathies, hearing loss, nausea, vomiting, and myelosuppression. Severe nephrotoxicity was the most important dose limiting finding in early clinical trials. With forced diuresis, this side effect has become more manageable, leaving peripheral neuropathies and ototoxicity as the major side effects of concern.

Cisplatin-induced sensory peripheral neuropathy

Peripheral neuropathy is one of the most commonly encountered side effects of cisplatin. It is dose dependent and may occur upon exposure to amounts as low as 150 mg/m^2 (Kopelman et al., 1988; Laurell and Borg, 1988). The extent of the neurotoxic effects is closely related to the total cumulative drug dose and dosage schedule, but it also depends on the concentration of the single dose administered. Significant neurotoxicity will always occur when patients receive more than 300 mg/m^2 cisplatin (Walsh et al., 1982; Cersosimo, 1989). The first clinical signs indicating cisplatin-induced peripheral neuropathy are numbness, tingling, loss of ankle jerks and painful paresthesia in the hands and feet. With further treatment loss of vibration sense, reduction in sensibility to touch or pain and decrease in position sense of the affected areas may develop (Thompson et al., 1984; Cersosimo, 1989). No damage to the motor system has been observed. The neurotoxic effect of cisplatin is limited to the sensory system (Roelofs et al., 1984; Thompson et al., 1984). Cisplatin-induced sensory neuropathy shows a typical delayed time-course, which often reaches its maximum 1-4 months after the last cycle of cisplatin chemotherapy (Hovestadt et al., 1992). Neurophysiological studies have demonstrated that cisplatin causes decreased amplitudes of the sensory nerve action potential, slowing down of sensory nerve conduction velocity and prolongation of sensory nerve latency (Cersosimo, 1989). Histological studies have shown sensory root ganglia disruption, loss of large myelinated fibers, axonal degeneration and degeneration of myelin sheaths (Roelofs et al., 1984; Thompson et al., 1984; Gregg et al., 1992). In animal studies, the electrophysiological and pathophysiological pattern of cisplatin-induced peripheral neuropathy is similar to that seen in patients. Cisplatin largely affects sensory nerve structure and function. Preferential toxicity is found for large-diameter neurons and proprioceptive sensory modalities, while motor nerves are spared (Muller et al., 1990; Apfel et al., 1992; Cavaletti et al., 1994; Cece et al., 1995).

Cisplatin-induced ototoxicity

Clinical studies

The ototoxic effect caused by cisplatin in humans is characterized by a bilateral, high-frequency sensorineural hearing loss (changes in thresholds at 4 to 8 kHz), usually associated with tinnitus. After prolonged drug use, hearing loss can progress to the speech frequency range, which is from 1-4 kHz (De Oliviera, 1989; Schweitzer, 1993). The incidence of cisplatin-induced ototoxicity ranges from 11 to 91%, depending on the mode of drug administra-

tion, dosage per treatment and cumulative dose (De Oliviera, 1989; Waters et al., 1991). Also, age and pre-existing hearing loss can influence the severity of cisplatin ototoxicity (Fausti et al., 1984). Bolus injections of 60 mg/m^2, administered once a week, have been shown to cause significant threshold differences after 6-12 months of treatment (Aguilar-Markulis et al., 1981). At cumulative doses of 270 mg/m^2 the first significant changes in auditory threshold appear, especially at the high frequencies (Schaefer et al., 1985). At doses of more than 450 mg/m^2, 88% of the patients show a high-frequency hearing loss (> 4 kHz) (McHaney et al., 1983). Only sporadically (incomplete) recovery of cisplatin-induced hearing loss has been reported (Aguilar-Markulis et al., 1981; Vermorken et al., 1983; Melamed et al., 1985; Laurell and Jungnelius, 1990). Histopathological studies in humans have shown loss of OHCs and IHCs in the basal turn of the cochlea, degeneration of the stria vascularis, significant decrease in the number of spiral ganglion cells, and damage to the cuticular plate (Wright and Schaefer, 1982; Strauss et al., 1983; Hinojosa et al., 1995; Hoistad et al., 1998).

Experimental studies

In vitro cisplatin-models generally concern the toxicity of cisplatin with respect to isolated cochlear OHCs (Saito et al., 1991, 1996; Sha et al., 2001; Devarajan et al., 2002) and cochlear explants (Clerici et al., 1996; Zheng and Gao, 1996; Kopke et al., 1997; Liu et al., 1998). However, most of the studies about the ototoxic effects of cisplatin have been performed *in vivo* in rodents: e.g., hamsters (Melamed et al., 2000; Kaltenbach et al., 2002), chinchillas (Ford et al., 1997; Tsukasaki et al., 2000), gerbils (Sie et al., 1997, 1999; Alam et al., 2000), rats (Laurell et al., 1995, 1997; Meech et al., 1998; Hatzopoulos et al., 1999, 2001, 2002), guinea pigs (Tange, 1984; Schweitzer et al., 1986; Kohn et al., 1988; Laurell and Engström, 1989, Laurell and Bagger-Sjöbäck 1991b; Schweitzer, 1993; Saito et al., 1994a, b; 1997a, b; Kohn et al., 1997; De Groot et al., 1997; Cardinaal et al., 2000a-c; Klis et al., 2000, 2002), and sporadically in other mammals such as dogs (Sockalingam et al., 2002) and rhesus monkeys (Stadnicki et al., 1975). In these studies cisplatin was administered by intraperitoneal injection at doses ranging from 0.75 to 4 mg/kg given repeatedly one to five times per week for a total of 1-8 weeks or as a single dose of 5-18 mg/kg by intraperitoneal injection or intravenous infusion.

The estimation of the onset of ototoxicity has been performed by measuring the auditory brain stem response (ABR), electrocochleography (ECochG) or by measuring the otoacoustic emissions (OAE). In animals the electrophysiological and pathophysiological pattern of cisplatin-induced ototoxicity is simi-

lar to that seen in patients. Cisplatin induces a dose-related permanent sensorineural hearing loss starting at the high frequencies. Pathophysiological studies in guinea pigs have shown that chronic cisplatin administration leads to loss of OHCs, and at high doses also to loss of IHCs, with those in the basal turn more severely affected than the ones in the middle and apical turns (Nakai et al., 1982; Tange, 1984, Hoeve et al., 1988; Saito and Aran, 1994b; Cardinaal et al., 2000a). Laurell and Bagger-Sjöbäck (1991a) have shown that the morphological changes in the cochlea of guinea pigs after cisplatin exposure occur in three stages. The first stage includes disturbance of the supporting cells surrounding the OHCs. The second stage was characterized by degeneration of the OHCs; one of the first signs is loss of stereocilia and intracellular vacuolation. The IHCs usually remain intact until all the OHCs have degenerated. In the final stage collapse of the entire organ of Corti occurs. The effects of cisplatin are not limited to the hair cells. Boheim and Bichler (1985) have shown that cisplatin destroys the efferent auditory nerve fibers near the OHCs. Others have found histological changes in the spiral ganglion cells of guinea pigs, consisting of vacuolation of their cytoplasm (Cardinaal et al., 2000b) and cell shrinkage (Van Ruijven et al., personal communication). Furthermore, damage to the stria vascularis was observed in several studies in rats and guinea pigs (Kohn et al., 1988, 1997; Meech et al., 1998; Campbell et al., 1999; Cardinaal et al., 2000a, b). This damage consisted of blebbing and vacuolation of the marginal cells and atrophy of the intermediate cells. Besides these morphological changes, a smaller than normal endocochlear potential (EP) was observed after administration of cisplatin to chinchillas (Ford et al., 1997) and guinea pigs (Komune et al., 1981; Konishi et al., 1983; Laurell and Engström 1989; Klis et al., 2000, 2002).

A number of animal studies (Stadnicki et al., 1975; Nakai et al., 1982; Stengs et al., 1997; Cardinaal et al., 2000b; Klis et al., 2000, 2002) demonstrated that several of the cisplatin-induced ototoxic effects (OHC damage, increase of hearing threshold, decease of EP) recover after cessation of the cisplatin treatment. Summarizing, cisplatin seems to have at least three targets in the cochlea, the organ of Corti, the stria vascularis and the spiral ganglion cells. Presently, the relation between the respective effects on these targets, *e.g.*, whether one is causally related to the other and how these targets are involved in recovery, is unknown.

Protection against cisplatin-induced side effects

Several attempts have been made to prevent cisplatin-induced side effects, *e.g.* by changing the dose, the method of administration or even by replacing cisplatin with a non-toxic analogue (*e.g.* carboplatin). However, these efforts proved to be unsatisfactory. Thus, another approach was investigated to overcome the toxic effects of cisplatin: pharmacological intervention. Several classes of compounds, such as neurotrophins and sulphur-containing compounds, have been found to protect against cisplatin-induced neuro- and ototoxicity. In this section the most significant results from experiments with different classes of compounds that protect against cisplatin-induced ototoxicity will be reviewed.

Fosfomycin

The first agent tested for its possible protection against cisplatin ototoxicity was fosfomycin. Both Ohtani et al. (1985) and Schweitzer et al. (1986, 1993) showed that significantly less OHC loss occurs when animals are treated with 1 mg/kg/day cisplatin in combination with 300 mg/kg/day fosfomycin. However, these results could not be reproduced in later studies performed by Church et al. (1995) and Kaltenbach et al. (1997), in which higher doses of cisplatin (3 mg/kg/day, once every other day) were used in combination with fosfomycin co-treatment. These seemingly contradictory results could be explained by the observation of Ohtani et al. (1985) that at high concentrations of cisplatin (5 mg/kg/day) fosfomycin has no protective effect.

Sulphur-containing compounds

The application of sulphur-containing compounds was based on the hypothesis that damage caused by cisplatin is due to formation of free radicals, which interfere with the antioxidant defense system, resulting in oxidative stress (Rybak et al., 1995, Ravi et al., 1995). Severe oxidative stress produces major disruption of cell metabolism, resulting in cell death (Evans and Halliwell, 1999). Several compounds that may reduce free radical formation, have been described, *e.g.* sodium thiosulfate (STS), diethyldithiocarbamate (DDTC), 4-methylthiobenzoic acid (MTBA), L- and D-methionine, lipoic acid, and salicylate. In 1985, it was shown that sodium thiosulfate, already known to prevent cisplatin nephrotoxicity, prevents from neurotoxicity when administered simultaneously with cisplatin in patients (Markman et al., 1985). This compound also seemed to protect guinea pigs from cisplatin-induced hearing loss (Otto et al., 1988; Church et al., 1995; Kaltenbach et al., 1997; Saito et al., 1997a). However, it has been shown that STS reacts with cisplatin to form

covalently bound complexes, thus hampering cisplatin's anti-tumor activity (Howell et al., 1982). Therefore, recent experiments have focused on the direct application of STS into the inner ear in order to selectively bind cisplatin in the cochlea (Wang et al., 2002). Another protective agent is the sulphur-containing amino acid methionine. Both the naturally occurring L-methionine and its synthetic analog D-methionine have been tested for their otoprotective properties. Campbell et al. (1996, 1999) have showed excellent protection by D-methionine from cisplatin ototoxicity in rats. Unfortunately, also L- and D-methionine lowered the systemic exposure to cisplatin (Reser et al., 1999; Ekborn et al., 2002; Vrana and Brabec, 2002). Therefore, topical administration of L- or D-methionine directly onto the round window membrane has been studied (Li et al., 2001; Korver et al., 2002). With this approach both compounds completely protect the inner ear from cisplatin-induced ototoxicity. Other sulphur-containing compounds that provide protection against cisplatin ototoxicity after systemic application are diethyldithiocarbamate (Church et al., 1995; Kaltenbach et al., 1997; Rybak et al., 1995; Walker et al., 1994), 4-methylthiobenzoic acid (Rybak et al., 1997; Kamimura et al., 1999), salicylate (Li et al., 2002) and lipoic acid (Rybak et al., 1999a-c). However, excessive amounts of these compounds are necessary to realize a protective effect against cisplatin ototoxicity; the concentration of the sulphur-containing compounds exceeds the cisplatin dose by 5 to 100 times (*e.g.*, 300 mg/kg D-methionine versus 8 mg/kg cisplatin). At these concentrations the sulphur-containing compounds are known to react directly with cisplatin resulting in a lowered cytotoxic effect of the drug (Ekborn et al., 2002).

Neurotrophic factors

In 1992 a group of neuropeptides was shown to have protective effects on cisplatin neuropathy (Apfel et al., 1992). These neurotrophic factors are, among others, nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF). Each of them signals through a specific high-affinity Trk receptor (Gao, 1999). NGF specifically acts on TrkA, BDNF and NT-4/5 on TrkB, and NT-3 selectively activates TrkC (Gao, 1999). All of the neurotrophins also bind to the NGF low-affinity receptor p75 (Chao, 1994). Three of these receptors, *i.e.*, TrkB, TrkC, and p75 have been identified in spiral ganglion and hair cells (Ylikosky et al., 1993; Pirvola et al., 1994). Recent experiments have reported that rats and patients treated with cisplatin show a significant reduction in the level of circulating NGF (De Santis, 2000; Cavaletti et al., 2002). Since NGF suppresses the generation of reaction oxygen species (ROS)

(Dugan et al., 1997), reduction of NGF could result in an increased generation of ROS, eventually leading to peripheral neuropathy or ototoxicity. Most of the experiments with growth factors have been performed *in vitro* with organotypic culture of cochlear explants or vestibular neuro-epithelia. BDNF and NT-4/5 were found to delay further degeneration of spiral ganglion cells. This protective effect, although somewhat smaller, was also observed when NT-3 was administered to spiral ganglion cells (Zheng et al., 1995, 1996). However, no attenuation of cisplatin-induced hair cell loss was observed with these compounds (Zheng and Gao, 1996). The results of studies performed with NGF are rather conflicting. Some authors have reported that NGF protects spiral ganglion cells from cisplatin-induced toxicity (Malgrange et al., 1994), while others consider NGF to be ineffective (Zheng et al., 1995, 1996). Kuang et al. (1999) recently showed that locally delivered GDNF protects guinea pig cochleas when it was administered in combination with systemically applied cisplatin. This is the only study known to show protection *in vivo* by one of the above co-treatments at a dose in the μg-range, suggesting a specific mechanism against ototoxicity at the hair cell level. Recently, another group of peptides known as melanocortins has been demonstrated to protect against cisplatin-induced ototoxicity *in vivo* at this dose range.

Melanocortins

Already in 1987, it was reported that the class of peptides known as melanocortins, is able to protect against cisplatin-induced neurotoxicity. Melanocortins are derived from the pituitary peptide AdrenoCorticoTropic hormone (ACTH) and include α–Melanocyte Stimulating Hormone (α–MSH), the $ACTH_{(4\text{-}9)}$ analog ORG 2766 and the synthetic cyclic peptide melanotan-II (MT-II) (Fig. 3). *In vitro* experiments with neurons from the dorsal root ganglia (DRG) showed that both α–MSH and ORG 2766 prevent the outgrowth-inhibiting action of cisplatin. However, they do not increase survival of DRG neurons nor do they appear to have any effect upon the death of supporting cells (Bär et al., 1993; Hol et al., 1994a; Windebank et al., 1994).

In vivo experiments in rats showed a decrease in the sensory nerve conduction velocity (SNCV) as well as the number of thick myelinated fibers after treatment with cisplatin. Concurrent treatment with MT-II prevents the decrease of SNCV (Ter Laak et al., 2003), while ORG 2766 prevents both the decrease in SNCV (De Koning et al., 1987; Gerritsen van der Hoop et al., 1988; Hamers et al., 1991a, 1993a) and the decrease in number of thick fibers (Gerritsen van der Hoop et al., 1994).

A clinical trial, in which patients with ovarian carcinomas were concomitantly treated with cisplatin and ORG 2766, showed that ORG 2766 prevented part

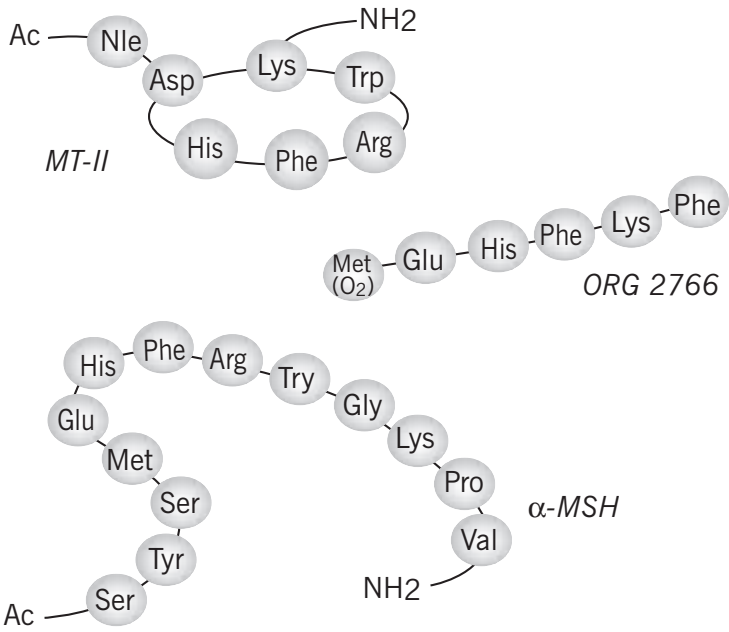

Figure 3: Structures of MT-II, ORG 2766 and α–MSH

of the cisplatin-induced decrease of the vibration perception threshold (VPT) (Gerritsen van der Hoop et al., 1990). Unfortunately, 4 months after cessation of the cisplatin treatment the majority of patients still had abnormal VPT values and showed a continued increase of clinical complaints (numbness, loss of strength, pain, etc.). Nevertheless, these effects were less pronounced in patients previously treated with ORG 2766 (Hovestadt et al., 1992). In a more recent clinical study, in which more patients were included, this protective effect of ORG 2766 could not be reproduced; It was observed that ORG 2766 causes an increase in the onset and degree of neuropathies (Roberts et al., 1997).

Since the melanocortins α–MSH, ORG 2766, and MT-II showed promising neuroprotective effects in animal studies, we decided to test for the possible otoprotective action of these neuropeptides. Hamers et al. (1994) and Stengs et al. (1998b) showed a partially protective effect of 75 μg/kg/day ORG 2766 upon cisplatin-induced ototoxicity in guinea pigs as shown from changes in ECochG thresholds. Major threshold shifts were observed in all saline co-treated animals but not in all ORG 2766 co-treated animals after 8 daily doses of 2.0 mg cisplatin/kg (Hamers et al., 1994). Animals treated with ORG 2766 that showed

no protection, tended to have slightly worse CAP input-output curves than the saline co-treated ones, but OHC survival was significantly better in these 'non-responders' than in saline-treated controls (De Groot et al., 1997). Heijmen et al. (1999) demonstrated that treatment of albino guinea pigs with daily injections of cisplatin (2 mg/kg/day i.p. for 8 days) and concomitant injections of α–MSH (75 μg/kg/day s.c. for 9 days) results in a considerable number of animals with preserved hearing after cessation of cisplatin treatment. This was not found in the cisplatin/saline treated group.
Thus, these experiments have demonstrated that ACTH-derived neuropeptides (melanocortins) are able to protect against cisplatin-induced ototoxicity. However, the mechanism by which these neuropeptides exert their otoprotective effect is not yet known. Their protective effect cannot be due to a direct interaction between the melanocortins and cisplatin, since both α–MSH and ORG 2766 were administered in much smaller doses than cisplatin. The actual target might be the intermediate cells in the stria vascularis. Meyer zum Gottesberge (2000) has suggested that these intermediate cells, which are actually melanocytes (Hilding and Ginzberg, 1977), are under α–MSH control. α–MSH may act as an emergency system in the regulation of inner ear homeostasis and function. ACTH-derived neuropeptides, such as α–MSH, are known to strongly bind to G-protein-coupled melanocortin (MC) receptors (Mountjoy et al., 1992). Therefore, α–MSH and ORG 2766 may exert their protective action by activating a MC-like receptor in the intermediate cells, thus preventing cisplatin from damaging the stria vascularis. Although the epidermal melanocytes have been shown to contain a MC-receptor that specifically binds to α–MSH (Tsatmali et al., 2002), the presence of such a MC-receptor has yet to be demonstrated in the inner ear. Moreover, none of the known MC-receptors binds to ORG 2766. So the actual mechanism of action in the prevention of cisplatin-induced ototoxicity of the ACTH-derived neuropeptides is still unclear.

Methods for studying ototoxicity

In this thesis we are mostly interested in the prevention of cisplatin-induced ototoxicity. Cisplatin causes structural damage and functional loss in several tissues of the cochlea and the auditory nerve. In order to study these effects experimentally we have used two approaches: electrocochleography and histology.

Electrocochleography

Electrocochleography (ECochG) is a method to record the stimulus-related potentials of the cochlea and auditory nerve (Ruth et al., 1988). The response that is measured with ECochG occurs within the first 2-3 ms after an abrupt stimulus, and includes the following components (Fig. 4):

- The cochlear microphonics (CM), which is a stimulus-related alternating current (AC) potential that closely mimics the frequency of the stimulus. It is primarily generated by the OHCs, and it represents the displacement of the basilar membrane in response to an acoustic stimulus (Sellick et al., 1982). When the electrode is placed on the round window membrane the recordings reflect the activity of the OHCs in the basal turn.
- The summating potential (SP), a positive or negative stimulus-related direct current (DC) potential that reflects the time-related displacement of the cochlear partition. It is generated mostly by the IHCs, although a significant contribution is made by the OHCs (Durrant et al., 1998).
- The compound action potential (CAP), which is a transient response that is generated by peripheral eighth cranial nerve. It represents the summed response of the synchronous firing of thousands of auditory nerve fibers (Goldstein and Kiang, 1958; Ferraro et al., 1983). The amplitude of the CAP reflects the number of nerve fibers that are firing simultaneously

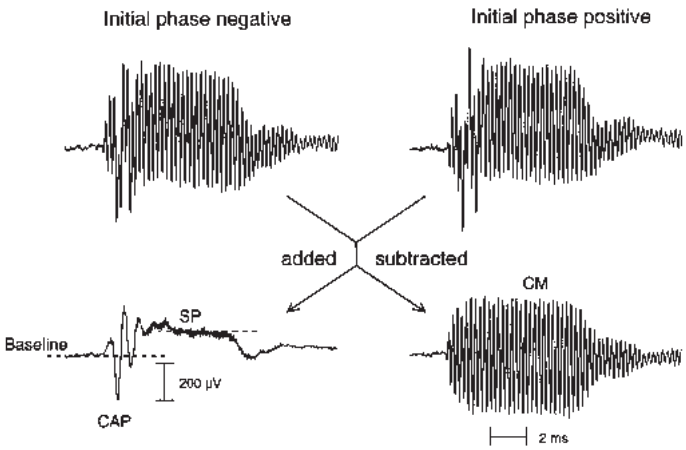

Figure 4: Principles of an ECochG measurement. All recordings were performed at the round window. Traces obtained on stimuli with 180º phase difference are averaged separately. Adding these averages cancels CM so that CAP and SP are clearly distinguished. Subtracting these averages removes CAP and SP while the CM remains.

Histology

Another method to investigate cisplatin-induced cochlear damage is by studying the histopathological effects of the various functional entities of the cochlea, *e.g.* outer hair cells (OHCs), inner hair cells (IHCs), spiral ganglion cells and stria vascularis. In this thesis the quantitative analysis of OHCs and IHCs was performed in midmodiolar sections. The cochlea is divided into two halves after which 1 μm thick slices (sections) are taken from the cut surface (Fig. 5).

In these sections the number of hair cells were counted at seven different locations along the basilar membrane (2 transections for the basal turn; 2 transections for the middle turn and 3 transections for the apical turn).

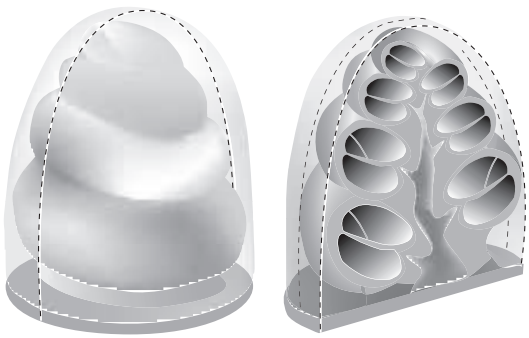

Figure 5: Principle of midmodiolar sectioning

The number of hair cells missing, relative to the expected 3 OHCs per transection, is a measure of cochlear damage. This hair-cell damage can be compared and correlated to the impairments found in the electrophysiological data.

Outline of the thesis

As outlined in the previous paragraphs, many advances have been made in the management of cisplatin-induced side effects. However, the neurotoxic and ototoxic side effects can not be treated effectively without hampering the cytotoxic action of cisplatin. Since ACTH-derived neuropeptides have shown to be effective as neuroprotective compounds that do not interfere with the antineoplastic properties of cisplatin, our group has tested these peptides for their

possible otoprotective action. In previous studies, in which the drugs were administered during a fixed number of days, it was shown that both α–MSH and ORG 2766 have a beneficial effect on cisplatin-induced ototoxicity. However, since only part of the animals was protected, further optimization of the melanocortin treatment was considered to be necessary. Furthermore, the mechanism underlying the action of melanocortins had to be investigated.

This thesis is based on a longitudinal animal model in which an implanted electrode allows repeated measurements of cochlear sensitivity. In the first part the compounds are administrated systemically, in the second part we focus on a cochlear model in which the compounds can be delivered directly into the cochlea via a mini-osmotic pump system.

In the first study (**Chapter 2**) the longitudinal animal model is used to investigate the otoprotective effects of the new and more potent melanocortin-receptor agonist melanotan-II (MT-II). This synthetic melanocortin has been effective in the protection of cisplatin-induced peripheral neuropathy (Ter Laak et al., 2003). The objective of the study was to investigate whether MT-II is able to delay the occurrence of cisplatin-induced ototoxicity and to effect subsequent recovery. Animals were implanted with a permanent electrode and treated daily with cisplatin and MT-II until a 40 dB CAP threshold shift at 8 kHz occurred. Subsequently, cisplatin treatment was stopped and CAP recovery was studied for another 2 weeks.

A subsequent study was performed to test the protective effects of the melanocortin peptides α–MSH and ORG 2766 in the same longitudinal animal model (**Chapter 3**). We investigated whether these peptides delay the occurrence of the cisplatin-induced shift in auditory threshold, and whether they effect the subsequent recovery of the cochlear action potential (CAP) and endocochlear potential (EP). Both peptides showed significant ameliorating effects on the recovery of the CAP.

Since it was suggested that this recovery might be due to reversible strial failure and EP recovery, in the third study (**Chapter 4**) we have investigated the time course of EP recovery and whether the recovery of the EP is influenced by α–MSH co-treatment. In an experimental set-up similar to that of the second study, the EP and CAP were measured 1, 2, or 3 days after the criterion threshold shift at 8 kHz was reached.

To gain more insight into the mechanism underlying α–MSH action, the second objective of this thesis, we switched to the cochlear model in which effects of cisplatin and melanocortins could be studied without the possibly confounding influence of systemic administration. In the fourth study (**Chapter 5**) this animal model was used; cisplatin was administered directly into the cochlea via a mini-osmotic pump system while α–MSH or saline were administered daily by systemic injection. This approach decreased interanimal variability, which made it easier to quantify the efficacy of systemic α–MSH co-treatment. Furthermore, since cisplatin was delivered directly to the cochlea, any ameliorating effects of α–MSH would indicate that treatment with α–MSH probably involves a cochlear target.

To complement the previous study a mirror experiment was performed in the fifth study (**Chapter 6**). Guinea pigs that were implanted with a permanent electrode and a mini-osmotic pump, pumping either saline or α–MSH, were co-treated systemically with cisplatin until the 40 dB threshold shift at 8 kHz was reached. Then, cisplatin treatment was stopped, but intracochlear perfusion and electrocochleography were continued for 10 days to evaluate possible effects of local α–MSH treatment on recovery.

Chapter 2

Co-treatment with melanotan-II, a potent melanocortin, does not protect against cisplatin ototoxicity

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Summary

Cisplatin, an important chemotherapeutic agent, has severe dose-limiting side effects including peripheral neurotoxicity and ototoxicity. Peripheral neurotoxicity can be delayed or prevented by simultaneous treatment with a class of neuropeptides known as melanocortins. Examples are ORG 2766, α–melanocyte stimulating hormone (α–MSH) and melanotan-II (MT-II). In albino guinea pigs, our group has found that ORG 2766 and α–MSH can also reduce cisplatin-induced ototoxicity. In this study we investigated the possibly protective effects of MT-II upon cisplatin ototoxicity.

Guinea pigs, equipped with a permanent round window electrode for electrocochleography, were treated with cisplatin (1.5 mg/kg/day i.p.) and simultaneously with MT-II (30 or 3 μg/kg/day s.c.) or saline until a 40 dB suppression of the compound action potential (CAP) threshold (3 μV criterion) at 8 kHz occurred. This 40 dB criterion was reached after 5 to 18 days. Thereafter, the treatment was stopped, but electrocochleography was continued for another four weeks.

The number of days in which the 40 dB criterion threshold shift was reached in the MT-II co-treated group did not differ from the period in the saline group. Ten days after the end of the treatment a spontaneous recovery of the CAP was observed in all groups and at all frequencies, although it was more pronounced at lower frequencies. Also with respect to recovery, no differences were found between the saline and the MT-II co-treated group. Thus, in contrast with the otoprotective properties of other melanocortins, MT-II has no protective properties against cisplatin-induced ototoxicity, at least not with the doses applied here.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum (II)), an important chemotherapeutic drug, is used in the treatment of different types of malignancies such as ovarian and testicular carcinoma and in cases of cancer of the head, neck, bladder and lung. Unfortunately, cisplatin has severe dose-limiting side effects including nephrotoxicity, peripheral neuropathy and ototoxicity.

The ototoxic effect of cisplatin is characterized by a bilateral, high frequency sensorineural hearing loss, usually associated with tinnitus (De Oliviera, 1989; Schweitzer, 1993). Laurell and Bagger-Sjöbäck (1991a) showed that the morphological changes in the cochlea of guinea pigs after cisplatin exposure occur in three stages. The first stage included disturbance of the supporting cells surrounding the outer hair cells (OHCs). The second stage was characterized by degeneration of the OHCs, which previously had been shown to progress from base to apex (Nakai et al., 1982); One of the first signs was the loss of stereocilia and intracellular vacuolization. The inner hair cells (IHCs) usually remained intact until all the OHCs had degenerated. In the final stage the entire organ of Corti had collapsed. Other studies, in rats and guinea pigs (Kohn et al., 1997; Meech et al., 1998; Campbell et al., 1999; Cardinaal et al., 2000a,b), focused on damage to the stria vascularis. This damage consisted of blebbing and vacuolization of the marginal cells and atrophy of the intermediate cells. The stria vascularis gives rise to the endocochlear potential (EP). In line with the morphological damage to the stria, a smaller than normal EP was observed after administration of cisplatin to guinea pigs (Klis et al., 2000, 2002). Summarizing, cisplatin seems to have at least two targets in the cochlea: the organ of Corti and the stria vascularis. Presently, the relation between the respective effects on these targets, *e.g.* whether one is causally related to the other, is unknown.

Several compounds that are known for their nephroprotective and neuroprotective effects also seem to be able to protect the inner ear from cisplatin toxicity. These compounds include anti-oxidants, *e.g.* D-methionine (Reser et al., 1999; Campbell et al., 1999), 4-methylthiobenzoic acid (Kamimura et al., 1999; Rybak et al., 1999a) and diethyldithiocarbamate (Kaltenbach et al., 1997; Rybak et al., 1999a). The application of these compounds is based on the demonstration that reactive oxygen species generation is increased in the cochlea after administration of cisplatin (Clerici et al., 1996; Kopke et al., 1997). Another group of protective compounds is a class of neuropeptides known as the melanocortins. Melanocortins are compounds related to the mother compounds α–Melanocyte Stimulating Hormone (α–MSH) and AdrenoCorticoTropic Hormone (ACTH). α–MSH and the synthetic $ACTH_{(4-9)}$

analog ORG 2766 have shown to enhance recovery after peripheral nerve trauma at both histological and functional levels (Bijlsma et al., 1984; De Koning et al., 1986; Van der Zee et al., 1991), and ORG 2766 specifically has shown to prevent the development of cisplatin-induced neuropathy both in animals (De Koning et al., 1987; Hamers et al, 1993a) and in humans (Gerritsen van der Hoop et al., 1990). Our group showed that, in guinea pigs, these peptides can also provide protection against cisplatin ototoxicity (Hamers et al., 1994; De Groot et al., 1997; Stengs et al., 1998b; Heijmen et al., 1999; Smoorenburg et al., 1999; Cardinaal et al., 2000c).

Melanotan-II (MT-II) is a cyclic melanocortin (MC) with very high affinity for the MC1-, MC4- and MC5-receptor (Schiöth et al., 1997; Yang et al., 1997; Hadley et al., 1998; Haskell-Luevano et al., 2000). The compound has already been administered to humans and showed little side effects (Wessels et al., 2000). *In vitro* experiments showed that MT-II is a ten times more potent agonist for the human MC1-receptor than α–MSH (Yang et al., 1997; Haskell-Luevano et al., 2000). The MC1-receptor is found in melanocytes in the skin; MC4- and MC5-receptors have been localized in the nervous system. Concerning the inner ear, it has been suggested that a receptor for α–MSH, probably the MC1-receptor, is present in the intermediate cells (which are melanocytes) of the stria vascularis (Meyer zum Gottesberge, 2000). MT-II has proven to be effective in the prevention of cisplatin-induced peripheral neuropathy in rats; Ter Laak et al. (2003) showed that MT-II ameliorates cisplatin-induced neuropathy after subcutaneous administration at 1.0 μg/kg.

The present study was designed to investigate the possibly protective effect of MT-II on cisplatin ototoxicity in guinea pigs that were equipped with a permanent round window electrode. This animal model allowed us to track putative effects of MT-II before, during and after cisplatin-treatment. The post-treatment measurements are important with regard to recovery. Our group has shown that cisplatin-induced ototoxicity is partly reversible in guinea pigs (Stengs et al., 1997; Klis et al., 2000, 2002), *i.e.* the cochlea has the ability to recover from cisplatin insults. MT-II might enhance this recovery.

Materials and methods

Animals and experimental design

Thirty-two female albino guinea pigs (strain: Dunkin-Hartley, Harlan Laboratories, Horst, The Netherlands; weight 350-650 g), equipped with a permanent round window electrode were treated with cisplatin and one of three co-treatments. These co-treatments consisted of either MT-II in saline

(3 μg/kg/day; n=9 or 30 μg/kg/day; n=11) or approximately equal volumes of plain saline (n=12). The animals were housed, four together, in macrolon cages and had free access to food and water. They were maintained on a 12:12 h dark/light cycle. The animals were treated daily with cisplatin and the relevant co-treatment until the electrocochleogram showed a 40 dB reduction of the Compound Action Potential (CAP) threshold at 8 kHz stimulation. This threshold, more appropriately called iso-response level, was defined as the sound level required to evoke a CAP of 3 μV. One day after 40 dB reduction of the CAP threshold was reached, an additional last dose of MT-II or saline was given without administration of cisplatin. After the end of the cisplatin treatment electrocochleography was continued for four weeks to evaluate the possible effects of MT-II on the expected recovery.
The care and use of the animals reported in this study were approved by the Animal Care and Use Committee of the University of Utrecht (DEC-UMC #91035).

Drugs

Cisplatin (Platosin®; Pharmachemie B.V., Haarlem, The Netherlands) was diluted with physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml. It was administered intraperitoneally at a daily dose of 1.5 mg/kg body weight/day. This dose was based upon the previous experiments with cisplatin by Stengs et al. (1998a, b) and Heijmen et al. (1999). The high dilution was chosen to stimulate diuresis and thus to minimize renal effects. Melanotan-II (MT-II; Ac-Nle-cyclic-[Asp-His-D-Phe-Arg-Trp-Lys]NH_2; Bachem, Bubendorf, Switzerland) was dissolved in saline. MT-II was administered subcutaneously in a daily dose of 3 or 30 μg/kg body weight/day. The argument for these doses is as follows. Ter Laak et al. (2003) found protection against cisplatin neurotoxicity at 1.0 μg/kg MT-II, not at 0.1 μg/kg. α–MSH protection against cisplatin neurotoxicity works best between 7.5 and 75 μg/kg/day (Van der Zee et al., 1991). MT-II has a 10 times greater affinity for the MC1-receptor than α–MSH. Thus, 3 μg/kg/day is right in the middle of the predicted effective dose window for MT-II (0.75-7.5 μg/kg/day). We also did not want to risk too low a concentration (see effect of 0.1 μg/kg/day in neuroprotective studies). Thus, in order not to miss the protective effect we also chose a concentration 10 times higher (30 μg/kg/day).

Surgical techniques

Prior to surgery the animals were injected with an antibiotic (chloramphenicol sodium succinate; 60 mg/kg) and then anaesthetized with 50 mg/kg ketamine

(Parke Davis, Hoofddorp, The Netherlands) and 1 ml/kg Thalamonal (a mixture of fentanyl and droperidol: 0.05/2.5 mg/ml; Janssen Pharmaceutica, Tilburg, The Netherlands). Local anaesthetic (lidocaine 1%; Adrenaline 1:100.000; 0.3 ml) was used in areas to be incised. Under sterile conditions the bulla of the right ear was opened retro-aurically and the skull was exposed around the bregma. The round window electrode was made of insulated stainless-steel wire (diameter 0.175 mm including teflon insulation; Advent, Halesworth, UK) with a 0.5 mm diameter gold ball micro-welded (Unitek 80F; Unitek Equipment, Monrovia, CA, USA) to the exposed and flattened tip. The wire was soldered to a Berg 22-26 gold terminal that fitted into a Berg 2x3 mini-latch housing (Farnell, Maarssen, The Netherlands). Stainless-steel screws were inserted through the skull and connected to the mini-latch housing via two silver wires also connected to a gold terminal. The electrode was positioned on the round window and secured to the bulla with polymaleinate glass-ionomer cement (Ketac-Cem Aplicap, ESPE dental supplies, Utrecht, The Netherlands). The mini-latch housing was connected to the skull with dental acrylic cement, which also covered and insulated the stainless-steel screws and the electrodes. The wound was closed in two layers with vicryl.

Electrocochleography

Measurements were performed differentially with the round window electrode as the active electrode and two screws on the skull as reference and ground electrodes, respectively. Trains of tone bursts of 2, 4, 8, and 16 kHz were used as stimuli. The tone bursts were constructed with cosine-shaped rise-and-fall times of 1 ms (1.5 ms at 2 kHz) and had a duration of 8 ms. The sound stimuli were produced in an open field configuration with a Fame tweeter (Staffhorst Electronics, Utrecht, The Netherlands) positioned at 10 cm from the pinna. Consecutive tone bursts were presented with alternating polarity at 99 ms intervals in order to avoid synchronization with the mains frequency of 50 Hz. The responses were amplified (EG&G Instruments model 5113 amplifier, Te Lintelo Systems, Zevenaar, The Netherlands), bandpass filtered between 1 Hz and 30 kHz, AD converted and stored on disk for off-line analysis. CAP and Summating Potentials (SP) were obtained by adding the responses evoked by tone bursts of opposite polarity, Cochlear Microphonics (CM) by subtracting these responses. The CAP was measured relative to the SP and not relative to the baseline of the recording since, in principle, the CAP is superimposed on the SP. The CM was measured as the peak-to-peak amplitude in the middle of the sinusoidal response. Electrocochleography was continued until four weeks after the end of the treatment. Animals that did not

have a normal threshold at 8 kHz (defined as a threshold at less than 25 dB SPL stimulus level) or that showed signs of otitis media, during surgery or when they were sacrificed, were excluded from this study. Statistical analysis was performed by means of analysis of variance (ANOVA), using STATISTICA software.

Results

General findings

In the follow-up after cisplatin-treatment, four animals were lost because of a failing electrical connection (2 in each MT-II co-treated group). Fatalities did not occur, neither during treatment nor in the four weeks post-treatment survival period. Most animals treated with cisplatin, in combination with MT-II or plain saline, showed loss of weight (Table 1).

Table 1: Mean change in body weight ± s.d. at the end of the cisplatin-treatment

Co-treatment	Number of animals	Change of bodyweight	
		Grams	%
MT-II 30 μg/kg/day	N=11	-8 ± 14 (range -31 to +12)	-1.8 ± 3.3
MT-II 3 μg/kg/day	N=9	-20 ± 25 (range -57 to +8)	-3.7 ± 4.6
Saline	N=12	-17 ± 30 (range -87 to +30)	-3.6 ± 7.2

When the cisplatin-treatment was terminated the animals started gaining weight again at their normal rate. ANOVA showed a significant effect of time ($F_{(2,42)}=270$, $P<0.001$), but no interaction with co-treatment ($F_{(2,42)}=0.62$, $P=0.93$). This indicates that weight changed significantly over time, which is trivial, but that weight changes during and after cisplatin-treatment was similar in all three groups. Thus, weight changes cannot account for possible effects the MT-II co-treatment.

Cisplatin effects on CAP thresholds in relation to co-treatment

During the first days of treatment no dramatic change in CAP threshold was observed. As previously reported (Klis et al., 2000) a threshold shift occurred rather suddenly after several days of treatment (*cf.*, Fig. 3). The number of days necessary to evoke a threshold shift of ≥ 40 dB at 8 kHz was 5 to 18 days of treatment (Fig. 1).

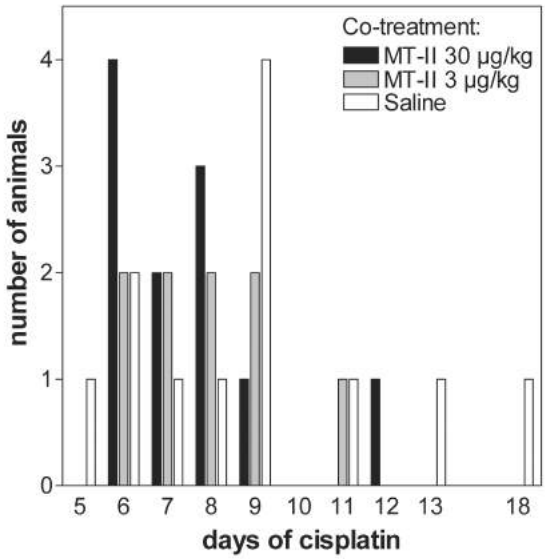

Figure 1: The time necessary to evoke a hearing loss of ≥ 40 dB in the 3 μV iso-response level (CAP threshold) at 8 kHz for the MT-II 30 μg/kg/day (black bar), MT-II 3 μg/kg/day (grey bar) and the saline (white bar) co-treated groups.

Figure 1 suggests that there is no effect of co-treatment with MT-II on the number of days necessary to reach criterion loss. ANOVA confirmed this lack of effect of co-treatment ($F_{(2,29)}=0.12$, $P=0.89$), even when excluding the atypical animal in the control group ($F_{(2,28)}=0.07$, $P=0.93$), which required 18 injections to reach criterion threshold shift. Maximum CAP threshold shifts occurred either at the day cisplatin administration was terminated or 1 to 2 days later (*cf.*, Fig. 3). Maximum threshold shift in dB as a function of frequency is shown in Figure 2 for the three treatments. The hearing loss occurred in a broad frequency range, although it clearly increased as a function of frequency. ANOVA showed a significant main effect of frequency ($F_{(2,12)}=40.3$, $P<0.001$), but not of co-treatment ($F_{(2,12)}=0.04$, $P=0.96$).

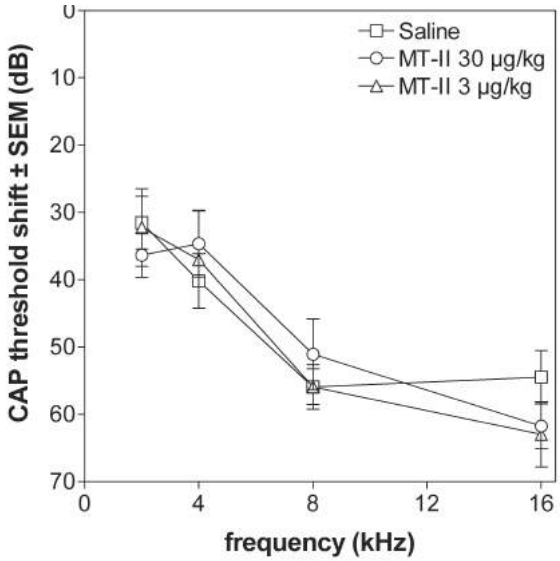

Figure 2: Maximal Compound Action Potential (CAP) threshold shift at the end of the cisplatin-treatment as a function of stimulus frequency for (○) MT-II 30 μg/kg/day (n=11), (△) MT-II 3 μg/kg/day (n=9) and (□) saline (n=12) co-treated groups.

Recovery of CAP thresholds in relation to co-treatment

Pronounced recovery of the CAP threshold was observed in all three groups after cessation of the cisplatin-treatment. This recovery was observed at all frequencies, although it was more pronounced at the lower frequencies. Every individual animal showed recovery; there were no signs of a dichotomous distribution in any of the three groups. The recovery started to level off at about 10 days post-treatment. Figure 3 shows the build-up and recovery of the CAP threshold as a percentage of the loss in dB at the time cisplatin-treatment was stopped (day 0). The measurements beyond 15 days post-treatment are not shown; they did not show any further systematic shift. Note the previously mentioned sudden onset of the hearing loss in Figure 3.

Statistical analysis (ANOVA) of the data starting at day 0 showed significant effects of time (≡recovery; $F_{(2,45)}=14.7$, $P<0.001$) and frequency ($F_{(2,45)}=3.76$, $P=0.032$). Co-treatment was not a significant main factor and there was no significant interaction between co-treatment and either frequency or time, indicating a lack of effect of MT-II co-treatment on threshold recovery. Recovery can also be expressed in terms of post-treatment growth of CAP amplitude at a fixed stimulus level (Klis et al., 2000).

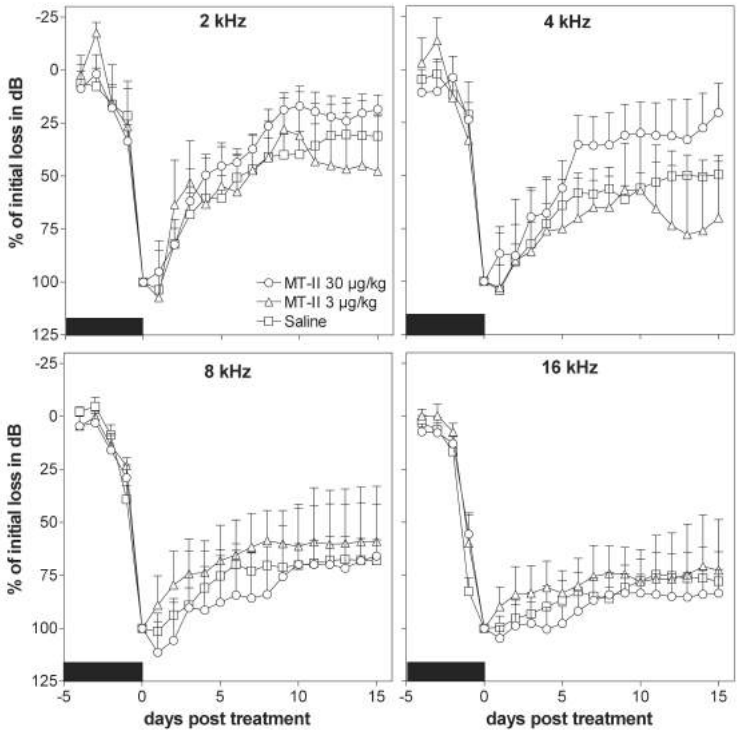

Figure 3: Build-up and recovery at 2, 4, 8, and 16 kHz (+ SEM) of the CAP threshold for (○) MT-II 30 μg/kg/day (n=9), (△) MT-II 3 μg/kg/day (n=7) and (□) saline (n=12) co-treated groups. The horizontal black bar represents the last 5 days of cisplatin-treatment. Day 0 represents the day that the treatment was stopped. 100% of initial loss in dB corresponds to the threshold shift at the time of cessation of cisplatin-treatment (average values in dB SPL are given in Fig. 2), 0% corresponds to the pre-treatment baseline.

We tested the effects of co-treatment on recovery of CAP-amplitudes at several stimulus levels and at all frequencies (2-16 kHz). This alternative approach also showed no significant effect of co-treatment (data not shown).

Recovery of CM in relation to co-treatment

The CM were also affected by cisplatin-treatment. As an example, Figure 4 shows the CM amplitude at 16 kHz, 66 dB SPL. This CM was lost at the same day the CAP criterion threshold shift was reached. At 16 kHz, the CM amplitude hardly recovers.

However, statistical analysis (ANOVA) of the 16 kHz CM data starting at day 0 showed a significant effect of time (≡recovery; $F_{(2,45)}=2.78$, $P<0.001$). Once again, co-treatment was not a significant main factor and there was no significant interaction between co-treatment and time, indicating a lack of effect of MT-II co-treatment on recovery. The same results were found at all other frequencies (2-8 kHz; not shown).

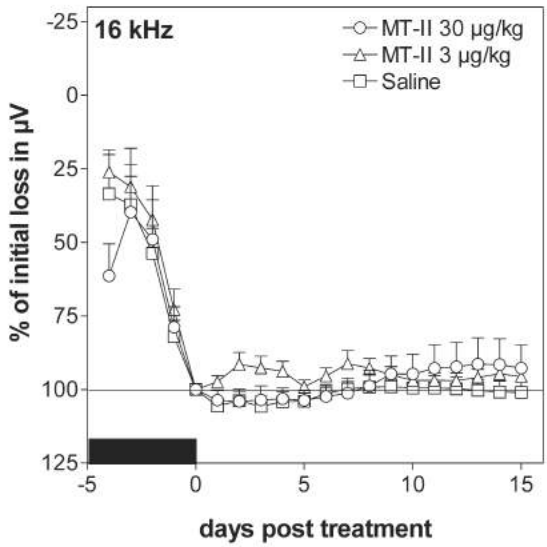

Figure 4: Build-up and recovery at 16 kHz, 66 dB SPL of the CM amplitude (+ SEM) for (○) MT-II 30 μg/kg/day (n=9), (△) MT-II 3 μg/kg/day (n=7) and (□) saline (n=12). The horizontal black bar represents the last 5 days of cisplatin-treatment. Day 0 represents the day that the treatment was stopped. 100% of initial loss in μV corresponds to the shift at the time of cessation of cisplatin. 0% corresponds to the pre-treatment baseline.

Discussion

Cisplatin ototoxicity

The chronic recording technique applied in this study allowed us to monitor cochlear sensitivity changes before, during and after cisplatin-treatment, with or without MT-II co-treatment, on a day-to-day basis. The effects of cisplatin alone on the CAP as a measure of cochlear sensitivity, were in accordance with previous results (Klis et al., 2000, 2002).

We observed that a physiological threshold shift occurred rather suddenly after several days of cisplatin administration. Further, we found that the number of injections necessary to evoke criterion threshold shift varied substantially between animals (Fig. 1). In the present study the number of days (=injections) necessary to reach the criterion threshold shift varied from 5 to 18 days, although the latter value is clearly an unexplained outlier. Hearing loss occurred over a broad frequency range (Fig. 2), although it was significantly larger at the higher frequencies. The marked recovery after cessation of cisplatin-treatment (Figs. 3, 4) was also found previously (Klis et al., 2000, 2002). Typically, recovery of CAP threshold was more prominent at lower frequencies (Fig. 3). Recovery of CM at all frequencies and CAP at high frequencies was far less pronounced. (Figs. 3, 4). It has to be kept in mind that the CM, recorded at the round window, derives from hair cells in the immediate vicinity of the round window, irrespective of stimulus frequency (Dallos, 1973). On the other hand, the CAP derives from its characteristic frequency location. Klis et al. (2000, 2002) hypothesized that the occurrence of hearing loss over a relatively broad frequency range and the subsequent recovery from cisplatin-evoked loss might be related to loss and recovery of the strial integrity, reflected in the EP. The less prominent recovery of the CM at all frequencies and the similarly less prominent recovery of the CAP at higher frequencies could be explained by simultaneously occurring permanent loss of OHCs in the basal cochlear turn near the electrode. We propose that similar events occurred in the present experiment.

MT-II does not change the characteristics of cisplatin intoxication and recovery

The main issue in this study was whether MT-II, a potent melanocortin with high affinity for the MC1-receptor, would be capable of protecting against cisplatin-induced ototoxicity. The rationale for this hypothesis was based on two sets of experimental observations. The first set of observations consisted of positive results from our laboratory with the related compounds ORG 2766 and α–MSH. Both compounds have shown to protect against cisplatin ototoxicity in various experiments (Hamers et al., 1994; De Groot et al., 1997; Stengs et al.,

1998b; Heijmen et al., 1999; Smoorenburg et al., 1999). The second observation, which led to the present experiment, was the effect of MT-II on cisplatin-induced peripheral neuropathy in rats. MT-II was found to protect against cisplatin-induced neuropathy (Ter Laak et al., 2003), a property which MT-II shares with ORG 2766 (Hamers et al., 1993a). Evidently, we did not find protecting effects of MT-II. At the two concentrations applied the compound had no effect on the number of cisplatin injections necessary to evoke criterion hearing loss (Fig. 1). Further, MT-II did not enhance recovery (Figs. 3, 4). One might assume that the compound would have had an effect at another dose. Melanocortins are known to have an inverted U-shaped or bell-shaped dose effect curve (De Koning et al., 1986). In other words low and high doses are inactive and only the intermediate dose results in enhanced recovery. Ter Laak et al. (2003) found that MT-II had protective effects on cisplatin-induced neuropathy at 1.0 μg/kg/day, but not at 0.1 μg/kg/day. We chose 3 and 30 μg/kg/day because MT-II, according to *in vitro* results, shows a ten times higher affinity for the human MC1-receptor than α–MSH (Yang et al, 1997). The latter compound and ORG 2766 are effective at daily doses of 75 μg/kg/day (Hamers et al., 1994; Stengs et al.,1998b; Heijmen et al., 1999; Smoorenburg et al., 1999). Thus, chances are small that we missed a possible protective effect of MT-II. Also, pharmacokinetic studies performed in the rat showed a half life of MT-II of 1.5 hours (Ugwu et al., 1994) compared to α–MSH, which has a half life of 7 minutes (Wilson and Harry, 1980). Considering these properties of MT-II we assumed that this compound should have the same or maybe even a larger effect if the effect of α–MSH is MC1-receptor mediated.

Another explanation for the lack of protecting effects could be that the compound simply does not reach its target. Although we do not know the exact target of the melanocortins, it is highly likely that the target is located in the inner ear. The actual target might be the intermediate cells in the stria vascularis, which presumably express receptors for melanocortins (Meyer zum Gottesberge, 2000). If so, the lack of effect of MT-II could be due to earlier binding of the compound to MC-receptors in the rest of the body. For instance, MT-II is believed to be more efficiently bound to MC4 and MC5-receptors in the brain than α–MSH because of its lower molecular weight and more lipophilic character (Yang et al., 1997; Schiöth et al. 1997). So MT-II could be intercepted on its way to the cochlea, whereas α–MSH would not be intercepted and would therefore reach the cochlea more efficiently. Experiments with local administration of melanocortins in the inner ear might shed more light on the issues discussed above. Finally, the ameliorating effects of α–MSH and ORG 2766 co-treatment on cisplatin-induced ototoxicity, found in previous studies, might be mediated by a mechanism that does not involve MC-receptors.

Conclusion

The potent melanocortin, melanotan-II, did not prevent cisplatin-induced ototoxicity in guinea pigs, at least not in the concentrations applied here. Also, no MT-II-induced enhancement of recovery of cochlear sensitivity after cisplatin-treatment was observed. In both these aspects, MT-II differs from its parent compound, α–melanocyte stimulating hormone and therefore appears less promising for future clinical applications.

Acknowledgements

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Chapter 3

Cisplatin ototoxicity involves organ of Corti and stria vascularis: modulation by α–MSH and ORG 2766

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Summary

Cisplatin, an important chemotherapeutic agent, has severe dose-limiting side effects, including peripheral neuropathy and ototoxicity. Peripheral neuropathy can be delayed or prevented by simultaneous treatment with a class of neuropeptides known as melanocortins. Examples are α–melanocyte stimulating hormone (α–MSH) and the non-melanotropic ACTH/$MSH_{(4-9)}$ analog ORG 2766. Here, we investigated whether these peptides delay the occurrence of the cisplatin-induced shift in auditory threshold, and whether they affect the subsequent recovery of cochlear potentials. Chronically implanted round window electrodes were used to obtain daily recordings of auditory nerve compound action potentials (CAP) and cochlear microphonics (CM) at frequencies ranging from 2 to 16 kHz. Cisplatin (1.5 mg/kg i.p.) plus α–MSH (75 μg/kg s.c.), ORG 2766 (75 μg/kg s.c.), or saline were injected daily until a 40 dB CAP threshold shift at 8 kHz was reached. Endocochlear potential (EP) was measured either 1-2 days or 28 days later, followed by histological analysis of the cochlea. Peptide co-treatment did not consistently delay the threshold shift. However, the CAP threshold recovered faster and to a greater extent, with the potency order being α–MSH>ORG 2766>saline. Significant recovery at the two highest frequencies was seen in the α–MSH-treated animals only. CAP amplitude at high sound pressures, which depends more on nerve function than on outer hair cell (OHC) function, decreased severely in all groups but recovered significantly in the α–MSH and completely in the ORG 2766 co-treated group. EP was significantly lower in the first days after the threshold shift but had completely recovered at 28 days. Both CM threshold and amplitude at 16 kHz decreased dramatically over the course of cisplatin treatment and remained very low afterwards, indicating severe loss of OHC function in the basal turn, although there was some recovery in both α–MSH and ORG 2766 co-treated animals. Histological analysis confirmed that OHC loss was most severe in the basal turn of saline treated animals. These data suggest that the cisplatin-induced acute threshold shift might be due to reversible strial failure, whereas subsequent OHC survival determines the final degree of functional recovery.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum-II) is one of the most potent anti-tumor agents available, but its use is hampered by severe toxic side effects, including ototoxicity and peripheral neuropathy. The main pathological finding in the inner ear is the degeneration of the outer hair cells (OHCs), with those in the first row and basal turn being most severely affected (Nakai et al., 1982; Laurell and Bagger-Sjöbäck, 1991b; De Groot et al., 1997). Loss of OHCs dramatically affects both hearing thresholds and the ability to discriminate between different frequencies. Clinically, cisplatin-induced hearing loss is usually irreversible, although evidence of recovery has been reported in both humans (De Oliveira, 1989; Laurell and Jungnelius, 1990) and experimental animals (Stengs et al., 1997; Klis et al., 2000, 2002).

Although cisplatin is one of the most potent OHC toxins known (Anniko and Sobin, 1986), it may also cause hearing loss by affecting other inner ear structures, such as spiral ganglion cells (Alam et al., 2000; Cardinaal et al., 2000b) and the stria vascularis (Nakai et al., 1982; Tange and Vuzevski, 1984; Kohn et al., 1988; Saito and Aran, 1994b; Zheng and Gao, 1996; Kohn et al., 1997; Meech et al., 1998; Cardinaal et al., 2000a, b; Watanabe et al., 2002). Indeed, a major decrease in the endocochlear potential, implying strial vascularis dysfunction, has been observed early after the occurrence of cisplatin-induced threshold shifts (Klis et al., 2000, 2002; Tsukasaki et al., 2000).

Several strategies have been shown to protect against cisplatin-induced neuro- and ototoxicity. One group of protective compounds is a class of neuropeptides known as the melanocortins. Melanocortins are compounds related to the mother compounds α–Melanocyte Stimulating Hormone (α–MSH) and AdrenoCorticoTropic Hormone (ACTH). α–MSH and the $ACTH_{(4-9)}$ analog ORG 2766 have been shown to enhance recovery after peripheral nerve trauma at both histological and functional level (Bijlsma et al., 1984; De Koning et al., 1986; Van der Zee et al., 1991), and ORG 2766 specifically has been shown to prevent the development of cisplatin-induced neuropathy in animals (De Koning et al., 1987; Hamers et al., 1993a) and in humans (Gerritsen Van der Hoop et al., 1990). Previous work from our laboratory has shown that co-treatment with neuroprotective doses of the $ACTH_{(4-9)}$ analog ORG 2766 (Hamers et al., 1994; Stengs et al., 1998b; Cardinaal et al., 2000c) and α–MSH (Heijmen et al., 1999) protects against cisplatin-induced hearing loss in guinea pigs. Both peptides either completely protected against loss of auditory thresholds and OHCs, or thresholds were decreased similar to the placebo co-treated animals. However, OHC counts in these 'non-responders' to ORG 2766 were significantly higher than in the saline-treated animals (De Groot et al.,

1997; Cardinaal et al., 2000c). In longitudinal studies spontaneous recovery of cochlear function from cisplatin-induced ototoxicity was observed (Klis et al., 2000, 2002). The longitudinal studies also demonstrated that threshold shifts initially develop slowly, followed by an abrupt threshold shift at 8 kHz between one cisplatin injection and the next (30-50 dB loss within 24 hours). On the basis of these data, we hypothesized that a slight delay in the onset of hearing loss might explain the dichotomy between either complete protection or severe hearing loss observed in our original protection studies in which we administered the drugs on a fixed number of days (Hamers et al., 1994; Stengs et al., 1998b, Heijmen et al., 1999). Furthermore, we also expected recovery to be faster and more complete in animals co-treated with ORG 2766 or α–MSH, since animals with severe threshold shifts, despite the fact that they were co-treated with ORG 2766, exhibited significantly less severe OHC loss than placebo-treated ones (De Groot et al., 1997; Cardinaal 2000c).
In the present study, we show that in guinea pigs α–MSH and ORG 2766 modulate the response of the cochlea to cisplatin and that they also enhance subsequent recovery of auditory thresholds and auditory nerve function.

Materials and methods

Animals and experimental design

Thirty-four female albino guinea pigs (strain: Dunkin-Hartley, Harlan Laboratories, Horst, The Netherlands; weight 330-560 g), equipped with a permanent round window electrode, were treated with cisplatin and one of three co-treatments. These co-treatments consisted of either ORG 2766, α–MSH or approximately equal volumes of saline. The animals were housed, four together, in macrolon cages and had free access to food and water. They were maintained on a 12:12 h dark/light cycle. After the stability of the cochlear responses was tested on at least 5 subsequent days, animals that displayed baseline Compound Action Potential (CAP) thresholds, defined as the sound level required to evoke a CAP of 3 μV at sound pressures of less than 25 dB SPL at 8 kHz, were given a daily intraperitoneal injection of cisplatin. This injection was immediately followed by a subcutaneous injection of the relevant co-treatment until the electrocochleograms showed a 40 dB suppression of CAP threshold at 8 kHz stimulation. One day after the 40 dB suppression was reached (day 0), an additional last dose of ORG 2766, α–MSH or saline was given without administration of cisplatin. Thereafter, electrocochleography was continued either for 1-2 days or for four weeks, followed by measurement of the endocochlear potential (EP) and harvesting of the cochleas for

histology. Animals were randomized to early (n=5/experimental group) or late (n=6/experimental group) EP measurements.
The care and use of the animals reported in this study were approved by the Animal Care and Use Committee of the University of Utrecht (DEC-UMC # 91035).

Drugs

Cisplatin (Platosin®; Pharmachemie B.V., Haarlem, The Netherlands) was diluted with saline (pH 7.4) to a final concentration of 0.1 mg/ml. It was administered intraperitoneally at a daily dose of 1.5 mg/kg body weight. This dose was based upon the previous experiments with cisplatin by Stengs et al. (1998a, b) and Heijmen et al. (1999). The high dilution was chosen to stimulate diuresis and thus to minimize renal effects. The cisplatin injection was immediately followed by a subcutaneous injection of either 60 μg/ml α–MSH (Bachem, Heidelberg, Germany) (75 μg/kg; n=11), 60 μg/ml ORG 2766 (Organon, Oss, The Netherlands) (75 μg/kg; n=12) or saline (n=11). The dose of 75 μg/kg α–MSH or ORG 2766 was based on experiments with a positive outcome performed previously by Heijmen et al. (1999).

Surgical techniques

Prior to surgery the animals were injected with an antibiotic (chloramphenicol sodium succinate; 60 mg/kg) and then anaesthetized with 50 mg/kg ketamine (Parke Davis, Hoofddorp, The Netherlands) and 1 ml/kg thalamonal (a mixture of fentanyl and droperidol: 0.05/2.5 mg/ml; Janssen Pharmaceutica, Tilburg, The Netherlands). Local anaesthetic (lidocaine 1%; Adrenaline 1:100.000; 0.3 ml) was used in areas to be incised. Under sterile conditions the bulla of the right ear was opened retro-aurically and the skull was exposed around the bregma. The round window electrode was made of insulated stainless-steel wire (diameter 0.175 mm including teflon insulation; Advent, Halesworth, UK) with a 0.5 mm diameter gold ball micro-welded (Unitek 80F; Unitek Equipment, Monrovia, CA) to the exposed and flattened tip. The wire was soldered to a Berg 22-26 gold terminal that fitted into a Berg 2x3 mini-latch housing (Farnell, Maarssen, The Netherlands). Stainless-steel screws were inserted through the skull and connected to the mini-latch housing via two silver wires also connected to a gold terminal. The electrode was positioned on the round window and secured to the bulla with polymaleinate glass-ionomer cement (Ketac-Cem Aplicap, ESPE dental supplies, Utrecht, The Netherlands). The mini-latch housing was connected to the skull with dental acrylic cement, which also covered and insulated the stainless-steel screws and the electrodes. The wound was closed in two layers with vicryl.

Electrocochleography

Measurements were performed differentially with the round window electrode as the active electrode and two screws on the skull as reference and ground electrode, respectively. Trains of tone bursts of 2, 4, 8, 11.3 (8√2), and 16 kHz were used as stimuli. The tone bursts were constructed with cosine-shaped rise-and-fall times of 1 ms (1.5 ms at 2 kHz) and had a duration of 8 ms. The sound stimuli were produced in an open field configuration with a Fame tweeter (Staffhorst Electronics, Utrecht, The Netherlands) positioned at 10 cm from the pinna. Consecutive tone bursts were presented with alternating polarity at 99 ms intervals in order to avoid synchronization with the mains frequency of 50 Hz. The responses were amplified (EG&G Instruments model 5113 amplifier, Te Lintelo Systems, Zevenaar, The Netherlands), bandpass filtered between 1 Hz and 30 kHz, AD converted and stored on disk for off-line analysis. CAP and Summating Potentials (SP) were obtained by adding the responses evoked by tone bursts of opposite polarity, Cochlear Microphonics (CM) by subtracting these responses (Fig. 1). The CAP was measured relative to the SP and not relative to the baseline of the recording since, in principle, the CAP is superimposed on the SP. The CM was measured as the peak-to-peak amplitude in the middle of the sinusoidal response. Complete input-out-

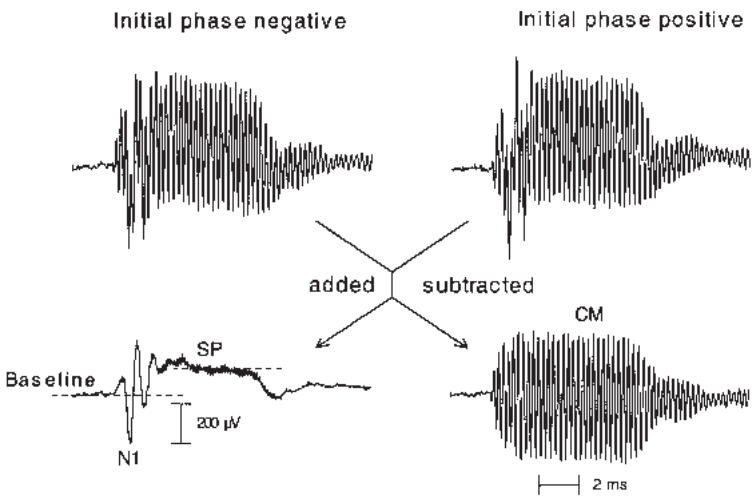

Figure 1: Principles of an ECochG measurement. All recordings were performed at the round window. Traces obtained on stimuli with 180° phase difference are averaged separately. Adding these averages cancels CM so that CAP and SP are clearly distinguished. Subtracting these averages removes CAP and SP while the CM remains. SP is measured relative to baseline, whereas CAP is the difference between N1 and SP.

put curves were produced at 10 dB resolution until no CAP or CM could be discerned in up to 128 stimulus pairs. Maximum sound pressure was between 80-90 dB SPL in order to prevent noise-induced hearing damage. Electrocochleography was continued either for 1-2 days or for four weeks (daily for the first 3-5 days, every other day for the next week, and bi-weekly thereafter), followed by measurement of the EP.

Measurements of the EP

The endocochlear potential was measured as described by Van Emst et al. (1997). Under halothane anesthesia (1.5% in N_2O/O_2) preceded by a thalamonal injection (1 ml/kg, i.m.) a tracheostomy was performed. During further surgery and experimental procedures the animals were anaesthetized by artificial ventilation through a tracheal cannula with a gas-mixture containing 33% O_2, 66% N_2O and 1% halothane. Heart frequency was monitored and body temperature was kept at 38°C. The cochlea was exposed using the ventrolateral approach. A small hole was shaven in the bony wall of the cochlea overlying the scala media of the second turn. A glass microelectrode filled with 150 mM KCl was placed just above the hole in the scala media. An Ag/AgCl pellet-containing microelectrode holder was connected to an Electro 705 preamplifier (WPI, New Haven, CT, USA). The EP was measured relatively to another Ag/AgCl pellet placed in the neck musculature. Using a micro-manipulator (Narishige, Tokyo, Japan), the microelectrode was advanced into the scala media while the EP was recorded. The recording potential was zeroed when the microelectrode came into contact with fluids just lateral to the stria vascularis. Then the microelectrode was advanced in small steps and a sudden positive voltage was measured when the electrode entered the scala media. On withdrawal of the electrode from the scala media the measured potential rapidly returned to zero.

Histological techniques

After the measurement of the EP the animal was deeply anaesthetized, temporal bones were removed, and the cochleas were fixed by intralabyrinthine perfusion with a tri-aldehyde fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2,5% dimethylsulfoxide in 0.1 M sodium cacodylate buffer (pH 7.4) followed by immersion in the same fixative for 3 h at room temperature. The cochleas were further processed according to the routine method for guinea pigs (De Groot et al., 1987). Semithin (1 μm) midmodiolar

sections were cut and stained with 1% methylene blue and 1% azur II in 1% sodium tetraborate. In the midmodiolar sections the organ of Corti was examined at seven (2 basal, 2 middle and 3 apical) locations, separated by a half turn spacing, and the number of OHCs present at each location was counted in 8-10 sequential 1 μm sections. If a hair cell was not seen in 8-10 sections, it was assumed to be absent. OHC counts from the right and left ears of each animal were averaged. All OHC counts were performed by two investigators, independently of one another, in a single-blind fashion (*cf.*, De Groot et al., 1997; Cardinaal et al., 2000a). An estimate was made of the characteristic frequencies of the locations along the basilar membrane at which the OHCs were counted (Fig. 2).

Turn		c.f. (kHz)
b1	Lower basal turn	26.2
b2	Upper basal turn	10.4
m1	Lower middle turn	5.1
m2	Upper middle turn	2.7
a1	Lower apical turn	1.3
a2	Middle apical turn	0.7
a3	Upper apical turn	0.3

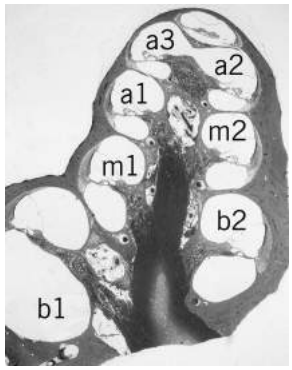

Figure 2: Midmodiolar section of the cochlea. Two transections each for basal (b1 and b2) and middle turns (m1 and m2), three transections for the apical turn (a1, a2 and a3). The characteristic frequency (c.f.) in kHz is computed based upon the frequency map by Greenwood (1990).

Data analysis and statistics

Animals that did not have a normal threshold at 8 kHz (defined as a threshold at less than 25 dB SPL stimulus level) or that showed signs of otitis media at the moment of EP measurement were discarded from the analysis. In addition, we discarded data from cochleas that were damaged during histological processing. Each electrocochleographic (ECochG) measurement consisted of complete input-output curves at 10 dB resolution for 2, 4, 8, 11.3 (8√2), and 16 kHz tone bursts. Thresholds (3 μV iso-response levels) were computed by linearly extrapolating the sound pressure at which the line connecting the two lowest sound pressures yielding responses larger than 3 μV crossed the 3 μV level. For assessment of recovery, all threshold shifts were expressed as the percentage difference from baseline (with threshold at criterion defined at

100%). This enabled comparison of differences in recovery between different frequencies (*e.g.*, the absolute 16 kHz threshold shifts were significantly larger than observed at 2 kHz). Differences in the number of cisplatin injections needed to generate the criterion threshold shift were analyzed by a one-way analysis of variance (ANOVA). Differences in acute threshold shift, EP and OHC counts were also evaluated by ANOVA, followed by Student-Newman-Keuls test if ANOVA produced evidence of group differences. Return of CAP and CM thresholds towards baseline were tested within each treatment group by paired t-tests at days 2 (early) and 14 (late) after criterion threshold shift was reached. Group differences in maximum CAP amplitude were analyzed by repeated measures ANOVA, followed by Student-Newman-Keuls test at 14 days. Differences were considered statistically significant if $P<0.05$.

Results

Number of days to rapid threshold deterioration

All saline co-treated animals displayed a sudden CAP threshold shift at 8 kHz after 7 to 9 daily doses of cisplatin. As expected, the development of hearing loss was delayed substantially (up to 14 injections needed) in several ORG 2766- and α–MSH-treated animals. However, in others the development of hearing loss was accelerated by 1 or 2 days. The mean cumulative cisplatin dose needed to cause hearing loss did not differ between the groups ($F_{(2,31)}=0.809$, ns), but the variance in the α–MSH- (Levine's test, $P<0.049$) and especially in the ORG 2766- (Levine's test, $P<0.035$) treated group was significantly higher than in the saline-treated group (Fig. 3).

Cisplatin effects on CAP and CM thresholds in relation to co-treatment.

The criterion to stop treatment was a CAP threshold shift of at least 40 dB at 8 kHz. The absolute CAP threshold shift at this criterion did not differ between the groups at any of the tested frequencies. As expected, the largest shifts were observed at 16 kHz (Fig. 4). The electrical activity of all OHCs with characteristic frequencies above the probe frequency contributes to the CM signal. Because the basal OHCs are affected most by cisplatin, we only analyzed the CM elicited by 16 kHz tone. Similarly to the CAP, CM threshold shifts (3 μV isoresponse levels) were equal in the three groups (Fig. 4).

Because the cumulative cisplatin dose needed to cause hearing loss differed widely between animals, especially in the α-MSH and ORG 2766 groups, and hearing loss became evident after as few as 5 doses in one case, we plotted the threshold shifts relative to baseline from 4 days before the sudden increase in CAP threshold.

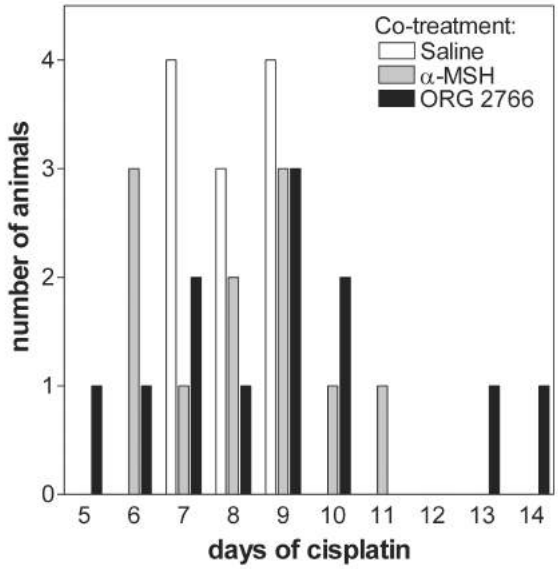

Figure 3: Number of animals reaching the criterion threshold shift (≥ 40 dB at 8 kHz compared to baseline) at a given day. White bar: Saline; Grey bar: α-MSH; Black bar: ORG 2766

Moreover, as absolute threshold shifts did not differ between experimental groups, but did differ between tone burst frequencies, the threshold shift at the day the threshold criterion was reached was normalized to 100% (per animal). The absolute threshold shift on the day criterion was reached at 8 kHz was about 50 dB compared with pre-treatment (*cf.*, Fig. 4). The larger part of this threshold shift developed within 24 hours after the last cisplatin injection (Fig. 5). A slight threshold shift was already present at day -1.

For all five frequencies tested, this threshold shift tended to be smaller in the α–MSH (but not ORG 2766) group than in the saline co-treated group (t-tests α–MSH versus saline: 2, 4 kHz, ns; 8 kHz, $P<0.05$; 11.313 kHz, $P<0.03$; 16 kHz, $P<0.004$).

CAP thresholds in relation to time

For the first 3 to 5 days of hearing loss, the ECochG was recorded daily. Thereafter, the ECochG was not recorded in all animals on the same recovery days. To facilitate visualization and statistical analysis, thresholds were linearly interpolated to give daily values. At 2 and 4 kHz, the relative CAP threshold loss increased even further after the criterion had been reached, even though no further cisplatin injections were given. This deterioration tended to be less

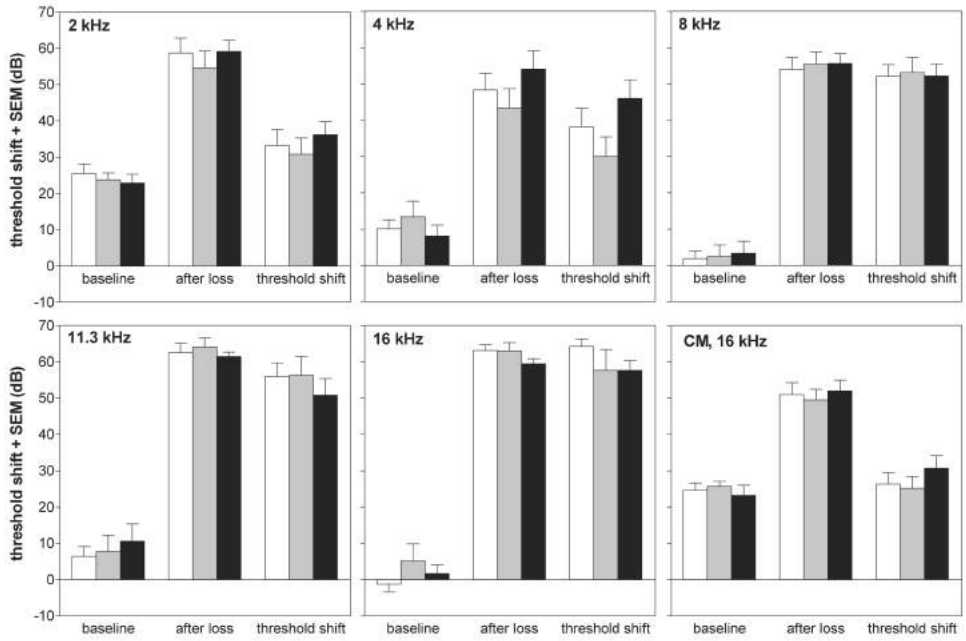

Figure 4: Mean 3 μV iso-response levels (dB SPL ± SEM) before the start of treatment (baseline), after reaching the criterion threshold shift at 8 kHz (after loss) and the difference between these (absolute threshold shift) for CAP at respectively 2, 4, 8, 11.3, and 16 kHz and CM at 16 kHz. White bar: Saline; Grey bar: α–MSH; Black bar: ORG 2766.

severe in the peptide-treated groups. Ultimately, slight (16 kHz) to major (2 kHz) recovery of the CAP threshold was observed (Fig. 5). Recovery progressed most rapidly in the α–MSH-treated animals, but eventually became similar in all groups, except at 11.3 and 16 kHz. At these high frequencies, the CAP threshold did not recover significantly in the saline-treated animals (88% and 92% residual loss at 14 days, respectively), whereas slight recovery was seen in ORG 2766-treated animals (76% and 83% residual loss at 14 days) and even more so in α–MSH-treated animals (45% and 61% residual loss at 14 days). The power to detect significant group differences was small due to the fact that half the animals were used for EP measurements between day 0 and day 2 and because some remaining animals developed problems with the round window electrode hereafter. As such, ANOVA indicates group differences at 2 days for 8 and 11.3 kHz only ($F_{(2,17)}=3.46$, $P<0.048$, and $F_{(2,17)}=3.74$, $P<0.045$ respectively; Student-Newman-Keuls: α–MSH differed from saline at the 5% level). Paired t-tests, which can be used to look for significant recovery from the original threshold shift, however, indicated that statistically significant

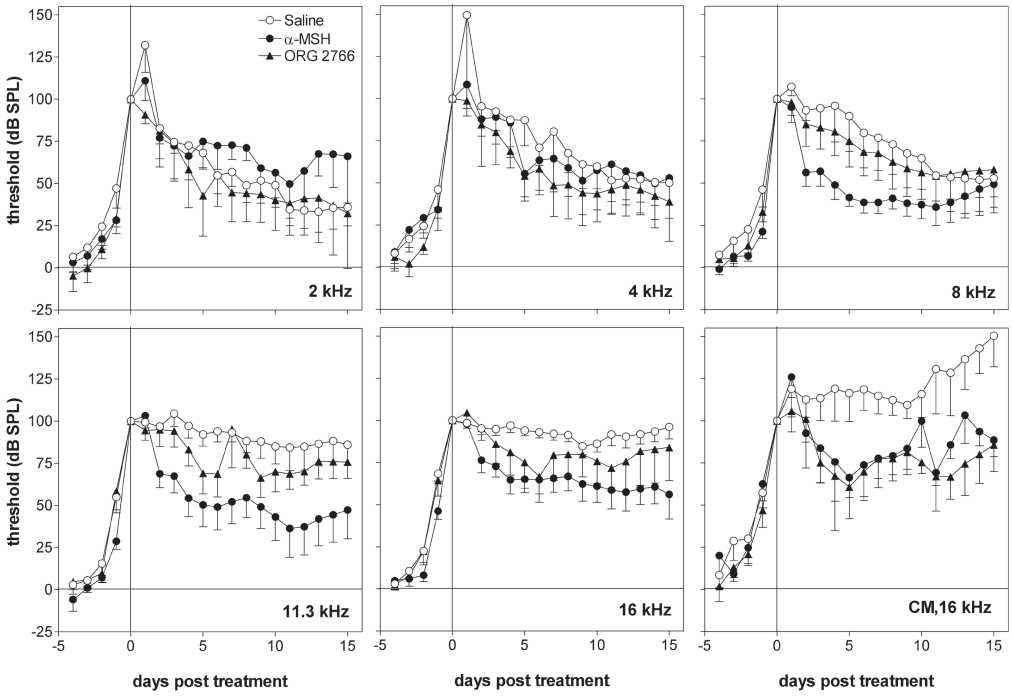

Figure 5: Normalized shifts in 3 μV iso-response levels for the CAP threshold shift at respectively 2, 4, 8, 11.3, and 16 kHz and the CM threshold shift at 16 kHz as function of time (% ± SEM). Day 0 is the day the criterion threshold shift (≥ 40 dB at 8 kHz, compared to pre-treatment) was observed. Threshold shift at the day criterion has been reached has been set to 100%. Note that because animals were used for EP measurements, the number of animals that contributed to the graph was halved between days 0 and 2. ○, Saline; ●, α–MSH; ▲, ORG 2766.

recovery of the CAP threshold was present in the saline-treated animals only at 2 and 4 kHz, in the ORG 2766-treated animals only at 4 kHz, but in the α–MSH-treated animals at 8, 11.3, and 16 kHz (Table 1).

To exclude the possibility that higher cumulative cisplatin doses impair the ability to recover, we tested for correlations between the cumulative dose and recovery 14 days after discontinuation of cisplatin treatment. No significant correlation was found (data not shown). A similar picture as for the CAP threshold emerged for the CM threshold. In the saline co-treated animals the 3 μV iso-response level of the CM at 16 kHz increased even further in the following 2 weeks, whereas it recovered in the α–MSH and ORG 2766 co-treated animals (14 days, ANOVA $F_{(2,10)}=4.87$, $P<0.033$; Student-Newman-Keuls: both α–MSH and ORG 2766 differed from saline at the 5% level) (Fig. 5).

Table 1: The presence or absence of significant recovery of CAP thresholds in individual treatment groups was tested by paired t-tests, comparing the threshold shifts at day 0 (criterion threshold shift reached) with those at days 2 and 14 for Saline, α–MSH and ORG 2766 co-treated animals. P-values are summarized in this table: P-values < 0.05 have been indicated in bold.

	Saline		α–MSH		ORG 2766	
Probe (kHz)	d0-d2	d0-d14	d0-d2	d0-d14	d0-d2	d0-d14
2	0.111	**0.015**	0.243	0.178	0.312	0.100
4	0.526	**0.035**	0.685	0.078	0.069	**0.040**
8	0.316	0.108	**0.003**	**0.010**	0.301	0.057
11.313	0.450	0.415	**0.012**	**0.027**	0.637	0.077
16	0.243	0.370	**0.034**	**0.018**	0.567	0.456
Number of animals	6	6	5	5	6	4

CAP amplitude in relation to co-treatment

The CAP amplitude, measured at high sound pressures (between 80-90 dB SPL), recovered differently than the CAP thresholds (Fig. 6).

During drug administration the CAP amplitude initially decreased to a similar extent in all three groups, even before significant changes in the CAP threshold occurred. Whereas saline-treated animals showed slight recovery to pretreatment levels, α–MSH treatment evoked a modest, and ORG 2766 treatment an almost complete recovery of the CAP amplitude (ANOVA for repeated measurements days 0-10: 2 kHz: $F_{(2,10)}=5.12$, $P<0.029$; 4 kHz: $F_{(2,10)}=5.71$, $P<0.022$; 8 kHz: $F_{(2,12)}=3.10$, $P<0.082$; 11.313 kHz: $F_{(2,11)}=1.74$, $P<0.221$; 16 kHz: $F_{(2,11)}=5.27$, $P<0.025$; Student-Newmans-Keuls test at day 10: 2 and 8 kHz: ORG 2766 differed from α–MSH and saline; 8 and 16 kHz: ORG 2766 differed from saline, α–MSH differed from neither ORG 2766 nor saline).

CM amplitude in relation to co-treatment

The maximum obtainable CM amplitude at 16 kHz decreased dramatically over the course of cisplatin treatment and remained at a very low level afterwards (Fig. 6), indicating severe loss of outer hair cell function in the basal turn. Although there was some recovery in both the α–MSH- and ORG 2766-treated groups, this recovery was significant only at day 2 after cessation of cisplatin, at which point the α–MSH-treated group differed significantly from the ORG 2766- and saline-treated groups (ANOVA $F_{(2,17)}=5.25$, $P<0.015$).

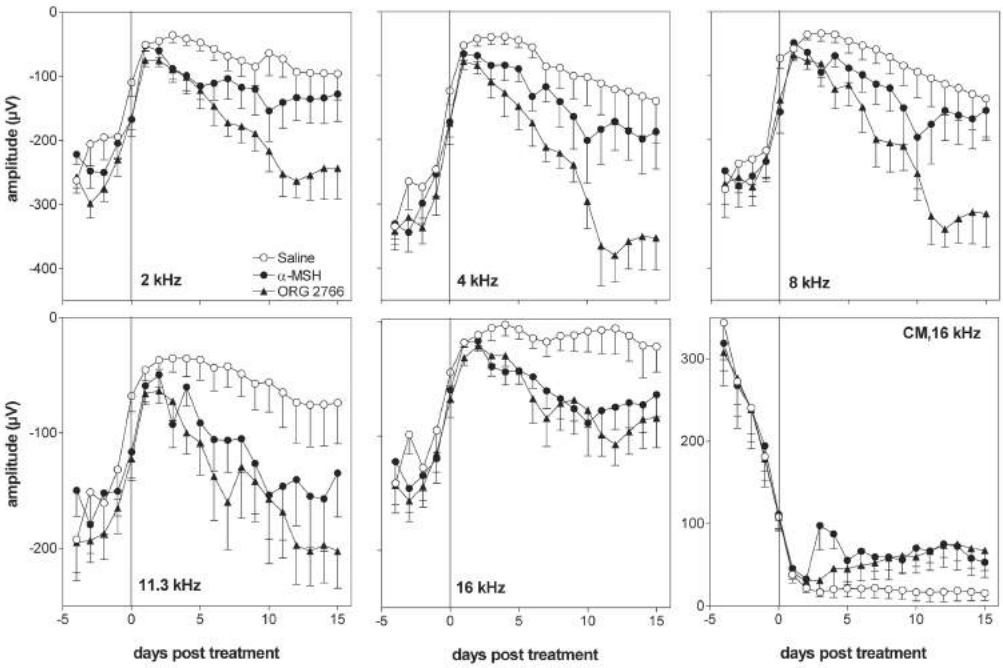

Figure 6: Maximum CAP and CM amplitudes obtained at high stimulus levels (80-90 dB SPL, exact level depending on tone burst frequency) (μV - SEM). Note that CAP amplitudes are expressed as negative voltages (measured against SP) so they go up as they deteriorate, in contrast to CM that goes down upon deterioration. ○ Saline; ●, α–MSH; ▲, ORG 2766.

Endocochlear potential measurements

Since longitudinal measurement of the EP is technically not feasible, EP was measured in half the animals between 0 and 2 days after the criterion was reached and in the remaining animals about 26-28 days later. In the first days after discontinuation of the cisplatin treatment, there was a severe reduction in EP (range 35-65 mV). Small group differences existed (ANOVA: $F_{(2,12)}=4.32$, $P<0.04$), with the EP after ORG 2766 co-treatment being slightly lower compared with saline or α–MSH co-treatment. The EP and absolute CAP or CM threshold shifts were not correlated. The EP was back to normal values (mean: 80 mV, range 72-87 mV) after 28 days in all three treatment groups.

OHC counts

The presence of OHCs was evaluated on midmodiolar sections through both left and right cochleas, and counts from the left and right ears of a given animal were averaged. The number of OHCs did not correlate with the cumulative cisplatin dose, nor was there a difference between OHC counts in the animals killed early versus late (*i.e.*, there was no evidence for either further degeneration or regeneration during the 4 weeks following discontinuation of cisplatin treatment, see also Klis et al. (2000)). Thus, the OHC counts from all animals were pooled over all survival times.

In cross-sections the normal number of OHCs is 3 and the most severe loss of these cells was observed in the basal turn of the saline co-treated animals (1.04 ± 0.21 (mean ± SEM) OHCs remaining). Both α–MSH and ORG 2766 co-treatment improved the survival of OHCs (1.86 ± 0.23 and 1.69 ± 0.20 OHCs remaining, respectively; ANOVA $F_{(2,28)}=3.76$, $P<0.038$). OHC loss in the middle and apical turn was less severe. Again, less OHCs remained in these turns in the saline-treated animals than in the α–MSH and ORG 2766-treated ones, but differences were not statistically significant (Fig. 7). There was no IHC loss.

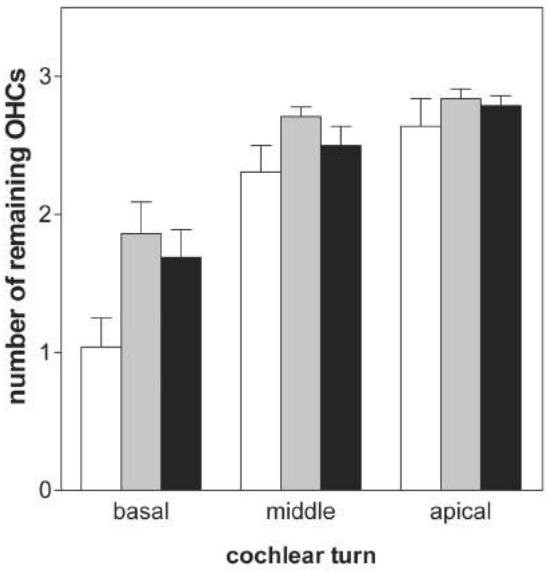

Figure 7: Number of OHCs remaining in the basal, middle and apical turns. As there were no differences in OHC counts between animals sacrificed early (0-2 days after reaching criterion threshold shift) versus late (> 22 days) data from all animals were pooled. Bars represent the mean OHC counts in the 2 basal, 2 middle and 3 apical sections. White bar: Saline; Grey bar: α–MSH; Black bar: ORG 2766.

Discussion

Cisplatin ototoxicity

This study demonstrates that cisplatin-induced ototoxicity is partly reversible at a functional level (Klis et al., 2000, 2002) and that co-treatment with α-MSH and ORG 2766 positively affects OHC survival and function. Neither peptide consistently delayed the occurrence of ototoxicity, but recovery from ototoxicity was faster and more extensive in peptide-treated animals. Moreover, cisplatin affected cochlear function at two levels, the stria vascularis and the OHCs. The EP, which is generated by the stria vascularis and critical for sound transduction, initially decreased in all three experimental groups but recovered completely. The initial decrease in the EP can explain both the observed losses of CM and the CAP threshold shift. However, whereas the EP recovered completely, the CAP threshold did not. Recovery was especially poor after saline co-treatment and at the highest frequencies, probably because of the severe OHC loss observed in the basal turn. The finding that no statistically significant recovery of the CAP threshold was observed at 4 and 8 kHz might seem to contradict earlier observations (Klis et al., 2000), but is probably caused by the low number of animals monitored over an extended period in this study, as compared to the previous one, diminishing statistical power. The strong decrease in CAP amplitude at high sound pressures indicates either disruption of the transduction machinery (IHC to afferent axon), neuropathy of the cochlear ganglion, or both. Because quantitative measures indicated that cisplatin adversely affected the morphology of spiral ganglion cells (Alam et al., 2000; Cardinaal et al., 2000b), we suggest that cisplatin also causes neuropathy of the auditory nerve, analogous to the peripheral sensory neuropathy caused by the drug (Hamers et al., 1991b).

Pharmacological intervention with both ORG 2766 and α–MSH provided partial protection against most of the adverse effects of cisplatin. Both OHC survival and recovery of the CAP threshold were significantly enhanced by both peptides. The recovery of CAP amplitude at high sound pressures was enhanced by both ORG 2766 and α–MSH. Unexpectedly, neither of the peptides consistently delayed the occurrence of hearing loss. Although in several animals ototoxicity was considerably delayed (up to 14 doses needed instead of 9), in other animals it occurred earlier (5 doses instead of 7). It should be noted that there is no clear relationship between the cumulative cisplatin dose and ototoxic symptoms in either humans (Black et al., 2001) or experimental animals, and that in the latter the amount of cisplatin needed to elicit pathology may vary widely between experiments (Klis et al., 2002). Nonetheless, the observa-

tions in this study demonstrate that the peptides do affect the response of the cochlea to cisplatin. The findings also help to explain the results of previous studies in which fixed cumulative cisplatin doses were used. Major threshold shifts were observed in all saline but not all ORG 2766 co-treated animals after 8 (2.0 mg/kg/day) (Hamers et al., 1994) or 10 (1.6 mg/kg/day, Hamers and Klis: unpublished observations) doses. In view of the present data, we suppose that in these previous studies the cumulative cisplatin dose sufficed to affect all saline- but not all peptide-treated animals. The 'non-responders' to ORG 2766 tended to have slightly worse CAP input-output curves than the saline co-treated ones, but OHC survival was significantly better in these 'non-responders' than in saline-treated controls (De Groot et al., 1997). Similar observations were reported by Stengs et al. (1998b): 8 doses of 1.0 mg cisplatin/kg induced significant CAP threshold shifts in only 1 of 6 of saline co-treated animals, whereas it did so in 3 of 6 ORG 2766 co-treated animals. In contrast, 8 injections with 1.5 mg cisplatin/kg led to severe threshold shifts in 6 of 6 saline co-treated animals but only in 3/6 ORG 2766 co-treated animals. These results suggest that, as in the current study, co-treatment with ORG 2766 might both sensitize and desensitize animals for cisplatin-induced hearing loss.

Little is known about the sub-cellular mechanism by which cisplatin exerts its side effects. Cisplatin easily crosses the cell membrane, but once within the cell it is hydrated and platinates proteins and nucleic acids; its antineoplastic activity is due to intra-strand DNA cross-linking inside the tumor cells. Transplatin, the isomer of cisplatin, does not cause intra-strand DNA/RNA cross-links and does not display anti-tumor activity or any of the other side effects (Saito et al., 1997b). Therefore, it is likely that the anti-tumor efficacy and the side effects have a similar background. Actively dividing cells with cisplatin-caused DNA damage eventually proceed into apoptosis, but the question remains why terminally differentiated cells such as OHCs or neurons are killed by cisplatin-induced genomic DNA damage. One reason may be that the drug negatively affects transcription (by DNA cross-linking and inhibition of rRNA synthesis (Jordan and Carmo-Fonseca, 2000)) and translation. Another reason is that mitochondrial DNA is especially vulnerable to cisplatin because it lacks efficient repair mechanisms (Olivero et al., 1995). Indeed, mitochondrial damage is an early event in cisplatin nephrotoxicity (Brady et al., 1990). Caspase-9 (mitochondrial apoptosis pathway) is activated in an *in vitro* model of cisplatin-induced cochlear hair cell damage (Devarajan et al., 2002), and severe swelling of mitochondria can sometimes be observed in spiral ganglion cells of animals treated with cisplatin (Cardinaal and De Groot, unpublished observations). Cisplatin also affects mitochondrial function by

directly inhibiting the respiratory chain (Kruidering et al., 1997). In kidney proximal tubular cells, inhibition of mitochondrial function precedes cell death. Mitochondrial dysfunction can increase free radical production (and so promote cell damage) on the one hand and initiate apoptosis through release of cytochrome-c on the other (Li et al., 1997), as reported in the stria vascularis (Watanabe et al., 2001). The former may explain why radical scavengers can prevent at least some of the cisplatin-induced damage (Hamers et al., 1993b, Kamimura et al., 1999, Huang et al., 2000, Feghali et al., 2001), and the latter explains why the same holds true for trophic compounds such as α–MSH and ORG 2766 but also classical neurotrophic substances such as NGF (Apfel et al., 1992), which in general enhance the ability of cells to counter a-specific insults.

On the basis of these and earlier data (Hamers et al., 1994; De Groot et al., 1997; Klis et al., 2000), we suggest that cisplatin toxicity in the inner ear first occurs in the stria vascularis because this structure is more exposed to cisplatin than the OHCs (Laurell et al., 1995). Interference with mitochondrial function in the stria vascularis eventually impairs its electrogenic activity, leading to a precipitous drop of the EP and a strong increase in CAP thresholds. Until then, OHCs function relatively normal and no OHC loss occurs (De Groot et al., 1997). Further strial failure, due to either the decrease in the EP or the release of toxins in the endolymph, then causes the death of the most active (*i.e.*, basal) OHCs, even though OHC intracellular levels of cisplatin are not high enough to cause damage per se. Discontinuation of cisplatin treatment enables the stria vascularis and thus the EP to recover, with concomitant CAP threshold recovery, provided that a sufficient number of OHCs have survived and are functional.

According to this hypothesis, the peptides affect the ability of the stria vascularis to withstand the toxicity of cisplatin, although this effect is not consistent among individuals. The peptides also consistently protect against OHC death once strial failure has developed. In principle, α–MSH might modulate strial function because the intermediate cells necessary for normal strial function are melanocytes (Motohashi et al., 1994). ORG 2766, however, has no melanotrophic or corticotrophic activity (Greven and De Wied, 1973) and is known for sure not to activate any of the five currently known melanocortin (MC) receptor subtypes (Adan et al., 1994, 1996). As both peptides have similar trophic activity in models of mechanical peripheral nerve damage (Bijlsma et al., 1984; Van der Zee et al., 1991) and ototoxicity (Hamers et al., 1994; De Groot et al., 1997; Stengs et al., 1998b; Heijmen et al., 1999), it is conceivable that there is an as yet unidentified receptor for ORG 2766 that can also be activated by α–

MSH, though less efficiently, because α–MSH and the potent MC1 and MC4 agonist MT-II seem to be less effective than ORG 2766 in preventing peripheral neuropathies (Hamers and Ter Laak, unpublished observations).
ORG 2766 and α–MSH also differed in their efficacy to protect against cisplatin-induced cochlear damage. Both provided comparable protection against OHC loss, but whereas α–MSH produced a robust recovery of the CAP threshold, ORG 2766 most strongly affected recovery of the CAP amplitude, or auditory nerve function. So, co-treatment with ORG 2766 might reverse the cochlear neuropathy, similar to its action in peripheral sensory neuropathy in both animals (De Koning et al., 1987; Hamers et al., 1991a, 1993a) and humans (Gerritsen Van der Hoop et al., 1990). However, to date, little evidence for the involvement of the auditory nerve in cisplatin-induced hearing loss has been published, although an animal study (Cardinaal et al., 2000b) and a human study of post-mortem temporal bone material (Hinojosa et al., 1995) described morphological damage to the spiral ganglion in addition to OHC and strial damage.

Conclusion

This study shows that cisplatin causes profound damage to the cochlea, affecting OHCs, stria vascularis and spiral ganglion neurons. α–MSH and ORG 2766 significantly prevent OHC loss and ameliorate other signs and symptoms of cisplatin-induced ototoxicity. The fact that co-treatment with these peptides did not consistently delay the occurrence of ototoxicity will probably limit its clinical use. Nonetheless, these peptides remain interesting tools to further investigate the cellular processes that determine susceptibility and resistance to cisplatin ototoxicity and perhaps also other types of hearing loss.

Acknowledgements

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Chapter 4

Cisplatin-induced reduction of the cochlear potentials and subsequent recovery: effects of α–MSH and time A preliminary study

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Summary

It has previously been demonstrated that systemic administration of cisplatin induces severe ototoxic effects such as an increase of the compound action potential (CAP) threshold, a reduction in CAP amplitude at high stimulus levels, cochlear microphonics (CM) and the endocochlear potential (EP). Concomitant administration of α–melanocyte stimulating hormone (α–MSH) has shown to stimulate recovery of both CAP threshold and CM. The objective of the present study was to investigate whether this recovery co-varies with the recovery of EP within the first three days after cessation of cisplatin treatment. Albino guinea pigs, equipped with a permanent round window electrode, which allowed daily monitoring of the cochlear potentials, were treated daily with a bolus injection of 1.5 mg/kg cisplatin (i.p.) and an injection of 75 μg/kg α–MSH (s.c.) or saline until the electrocochleogram showed a persistent decrease in CAP amplitude (≥ 40 dB threshold shift at 8 kHz). Then, cisplatin treatment was stopped, but α–MSH and saline treatment were continued for one extra day. Either one, two, or three days after cessation of the cisplatin treatment the EP was measured. The EP was depressed when measured one day after cessation of cisplatin and showed no tendency to recover in the subsequent two days. Co-treatment with α–MSH did not prevent nor stimulate recovery of the EP within this time-window. Nevertheless, recovery of the CAP threshold at 2 kHz was observed in both saline and α–MSH treated animals. Since no recovery of EP was observed in the measured time-window, the ameliorating effect of α–MSH in cisplatin-induced ototoxicity might be more complex than previously thought; α–MSH might exert its effect at levels other than the stria vascularis.

Introduction

Cisplatin is an important antineoplastic drug, effective in the treatment of solid epithelial tumors. Unfortunately, treatment with cisplatin evokes severe side effects. One of these side effects is ototoxicity, which usually presents itself in humans as a high-frequency hearing loss accompanied with tinnitus (Schweitzer, 1993). Animal studies have shown that chronic cisplatin administration results in degeneration of the outer hair cells (OHCs), predominantly in the basal turn of the cochlea. In addition to the toxic effect upon the hair cells, there is growing evidence for an effect on the stria vascularis (Nakai et al., 1982; Tange and Vuzevski, 1984; Kohn et al., 1988; Saito and Aran, 1994b; Zheng and Gao, 1996; Kohn et al., 1997; Meech et al., 1998; Cardinaal et al., 2000a, b; Watanabe et al., 2002). The damage of the stria vascularis consists of blebbing and vacuolation of the marginal cells and atrophy of the intermediate cells. Degeneration of these tissues is associated with a decrease in the endocochlear potential (EP) (Laurell and Engstrom, 1989; Tsukasaki et al., 2000; Klis et al., 2000, 2002; O'Leary and Klis, 2002). Since the EP provides the main driving force for hair cell transduction processes within the cochlea and, therefore, for normal auditory function (Wangemann and Schacht, 1996), the decrease of the EP in turn raises auditory thresholds. Previous experiments, performed by Klis et al. (2000, 2002), showed that both EP and auditory thresholds partially recover within 4 weeks after cisplatin treatment, while OHC counts do not improve in time. Another study, by O'Leary and Klis (2002), showed that in cisplatin-deafened albino guinea pigs the EP returns to normal within 5 to 7 days. Finally, some studies (Alam et al., 2000; Cardinaal et al., 2000b) suggest that cisplatin also affects the spiral ganglion cells. These findings raise the question whether cisplatin affects all three target-structures separately or that degeneration of one target-structure is related to the other.

Previous work within our group has shown that the melanocortin α–melanocyte stimulating hormone (α–MSH) may stimulate recovery (Hamers et al., 2003). Since epidermal melanocytes have shown to be primary targets for α–MSH and melanocytes are also present within the stria vascularis as intermediate cells (Hilding and Ginzberg, 1977), it seems likely that this is the site of action of the putative protective effect of α–MSH.

The purpose of this study is to further investigate whether recovery of auditory thresholds and EP are related and whether these two indicators of cochlear health are affected to the same extent by co-treatment with α–MSH.

Materials and Methods

Animals and experimental design

Fifty female albino guinea pigs (strain: Dunkin-Hartley, Harlan Laboratories, Horst, The Netherlands; weight 240-560 g), equipped with a permanent round window electrode were treated with cisplatin (i.p.) and one of two co-treatments (s.c.). These co-treatments consisted of either α–MSH or approximately equal volumes of saline. The animals were housed, five together, in macrolon cages and had free access to food and water. They were maintained on a 12:12 h dark/light cycle. The animals were treated daily with cisplatin and the relevant co-treatment until electrocochleograms showed at least 40 dB increase in the compound action potential (CAP) threshold at 8 kHz. This threshold was defined as the sound level required to evoke a CAP amplitude of 3 μV. One day after the 40 dB criterion was reached, an additional last dose of α–MSH or saline was given, but no cisplatin. After the end of the treatment electrocochleography was continued for one, two, or three days (*cf.*, Table 1) to evaluate possible effects of α–MSH on recovery, followed by measurement of the endocochlear potential (EP). The care and use of the animals reported in this study were approved by the Animal Care and Use Committee of the University of Utrecht (DEC-UMC # 91035).

Drugs

Cisplatin (1 mg/ml; Platosin®; Pharmachemie BV, Haarlem, The Netherlands) was diluted with physiological saline (pH 7.4) to a final concentration of 0.1 mg/ml. It was administered intraperitoneally at a daily dose of 1.5 mg/kg body weight. This dose was based upon the previous experiments of Stengs et al. (1998a, b) and Heijmen et al. (1999). The high dilution was chosen to stimulate diuresis and thus to minimize renal effects. The cisplatin injection was immediately followed by a subcutaneous injection of either 75 μg/kg α–MSH (Bachem, Heidelberg, Germany) or saline. The α–MSH dose of 75 μg/kg/day was based upon experiments with a positive outcome performed previously by Heijmen et al. (1999) and Hamers et al. (2003).

Surgical techniques

Prior to the operation the animals were injected with chloramphenicol sodium succinate (30 mg/kg i.m.) and then anaesthetized with 40 mg/kg ketamine and 10 mg/kg xylazine (i.m.). Local anaesthetic (1% lidocaine, 0.50 ml) was used in areas to be incised. A mid-line incision was made on the dorsal surface of the head starting 2.5 cm anterior of bregma and continued post-auricularly to the base of the pinna. Under sterile conditions the bulla of the left ear was opened

retro-aurically and the skull was exposed around the bregma. The round window electrode, made of insulated stainless-steel wire (diameter 0.175 mm including teflon insulation; Advent, Halesworth, UK) with a 0.5 mm diameter gold ball micro-welded (Unitek 80F; Unitek Equipment, Monrovia, CA, USA) to the exposed and flattened tip, was positioned on the round window and secured to the bulla with carboxylate cement (Durelon, ESPE dental supplies, Utrecht, The Netherlands). The wire was soldered to a Berg 22-26 gold terminal that fitted into a Berg 2x3 mini-latch housing (Farnell, Maarssen, The Netherlands). Stainless-steel screws were inserted through the skull and connected to the mini-latch housing via two silver wires connected to a gold terminal. The mini-latch housing was connected to the skull with dental acrylic cement, which also covered and insulated the stainless-steel screws and the electrodes. The wound was closed in two layers with vicryl.

Electrocochleography

Measurements were performed differentially with the round window electrode as the active electrode and two screws on the skull as reference and ground electrode, respectively.

Animals that did not have a normal threshold at 8 kHz (defined as a threshold at less than 25 dB SPL stimulus level) to begin with or that showed signs of otitis media at the moment of EP measurement were excluded from further analysis. Trains of tone bursts of 2, 4, 8, and 16 kHz were used as stimuli. The tone bursts were constructed with cosine-shaped rise-and-fall times of 1 ms (1.5 ms at 2 kHz) and had a duration of 8 ms. The sound stimuli were produced in an open-field configuration with a Fame tweeter (Staffhorst Electronics, Utrecht, The Netherlands) positioned at 10 cm from the pinna. Consecutive tone bursts were presented with alternating polarity at 99 ms intervals in order to avoid synchronization with the mains frequency of 50 Hz. The responses were amplified (EG&G Instruments model 5113 amplifier, Te Lintelo Systems, Zevenaar, The Netherlands), bandpass filtered between 1 Hz and 30 kHz, AD converted, and stored on disk for off-line analysis. The animals were lightly restrained, but remained awake during all measurements, avoiding the disadvantages of repeatedly having to anaesthetize the animals.

CAPs were obtained by adding the responses evoked by tone bursts of opposite polarity; Cochlear Microphonics (CM) by subtracting these responses. The CAP was measured relative to the positive summating potential (SP) and not relative to the baseline of the recording since, in principle, the CAP is superimposed upon the SP. The CM was measured as the peak-to-peak amplitude in the middle of the sinusoidal response. Complete input-output curves were produced at 10 dB resolution until no CAP or CM could be discerned in up to

128 stimulus pairs. Maximum sound pressure was between 80-90 dB SPL in order to prevent noise-induced hearing damage. Electrocochleography was continued either for one, two or three days followed by measurement of the endocochlear potential.
Statistical analysis was performed by means of analysis of variance (ANOVA), using STATISTICA software.

Measurement of the endocochlear potential

The endocochlear potential was measured as described in Van Emst et al. (1997). Under halothane anesthesia (1.5% in N_2O/O_2), preceded by a hypnorm injection (0.1 mg/kg, i.m.), a tracheostomy was performed. During further surgery and experimental procedures the animals were anaesthetized by artificial ventilation through a tracheal cannula with a gas-mixture containing 33% O_2, 66% N_2O and 1% halothane. Heart frequency was monitored and body temperature was kept at 38°C. The cochlea was exposed using the ventrolateral approach. A small hole was shaven in the bony wall of the cochlea overlying the scala media of the second turn. A glass microelectrode filled with 150 mM KCl was placed just above the hole in the scala media. An Ag/AgCl pellet-containing microelectrode holder was connected to an Electro 705 preamplifier (WPI, New Haven, CT, USA). The EP was measured relatively to another Ag/AgCl pellet placed in the neck musculature. Using a micromanipulator (Narishige, Tokyo, Japan), the microelectrode was advanced into the scala media while the EP was recorded. The recording potential was zeroed when the microelectrode came into contact with fluids just lateral to the stria vascularis. Then the microelectrode was advanced in small steps and a sudden positive voltage was measured when the electrode entered the scala media. On withdrawal of the electrode from the scala media the measured potential rapidly returned to zero.

Results

General findings

Eighteen of the 50 operated animals showed an abnormal threshold at 8 kHz directly after surgery or showed infections upon inspection of the left (operated) ear at the moment of EP measurement. These animals were excluded from the experiment. Table 1 shows the distribution of the remaining animals (n=32) over the six experimental groups.

Table 1: Distribution of the animals over the experimental groups

Time of EP measurement (days after threshold shift)	Co-treatment	
	Saline	α–MSH
Day 1	4	5
Day 2	5	6
Day 3	6	6

Number of days to rapid threshold deterioration

Figure 1 shows the time required to reach the criterion threshold shift of ≥ 40 dB at 8 kHz. It took the saline co-treated animals 5 to 10 days of cisplatin to evoke the criterion threshold loss, which corresponds to earlier findings (Klis et al., 2000; Hamers et al., 2003).

Figure 1 suggests that the development of hearing loss was not delayed in the α–MSH-treated animals. It took these animals 4 to 10 injections of cisplatin. A t-test confirmed this lack of effect of co-treatment (P=0.790).

Maximum threshold shift in dB as a function of frequency at the day cisplatin treatment was stopped is shown in Figure 2 for both co-treatments. The hearing loss occurred in a broad frequency range, although it tended to increase at higher stimulus frequencies. ANOVA showed a significant main effect of frequency ($F_{(3,90)}=33.88$, $P<0.001$), but not of co-treatment ($F_{(1,30)}=0.053$, $P=0.819$). We also analyzed the CM elicited by a 16 kHz tone (data not shown). Similar to the observed CAP threshold shifts, CM threshold shifts (3 μV iso-response levels) were equal in the two groups (ANOVA; $F_{(1,30)}=1.338$, $P=0.256$).

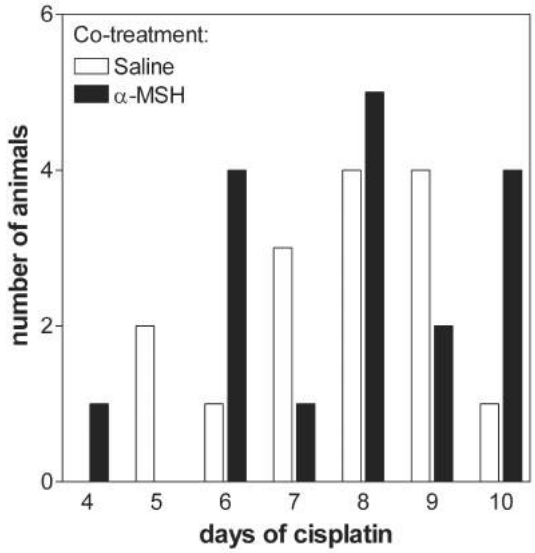

Figure 1: The time necessary to evoke a hearing loss of ≥ 40 dB in the 3 μV iso-response level (CAP threshold) at 8 kHz for the saline (white bar) and the 75 μg/kg/day α–MSH (black bar) co-treated animals.

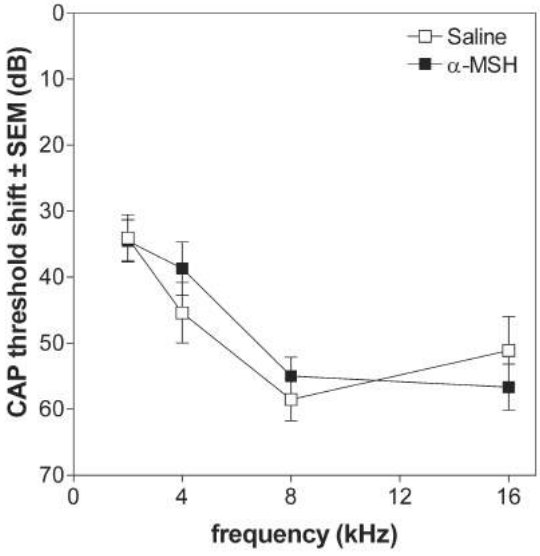

Figure 2: Maximal CAP threshold shift (± SEM) at the day the cisplatin treatment was stopped as a function of frequency for the saline (□) and the 75 μg/kg/day α–MSH (■) co-treated animals.

Effects on EP in relation to recovery time and co-treatment.

Both the saline and the α–MSH co-treated animals were divided into three separate groups in which the EP was measured 1, 2, or 3 days after cessation of the cisplatin treatment. Since the EP measurement is highly invasive, we used each day another group of animals. Figure 3 shows the results of the recording of the EP in the second turn of the cochlea.

For the CM thresholds (data not shown) no recovery was observed within the measured time-period in any of the co-treated groups.

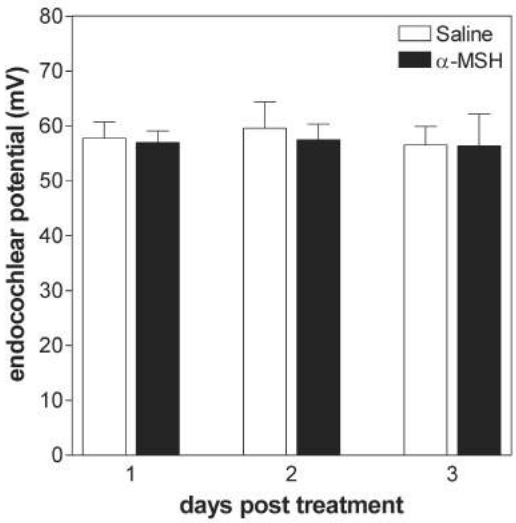

Figure 3: The endocochlear potential (± SEM) plotted at 1, 2 and 3 days following the last dose of cisplatin for the saline (white bar) and the 75 μg/kg/day α–MSH (black bar) co-treated animals.

At day 1 the EP has decreased from its normal value between 75 and 85 mV (Gill and Salt, 1997; Klis et al., 2002; O'Leary and Klis, 2002) to a value of 57.8 ± 2.9 mV (mean ± SEM). No recovery of the EP is observed at day 2 and 3 ($F_{(2,12)}$=0.175, P=0.842). Furthermore, co-treatment with α–MSH did not affect the decrease of EP nor its behavior in time.

Normalized recovery of CAP and CM thresholds in relation to stimulus frequency and co-treatment.

To facilitate comparisons across animals and across frequencies, threshold shifts and recovery were normalized by setting the threshold shift at the day cisplatin treatment was stopped to 0% and baseline values to 100%. Figure 4

shows the percentage recovery of CAP threshold during the first three days of recovery.

On days 2 and 3 after cessation of the cisplatin treatment a slight recovery of the CAP thresholds was observed. ANOVA showed a significant main effect of time (recovery) at 2 kHz ($F_{(2,20)}$=5.25, P=0.015), but no effect of co-treatment ($F_{(1,10)}$=0.165, P=0.693). At the other frequencies no significant recovery was observed. Neither of both co-treatments showed a significant correlation between the EP and the recovery of the CAP threshold at 2 kHz in the individual animals measured at day 2 and 3 (Fig. 5) (saline: r=0.577, P=0.063; α–MSH: r=-0.052, P=0.873).

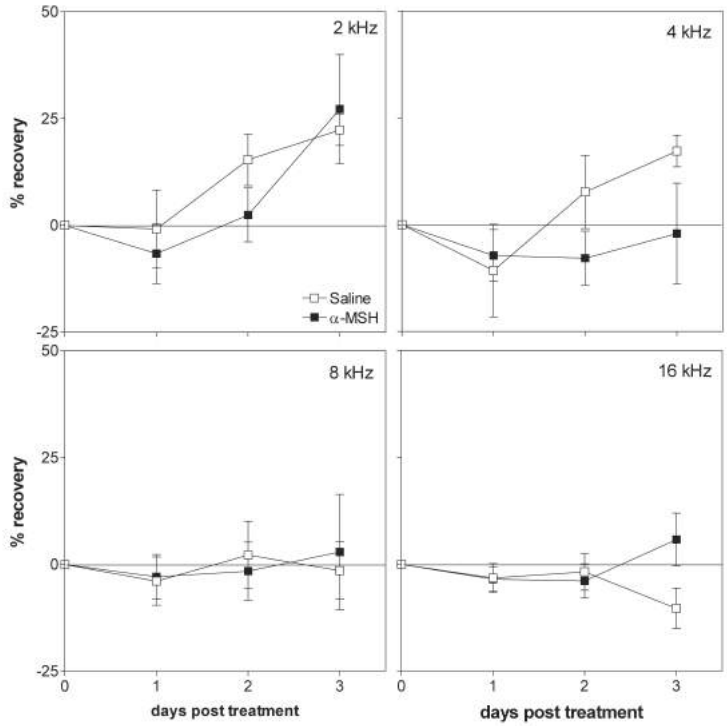

Figure 4: Recovery at 2, 4, 8, and 16 kHz (% ± SEM) of the CAP threshold for the saline (□) and the 75 μg/kg/day α–MSH (■) co-treated animals. Each day a group of animals was withdrawn to undergo EP measurements so the number of animals contributing decreased each subsequent day (*cf.*, Table 1).

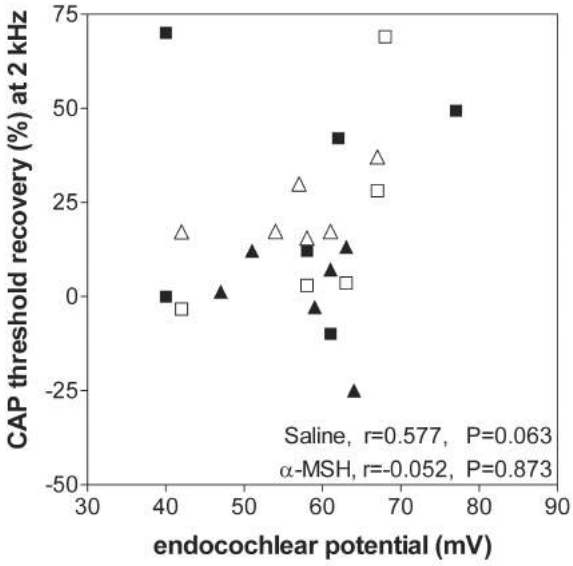

Figure 5: Recovery (%) of the CAP threshold at 2 kHz on day 2 (□) and 3 (△) after cessation of the cisplatin treatment as a function of EP (mV) for the individual saline (white) and the 75 μg/kg/day α–MSH (black) co-treated animals.

Recovery of CAP and CM amplitudes in relation to stimulus frequency and co-treatment

The CAP amplitude at high stimulus levels recovers differently from CAP thresholds in the sense that recovery is usually faster at higher stimulus levels (Klis et al., 2002). Figure 6 shows the (remaining) CAP amplitude, measured at high sound pressures (between 80-90 dB SPL), on the three days after the criterion threshold loss occurred. In contrast to the recovery of the CAP threshold, no recovery was observed for the CAP amplitude in any frequency in the studied time-window (ANOVA; $F_{(2,18)}=0.604$, $P=0.577$). However, after close inspection of the graphs a t-test was performed for day 3. This test showed a significantly higher CAP amplitude at 2 kHz for the α–MSH co-treated group ($P=0.040$).

The CM amplitude at 16 kHz (data not shown) also decreased dramatically over the course of cisplatin treatment. However, no recovery and no effect of co-treatment were found.

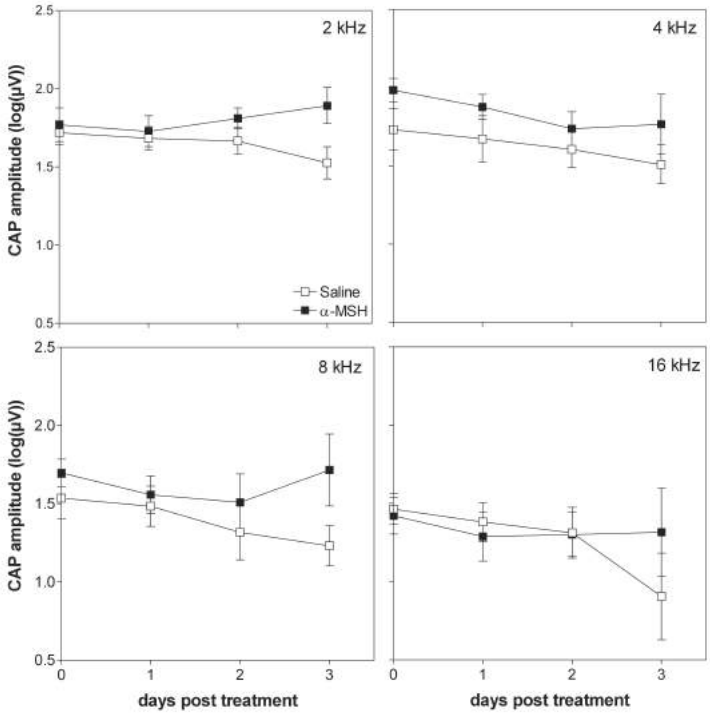

Figure 6: Remaining CAP amplitude (78-86 dB SPL) at 2, 4, 8, and 16 kHz (± SEM) plotted at 0, 1, 2, or 3 days following the last cisplatin treatment for the saline (□) and the 75 μg/kg/day α–MSH (■) co-treated animals. Day 0 represents the day that the cisplatin treatment was stopped. Each day a group of animals was withdrawn to undergo EP measurements so the number of animals contributing decreased each subsequent day (*cf.*, Table 1).

Discussion

The recovery of cochlear sensitivity and its relation to recovery of EP

Previously, several of our animal studies have shown statistically significant recovery of the auditory sensitivity after cisplatin-induced hearing loss (Stengs et al., 1997; Klis et al., 2000, 2002; O'Leary and Klis, 2002; Hamers et al., 2003). Recovery has also been reported in other experimental animal studies (Stadnicki et al., 1975; Nakai et al., 1982) and anecdotically in humans (Aguilar-Markulis et al., 1981; Vermorken et al., 1983; Melamed et al., 1985; Laurell and Jungnelius, 1990). An important issue is how to explain this recovery. Klis et al. (2002) showed that deteriorating CAP thresholds occurred in a broad frequency range, though the OHC loss was limited to the basal turn.

There was also no relation between recovery and OHC loss, *i.e.* OHC counts did not improve in time. So, the above findings suggested that a more global determinant of cochlear sensitivity had to be involved in the recovery of cochlear potentials. Klis et al. (2000, 2002) and O'Leary and Klis (2002) showed a depression of EP at the moment CAP threshold was increased, followed by a recovery of both EP and auditory sensitivity after cessation of the cisplatin treatment. Therefore, it was suggested that recovery of the cochlear sensitivity proceeds together with recovery of the EP up to a point determined by permanent OHC loss (Klis et al., 2002). O'Leary and Klis (2002) and Hamers et al. (2003) also proposed that both strial and hair cell injuries contribute to ototoxicity over a wide range of cisplatin doses, and that the effects upon both are temporally related. The present (preliminary) study suggests that cisplatin ototoxicity and subsequent recovery might be based on more complex processes. Our study concentrated on the recovery of the auditory thresholds within the first three days after cessation of the cisplatin treatment. In this time-window we only observed significant recovery of the CAP threshold at 2 kHz. However, in contrast to the other studies, in which also recovery of the EP was observed, we found a stable, but still decreased, EP value within this time-window. This observation seems to contradict the hypothesis that CAP threshold recovery and EP recovery are related. However, also in contrast with the previous studies, which used longer observation periods, recovery of CAP thresholds is only observed at 2 kHz and even at that frequency recovery is small. Furthermore, no recovery is observed in the CAP amplitudes at high stimulus levels. Since recovery was only observed occasionally in CAP threshold, CAP amplitude and EP, it was almost impossible to establish a correlation between the recovery of CAP and EP.

In other words, we may have missed an essential part of the recovery process by limiting our time-window to 3 days after cessation of cisplatin. Nevertheless, we cannot rule out a mechanism other than EP recovery that might contribute to recovery of cochlear sensitivity. Klis et al. (2002) have demonstrated that the OHCs do not contribute to the threshold recovery. Of course sub-cellular damage and subsequent repair of OHCs cannot be ruled out. Further, a morphological study by Cardinaal et al. (2000b) showed that cisplatin induced vacuolation of the spiral ganglion cells besides OHC loss and strial dysfunction. Another morphological study (van Ruijven et al., personal communication) showed severe shrinkage of spiral ganglion cells and a CAP threshold shift of 30-40 dB at 8 kHz after cisplatin treatment, even before any OHC loss was observed. Together these results suggest that recovery of the spiral ganglion cell function might be related to recovery of the CAP threshold

and CAP amplitude within the first days after cessation of cisplatin. Further research into the mechanism(s) of recovery, particularly expanding the time-window of analysis, will be necessary to settle this matter.

α–MSH treatment in relation to recovery of EP, CAP threshold and CAP amplitude.

In previous studies it was shown that α–MSH is not only able to postpone the ototoxic effects of cisplatin (Heijmen et al., 1999), but also to enhance recovery. A recent study performed by Hamers et al. (2003) showed that α–MSH provided partial protection against most of the adverse effects of cisplatin; OHC survival, recovery of the CAP threshold and recovery of the CAP amplitude at high sound pressures were significantly enhanced. Significant recovery of the CAP threshold at 8 kHz already revealed itself within two days after cessation of the cisplatin treatment, which is remarkably different from the situation in the present study. The recovery of the CAP amplitude at high sound pressures, observed especially in the low frequencies, was already enhanced by α–MSH three days after the end of the cisplatin administration similar to what was found here. Hamers et al. (2003) suggested that α–MSH enhanced recovery by affecting the ability of the stria vascularis to withstand the toxicity of cisplatin and therefore for the EP to recover faster. Since EP was only measured directly after cessation of the cisplatin administration or 28 days thereafter this supposedly faster recovery of EP as a result of α–MSH co-treatment could not be characterized. The results from the present study showed a significant recovery of the CAP threshold at 2 kHz within the first three days after cessation of cisplatin, but no ameliorating effect of α–MSH. Also EP did not show an enhanced recovery as result of the α–MSH treatment. We might conclude, as before, that the time-window in this experiment was too narrow to find α–MSH-enhanced recovery.

Furthermore, as concluded before when we discussed the relation between CAP recovery and EP recovery, we must conclude that the situation might be more complex than previously thought and that α–MSH might work also at levels other than the stria vascularis. Given the histological evidence for ganglion cell disruption (Cardinaal et al., 2000b; Hamers et al., 2003) we suggest that α–MSH might work at this level (too), similar to its action in peripheral sensory neuropathy in experimental animals (Bär et al., 1993; Windebank et al., 1994). The small recovery we found on day 3 in the CAP amplitude at 2 kHz of the α–MSH treated animals, in absence of any effect on the EP, might be due to these α–MSH effects on the eight nerve.

Acknowledgements

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Chapter 5

Systemic co-treatment with α–melanocyte stimulating hormone delays hearing loss caused by local cisplatin administration in guinea pigs

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Summary

It has previously been demonstrated that ototoxicity induced by systemic administration of cisplatin is reduced by concomitant administration of melanocortins, like α–melanocyte stimulating hormone (α–MSH). However, these experiments were hampered by large interanimal variability. Therefore, we re-investigated the effects of systemically administered α–MSH during local (intracochlear) administration of cisplatin. Guinea pigs, implanted with a round window electrode, allowing daily monitoring of the compound action potentials (CAP), and a mini-osmotic pump, pumping either 0.5 μl/h physiological saline or cisplatin solution (15 μg/ml), were co-treated daily with a subcutaneous bolus injection of either α–MSH (75 μg/kg) or physiological saline for one week or until the electrocochleogram showed a persistent decrease in CAP amplitude (40 dB threshold shift at 8 kHz). Next, the animals were sacrificed and the cochleas were processed for histology. After 2 to 3 days, cisplatin alone caused a threshold shift at all frequencies (2-16 kHz). Co-administration with α–MSH consistently delayed the criterion threshold shift by 1 day. When the 40 dB criterion had been reached, similar OHC losses in both the cisplatin/α–MSH and cisplatin/saline treated groups were observed.

This experiment confirms that direct administration of cisplatin into the cochlea results in considerably less interanimal variability than systemic administration and that co-treatment with α–MSH delays cisplatin ototoxicity. Since cisplatin was delivered directly to the cochlea, the ameliorating effect of α–MSH probably involves a cochlear target.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum (II)) is a widely used antineoplastic drug, effective in the treatment of solid epithelial tumors. Unfortunately, the therapeutic efficacy of cisplatin is limited because of its side effects, which include nephrotoxicity, peripheral neuropathy, and ototoxicity. In humans, the ototoxic effect usually presents itself as a high-frequency hearing loss accompanied by tinnitus (Schweitzer, 1993). Animal studies have shown that chronic cisplatin administration leads to loss of outer hair cells (OHCs), with those in the basal turn more severely affected than the OHCs in the medial and apical turn. At high doses or prolonged exposure, loss of inner hair cells (IHCs) has also been observed (Tange, 1984; Hoeve et al., 1988; Saito and Aran, 1994b; Cardinaal et al., 2000a). The ototoxic effects of cisplatin are not limited to the auditory hair cells; also the stria vascularis and spiral ganglion cells are affected (Tange and Vuzevski, 1984; Kohn et al., 1988; Zheng and Gao, 1996; Kohn et al., 1997; Meech et al., 1998; Cardinaal et al., 2000a,b).

During the past decade, a variety of compounds have been investigated in order to prevent or reduce the ototoxic effect of cisplatin without affecting its antineoplastic action. Compounds that are known for their nephro-, and neuroprotective actions, when administered together with cisplatin, also protect the inner ear from cisplatin toxicity. A well-investigated class of compounds are the anti-oxidants (Rybak et al., 1995, 1999a; Reser et al., 1999; Campbell et al., 1999; Li et al., 2001, 2002). The use of anti-oxidants is based upon the assumption that reactive oxygen species, generated in the cochlea after administration of cisplatin, are responsible for the ototoxic damage (Clerici et al., 1996; Kopke et al., 1997). However, excessive amounts of these anti-oxidants are necessary to realize a protective effect against cisplatin ototoxicity and most of these drugs probably bind directly to cisplatin, potentially inhibiting its anti-tumor action. Ekborn et al. (2002) showed that protection from cisplatin ototoxicity by systemic treatment with the anti-oxidant D-methionine could be explained by a decreased serum concentration of cisplatin.

Another class of compounds that ameliorates cisplatin ototoxicity is the group of melanocortins, such as the AdrenoCorticoTropic Hormone $(ACTH)_{(4\text{-}9)}$ -analog ORG 2766 and α–melanocyte stimulating hormone (α–MSH) (Hamers et al., 1994; De Groot et al., 1997; Stengs et al., 1998b; Heijmen et al., 1999; Smoorenburg et al., 1999; Cardinaal et al., 2000c). With both peptides, administered separately, either complete protection from cisplatin ototoxicity (normal electrophysiological auditory thresholds and OHC counts) was observed or thresholds were increased similar to those in saline co-treated animals (Hamers et al., 1994; Stengs et al., 1998b; Heijmen et al., 1999). Thus, a clear

dichotomy was found in the co-treated animals. Furthermore, a large inter-animal variability was found in the animals treated with cisplatin alone, hampering an effective comparison with those co-treated with ORG 2766 or α–MSH (Heijmen et al., 1999). Therefore, in the present study, a longitudinal model was used to study cisplatin ototoxicity. This model involved intra-cochlear administration of cisplatin into the scala tympani via a mini-osmotic pump system, while the protective compound (*i.e.*, α–MSH) was administered through a systemic route. Daily recordings of the compound action potential (CAP) were made from a permanent round window electrode.

It has been demonstrated that the use of this longitudinal model with local cisplatin treatment results in less interanimal variability (O'Leary et al., 2001) than observed with systemic application of cisplatin (Stengs et al., 1998b; Heijmen et al., 1999; Smoorenburg et al., 1999), thus allowing a better evaluation of possibly protective compounds. Another advantage of this approach is that if a protective effect is found, it cannot be due to accelerated clearance of cisplatin from the body resulting in a decrease in the amount of cisplatin reaching the cochlea (Ekborn et al., 2002).

Materials and Methods

Animals and experimental design

Thirty female albino guinea pigs (strain: Dunkin-Hartley, Harlan Laboratories, Indianapolis, IN, USA; weight 225-325 g) were implanted with a permanent round window electrode and a mini-osmotic pump containing either cisplatin solution or physiological saline (0.9% NaCl). The animals also received a daily injection of co-treatment, starting on the day of surgery, consisting of a subcutaneous injection of either α–MSH or equal volumes of physiological saline (*cf.*, Table 1). The animals were housed individually in micro-isolator cages and had free access to food and water. They were maintained on a 12:12 h dark/light cycle. Continuous intracochlear perfusion of both cisplatin and physiological saline continued for one week or less if electrocochleograms showed ≥ 40 dB suppression of the CAP threshold at 8 kHz stimulation. When the threshold shift was reached or when one week had passed, the animals were sacrificed and the ears were fixed and processed for histology.

The University of Michigan is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The animal protocol for this study was approved by the University Committee on Use and Care of Animals of the University of Michigan.

Preparation of cisplatin and α–MSH solutions

A stock cisplatin solution (American Pharmaceutical Partners Inc., Los Angeles, CA, USA) with a concentration of 1 mg/ml was diluted with physiological saline to a final concentration of 15 μg/ml. This concentration was chosen after interpolation of data obtained by O'Leary et al. (2001). These data suggested that after intracochlear application of 15 μg/ml cisplatin (pump rate 0.5 μl/h; 0.18 μg/day) it would take approximately 4 days to reach the criterion of 40 dB threshold shift at 8 kHz. It was assumed that within this time-window differences would become apparent between both co-treatments. The Alzet mini-osmotic pump (model #2002, Alza Corp., Palo Alto, CA, USA) was filled with either the cisplatin solution or physiological saline and placed in a saline bath (37°C) for 4 hours or more, using a sterile container. The cannula (length: 7.4 cm) was filled with 29 μl physiological saline. Given the cannula length and the flow rate of the pump, we calculated that it would take approximately 58 h after surgery before the saline in the cannula was completely replaced by cisplatin from the pump and, hence, for cisplatin to enter the cochlea. α–MSH (Sigma Chemical Co., St Louis, MO, USA) was dissolved in physiological saline at a final concentration of 60 μg/ml and administered subcutaneously, always in the morning, at a daily dose of 75 μg/kg. The first injection was given immediately after the surgery. The α–MSH dose of 75 μg/kg/day was based on experiments with a positive outcome performed previously by Heijmen et al. (1999).

Surgical techniques

Surgical implantation of the mini-osmotic pump and further techniques were performed according to the method described by Prieskorn and Miller (2000). Prior to the operation the animals were injected with chloramphenicol sodium succinate (30 mg/kg i.m.) and then anaesthetized with 40 mg/kg ketamine and 10 mg/kg xylazine (i.m.). Local anaesthetic (1% lidocaine, 0.75 ml) was used in areas to be incised and over the midline of the back (where the pump-pocket would be located). A mid-line incision was made on the surface of the head starting 2.5 cm anterior of bregma and continued post-auricularly to the base of the pinna. A superficial subcutaneous pocket was made in the back between the scapulae of the animal to accommodate the pump. Two stainless-steel screws were placed on the skull, the first 1 cm posterior to bregma to anchor the cannula and the other 2 cm anterior of bregma, which served as the ground electrode for the CAP measurements. Under sterile conditions the bulla of the left ear was opened. The round window electrode was made of insulated stainless-steel wire (diameter 0.175 mm including teflon insulation; Advent, Halesworth, UK) with a 0.5 mm diameter gold ball micro-welded (Unitek 80F; Unitek Equipment, Monrovia, CA, USA) to the exposed and

flattened tip. The wire was soldered to a connector (Samtec, New Albany, IN, USA), which was attached to the skull with methyl-methacrylate cement (Lang Jet Acrylic, Lang Dental MFG Co., Wheeling, IL, USA). The electrode was positioned on the round window and secured to the bulla with carboxylate cement (Durelon, ESPE Premier Sales Corp., Norristown, PA, USA). A small hole was made at the base of the cochlea, approximately 0.5 mm below the round window. The cannula tip was placed into the hole until a silicone ball, 0.5 mm from the tip, was seated against the cochlea, to prevent leakage. Carboxylate cement was used to cover the bulla defect and to secure the cannula in place. The pump was removed from the water-bath and attached to the cannula. Subsequently, the pump was placed in the subcutaneous pocket, the cannula was fixed to the skull, and the subcuticular layer was closed with a continuous absorbable suture; the skin was closed with nylon using interrupted sutures. The animals recovered overnight from surgery under a heating lamp.

Electrocochleography

Measurements were performed single-endedly with the round window electrode as the active electrode and the screw on the skull (2 cm anterior of bregma) as the ground electrode. Animals were excluded that did not have a normal threshold at 8 kHz (defined as a threshold at less than 30 dB SPL stimulus level) at the day after surgery or had a displaced cannula or signs of otitis media at sacrifice. Stimulus generation and data acquisition were performed with a TDT-II system (Tucker Davis, Gainesville, FL, USA) under control of custom-designed software. Trains of tone bursts of 2, 4, 8, and 16 kHz were used as stimuli. The tone bursts were constructed with cosine-shaped rise-and-fall times of 1 ms (1.5 ms at 2 kHz) and had a duration of 8 ms. The sound stimuli were produced in an open-field configuration with a speaker (Model GTO302, JBL, Woodbury, NY, USA), positioned at 10 cm from the pinna, driven by a Parasound Zamp/Zone amplifier (Parasound Products, San Francisco, CA, USA). Consecutive tone bursts were presented with alternating polarity at 99 ms. The responses were amplified (TDT DB4 bioamp system with HS4 head stage; Tucker Davis, Gainesville, FL, USA) at 300 Hz to 3 kHz bandwidth, AD converted, averaged and stored on disk for off-line analysis. The animals were lightly restrained, but remained awake during measurements, avoiding the disadvantages of repeatedly anaesthetizing the animals. The CAP (N1), measured relative to the P1 (first positive peak in the signal), was obtained by adding the responses evoked by tone bursts of opposite polarity. Cochlear Microphonics (CM) at 2 kHz were obtained by subtracting these responses. ANOVA was performed with co-treatment as a between-subject factor and frequency and level of stimulation as within-subject factors.

Histological techniques

One week after surgery or on the day the animals showed a 40 dB threshold shift at 8 kHz, the animal was deeply anaesthetised, temporal bones were removed, and the cochleas were fixed by intralabyrinthine perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) followed by immersion in the same fixative for 3 h at 4°C. After several rinses (2 x 15 min.) in 0.1 M sodium cacodylate buffer, the cochleas were stored in buffer at 4°C. The cochleas were further processed according to the routine method for guinea pigs (De Groot et al., 1987). Semi-thin (1 μm) midmodiolar sections were cut and stained with 1% methylene blue and 1% azur II in 1% sodiumtetraborate. In the midmodiolar sections of the left cochleas the organ of Corti was examined at 7 locations, separated by a half turn spacing. OHC loss was expressed as the percentage of remaining OHCs per cross sectioned half turn, relative to the expected 3 OHCs per transection. All OHC counts were performed by two investigators, independently of one another, in a blinded fashion (*cf.*, De Groot et al., 1997; Cardinaal et al., 2000a; Cappaert et al., 2001). ANOVA was used for statistical evaluation of the OHC counts. Co-treatment was a between-subjects factor and cochlear location a within-subject factor.

Results

General findings

Seven out of 30 animals were excluded from the study because they did not have a normal threshold at 8 kHz the day after surgery or showed a displaced cannula or signs of otitis media upon inspection of the left (operated) bulla when the animals were sacrificed. Two animals that developed the 40 dB criterion threshold shift at 8 kHz within the first 3 days after surgery were also excluded, since we assume that in those cases hearing loss was due to post-operative complications (arrival time of cisplatin was estimated at 58 h post-surgery). The remaining 21 animals were used in the experiment. Table 1 shows their distribution among the four experimental groups.

CAP thresholds as a function of time

Figure 1 shows CAP thresholds at 8 kHz, based on a 3 μV criterion, of all 21 individual animals as a function of time.

Table 1: Distribution of the animals over the experimental groups

Pump filling	Co-treatment (s.c. injection) Physiological saline	α–MSH
Cisplatin 15 μg/ml	7	8
Physiological saline	2	4

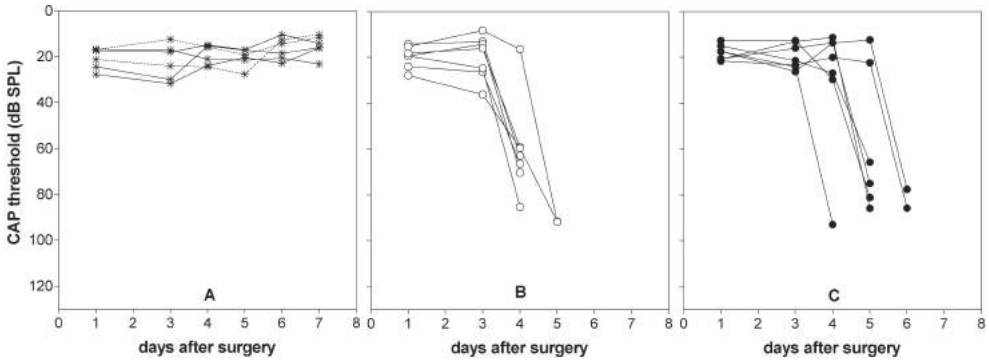

Figure 1: Development of CAP thresholds at 8 kHz stimulation during one week after the implantation of an osmotic pump (Alzet 2002; 0.5 μl/hr) at day 0. Each curve represents one individual animal. A: The pump was loaded with physiological saline. Co-treatment consisted of systemic physiological saline injections (interrupted lines) or α–MSH injections (75 μg/kg/day; continuous lines); B: The pump was loaded with cisplatin (15 μg/ml). Co-treatment consisted of physiological saline injections; C: The pump was loaded with cisplatin (15 μg/ml). Co-treatment consisted of α–MSH injections (75 μg/kg/day).

Figure 1A represents the animals that were treated with physiological saline in the pump (n=6). For this group, regardless of co-treatment (physiological saline, n=2 or α–MSH, n=4) the animals showed a stable CAP threshold during the observation period, which lasted 7 days. No significant differences were found between the two co-treatments in these animals at any frequency ($F_{(1,3)}$=0.65, P=0.48). Therefore the animals treated with saline in the pump were considered as one group (control group). Animals treated with 15 μg/ml cisplatin in the pump and physiological saline co-treatment (n=7) (Fig. 1B) showed thresholds equal to the control group during the first three days of treatment. This can be explained by the fact that it took approximately 58 hours for the saline in the cannula to be completely replaced by the cisplatin from the pump, which gave the animals time to recover from surgery.

Between day 3 and 4 after surgery a rapid deterioration of the threshold developed. The group of animals that received 15 μg/ml cisplatin in the pump and α–MSH as co-treatment (n=8) (Fig. 1C) also showed a stable baseline during the first three days after surgery followed by a rapid deterioration of the threshold between day 4 and 5 after surgery.

Number of days to rapid threshold deterioration

Cochlear susceptibility to perilymphatic administration of cisplatin can be expressed in terms of the number of days necessary to reach an ototoxic effect. Figure 2 shows the time necessary to reach a threshold shift of ≥ 40 dB at 8 kHz.

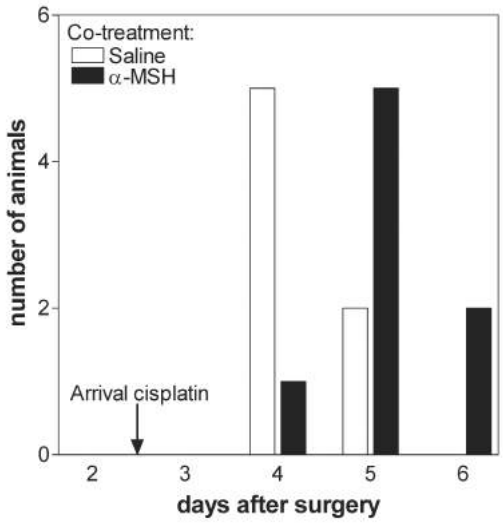

Figure 2: Number of animals reaching the CAP criterion threshold shift (≥ 40 dB at 8 kHz) at a given day. The arrow points to the calculated moment cisplatin from the pump enters the cochlea.

Since cisplatin arrived in the cochlea approximately 58 hours after surgery, this corresponds to 45 hours of intracochlear cisplatin infusion. For those animals co-treated with cisplatin/α–MSH it took significantly longer to reach the criterion threshold shift (t-test; P=0.017), on average 123 hours (5-6 days) after surgery, corresponding to 65 hours of cisplatin infusion. Thus, α–MSH co-treatment delayed the ototoxic effect of cisplatin by approximately 20 hours (44%).

CAP threshold shift as a function of frequency

The first animals reached the criterion threshold shift on the fourth day after surgery (Fig. 1). This was the last day a CAP measurement for all 21 animals was available. Figure 3 shows the CAP threshold in dB SPL as a function of frequency on day 4 for the three groups (cisplatin/α–MSH n=8; cisplatin/saline n=7; control (pooling the two groups that had physiological saline in the pump) n=6).

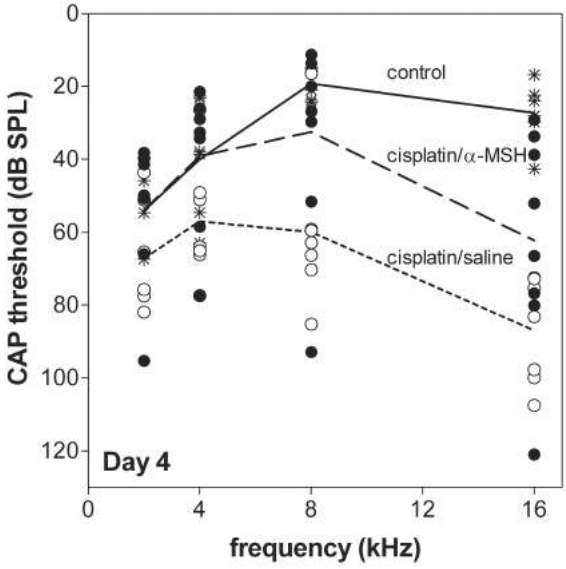

Figure 3: CAP threshold at 8 kHz (3 μV criterion) as a function of frequency at day 4 after the implantation of a mini-osmotic pump in the three groups of animals. Pumps were loaded with physiological saline (continuous line; saline/saline and saline/α–MSH treated groups were pooled) or cisplatin 15 μg/ml in combination with saline s.c. injections (dotted line) or α–MSH s.c. injections (interrupted line). The individual data are also shown; asterisk: pooled saline in pump groups; open circle: cisplatin/saline treated group; filled circle: cisplatin/α–MSH treated group.

On average, the threshold shift in the cisplatin/saline group is much larger than the shift in the cisplatin/α–MSH group, regardless of frequency. The animals in the cisplatin/α–MSH group show a similar CAP threshold in the low frequencies as compared to the control animals, although a threshold shift is apparent at the higher frequencies (8 and 16 kHz). Individual data are also depicted to illustrate the variation within the three groups. ANOVA showed a significant main effect of frequency ($F_{(2,18)}=22.43$, $P<0.001$) and treatment ($F_{(3,54)}=6.29$, $P=0.008$) and an interaction between both factors ($F_{(6,54)}=8.27$, $P<0.001$).

Post-hoc analysis (Tukey HSD) on treatment showed a significant difference between the cisplatin/saline group and the control group ($P=0.01$) for all frequencies, but not between the cisplatin/α–MSH group and the control group ($P=0.47$). The difference between the cisplatin/saline and cisplatin/α–MSH groups did not quite reach significance ($P=0.08$), which did not change when the analysis was done for each frequency separately.

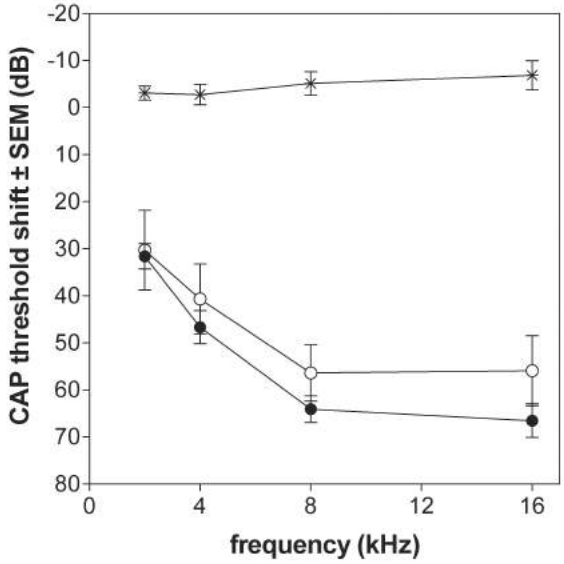

Figure 4: CAP threshold shift ± SEM at the time criterion threshold shift for 8 kHz was exceeded (≥ 40 dB) or after 7 days if this criterion was not reached (exclusively animals with saline in pump). Asterisk: pooled saline in pump groups; open circle: cisplatin/saline treated group; filled circle: cisplatin/α–MSH treated group.

Figure 4 shows the threshold shift in dB as a function of frequency on the day the animals were sacrificed, *i.e.*, on the day they passed criterion threshold shift (40 dB) at 8 kHz, or after 7 days if they did not pass this criterion (control animals only). The threshold shift was calculated for each animal separately, by subtracting the threshold measured on the last day from the threshold measured the day after surgery.

No threshold shift was observed in animals treated with physiological saline in the pump (*cf.*, Fig. 1A). The animals that received cisplatin in the pump displayed a threshold shift at all frequencies, although the higher frequencies were clearly most affected. ANOVA, comparing the cisplatin/saline and the cisplatin/α–MSH groups, showed a significant main effect of frequency ($F_{(3,39)}=17.55$, $P<0.001$), but no significant effect of co-treatment ($F_{(1,13)}=1.78$,

P=0.20). From one respect these data are partially trivial, because we used a criterion loss of ≥ 40 dB at 8 kHz to determine the day at which these measurements were made. Nevertheless, these data demonstrate that, regardless of co-treatment, hearing loss occurs over a broad frequency range.

CAP input-output curves

Figure 5 shows input-output curves for the CAP at 8 kHz stimulation on day 4 (the last day all 21 animals were still in the experiment). There is a large difference in CAP amplitudes between the control group (pooled) and the cisplatin/α–MSH group on the one hand and the cisplatin/saline group on the other hand, especially at low levels of stimulation.

ANOVA was performed on the logarithmically transformed CAP data to improve homogeneity of variance. Only the moderate-level stimuli (45-85 dB SPL) were tested in order to avoid floor effects and because of the recruitment-like shape of the input-output curves. Statistical analysis confirmed an effect of stimulus level (trivial) and treatment ($F_{(2, 18)}=2.72$, $P=0.006$) and a strong interaction between both factors ($F_{(8, 72)}=9.03$, $P<0.001$). A separate analysis of the difference between the cisplatin/α–MSH treated animals and the cisplatin/saline treated animals revealed a pronounced effect of α–MSH co-treatment ($F_{(1, 13)}=5.97$, $P=0.03$).

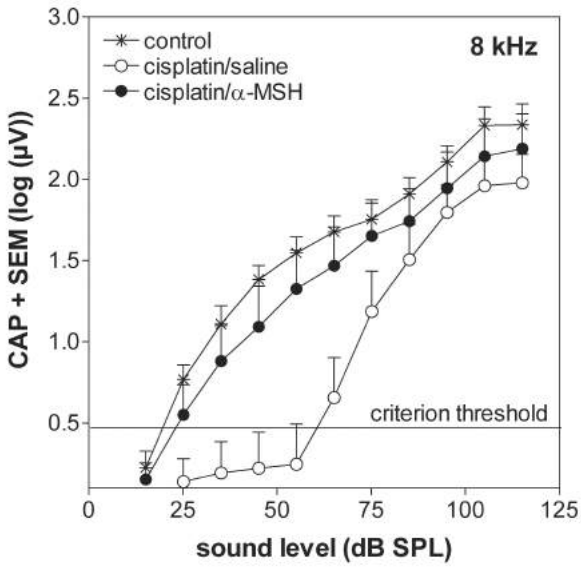

Figure 5: CAP growth curves at 8 kHz stimulation as a function of sound level obtained on day 4 after pump implantation. Asterisk: pooled saline in pump groups; open circle: cisplatin/saline treated group; filled circle: cisplatin/α–MSH treated group. Error bars represent SEM.

CM

A correlation was found between shifts in CAP threshold at 16 kHz and shifts in CM threshold at 2 kHz (r=0.80), also defined as the 3 μV iso-response level. Figure 6 illustrates that this correlation is not influenced by α–MSH co-treatment. The cisplatin/α–MSH treated animals required, on average, about 1 day of cisplatin treatment extra, but reached the same shift as the cisplatin/saline treated animals, both with regard to the CAP threshold shift as well as the CM threshold shift.

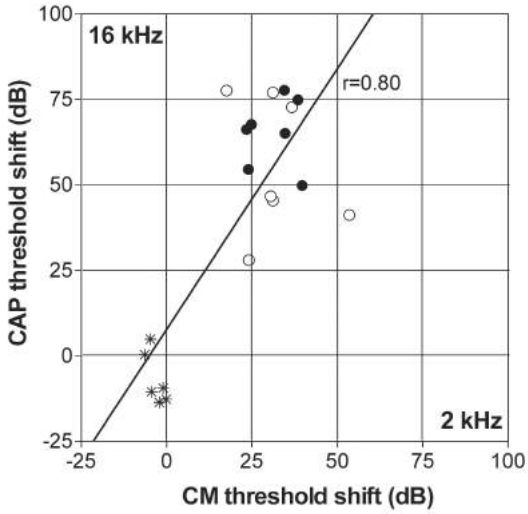

Figure 6: CAP threshold shifts at 16 kHz compared to CM threshold shifts at 2 kHz. Both shifts are expressed relative to their respective thresholds at day 0, when the pump was implanted. Each data-point represents the threshold shifts for one animal at the last day of treatment. Asterisk: pooled saline in pump groups; open circle: cisplatin/saline treated group; filled circle: cisplatin/α–MSH treated group.

OHC counts

Outer hair cells counts (*i.e.*, the percentage of remaining OHCs) of the three groups of animals are presented in Figure 7. The degree of OHC loss is presented for 7 different transections (at a half-turn spacing) along the basilar membrane. The control group, which was treated with physiological saline in the pump, showed almost no OHC loss. In animals treated with cisplatin in the pump both co-treatments (physiological saline and α–MSH) showed most pronounced OHC loss in the basal turn, 51% remaining OHCs versus 93% in the medial and 97% in the apical turn. Statistical analysis by means of ANOVA showed main effects of treatment ($F_{(2,18)}=6.75$, $P=0.007$) and location

($F_{(6,108)}$=33.29, P<0.001) and a significant interaction between both factors ($F_{(12,108)}$=5.9, P<0.001). Thus, an analysis of the effects of co-treatment was performed for each relevant transection separately. A significant effect of treatment (= less OHC loss) was found only in b1, the lower basal turn ($F_{(2,18)}$=16.19, P<0.001). Post-hoc analysis (Tukey HSD) on treatment showed that both cisplatin/saline and cisplatin/α–MSH treated groups were significantly different from the control group (α–MSH co-treatment, P<0.001; saline co-treatment, P<0.001). No significant difference was found between both co-treated groups (P=0.89). Thus, α–MSH co-treatment does delay, but not prevent the loss of OHCs caused by cisplatin treatment. Inner hair cell loss was not seen in this study.

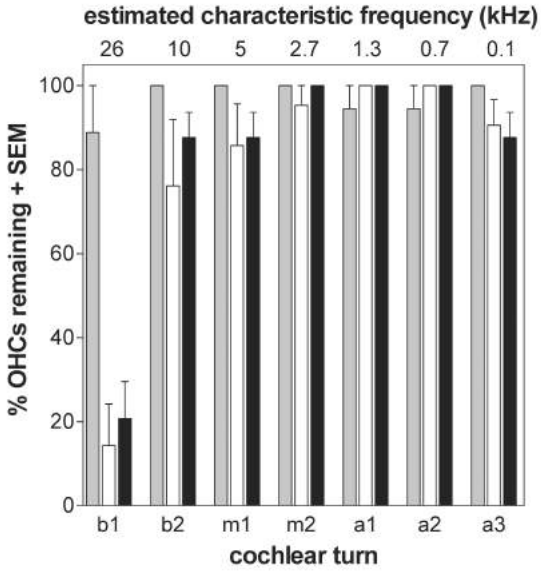

Figure 7: Outer hair cell counts (represented as percentage of remaining OHCs) as a function of cochlear position along the basilar membrane (b1, b2: basal turns; m1, m2: middle turns; a1, a2, a3: apical turns). Hatched bar: pooled saline in pump groups; open bar: cisplatin/saline treated group; filled bar: cisplatin/α–MSH treated group. The estimated characteristic frequencies (c.f.) according to the Greenwood (1996) frequency map are indicated above the graph. Error bars represent SEM.

Discussion

Perilymphatic cisplatin exerts an ototoxic effect

This study shows that intracochlear cisplatin application with a mini-osmotic pump (0.18 μg/day; 15 μg/ml in pump) results in loss of auditory sensitivity after 2-3 days of continuous perilymphatic infusion (Figs. 1-2). In comparison, O'Leary et al. (2001) demonstrated that local administration of cisplatin in the perilymph at a somewhat higher dose (0.36 μg/day; 30 μg/ml in pump) also resulted in a loss of auditory sensitivity after 2-3 days of continuous application with the same mini-osmotic pump system. They also found that at a lower concentration (0.036 μg/day; 3 μg/ml in pump) borderline loss of sensitivity occurs, whereas profound hearing loss is observed within 1 day in most of the animals treated with a higher concentration (3.60 μg/day; 300 μg/ml in pump). Our results are in general agreement with the results of O'Leary et al. (2001), although the concentration used (15 μg/ml in pump) did not result in the longer symptoms-free interval we desired. Our results also confirm that direct administration of cisplatin into the cochlea results in considerably less interanimal variability than systemic administration (*cf.*, Heijmen et al., 1999; Klis et al., 2000, 2002). This feature, together with the avoidance of systemic cisplatin toxicity, makes this animal model ideally suited for studies on potentially protective compounds.

α–MSH delays cisplatin ototoxicity

The most salient result in this study is that systemic co-treatment with α–MSH significantly alters the course of effects during perilymphatic application of cisplatin; co-treatment delays, but does not prevent, cisplatin ototoxicity. Thus, at the chosen endpoint (40 dB threshold shift at 8 kHz stimulation) similar frequency-dependent threshold shifts and position-dependent OHC losses were observed in both cisplatin/α–MSH and cisplatin/saline groups, but the threshold loss was found at a later time in the cisplatin/α–MSH treated group. Several recent studies, both morphologically and electrophysiologically, have shown that α–MSH reduces cisplatin ototoxicity. Heijmen et al. (1999) have demonstrated that treatment of albino guinea pigs with daily injections of cisplatin (2 mg/kg/day i.p. for 8 days) and concomitant injections of α–MSH (75 μg/kg/day s.c. for 9 days) resulted in a considerable number of animals with preserved hearing after cessation of cisplatin treatment. This was not found in the cisplatin/saline treated group. Hamers et al. (personal communication) have confirmed that α–MSH significantly enhances recovery of CAP thresholds and OHC survival after systemic cisplatin treatment. However, in the latter experiment the number of days necessary to reach criterion threshold shift (≥ 40 dB

at 8 kHz) did not differ in a statistically significant way between the cisplatin/α–MSH treated group and the cisplatin alone group. This discrepancy with our experiment might be explained by the higher interanimal variability, which is usually associated with systemic application of cisplatin.
An important issue is the mechanism of protection by α–MSH, which cannot be considered separately from the mechanism of cisplatin ototoxicity itself. Morphological effects of cisplatin upon OHCs (Komune et al., 1981; De Groot et al., 1997; Cardinaal et al., 2000a, 2000b), spiral ganglion cells (Cardinaal et al., 2000b; Zheng and Gao, 1996) and the stria vascularis (Nakai et al, 1982; Tange and Vuzevski, 1984; Kohn et al., 1988, 1997; Meech et al., 1998; Cardinaal et al., 2000a) have been described, but it is not known whether these effects are independent or causally related. Several recent studies emphasize that cisplatin affects the stria vascularis, both morphologically (Meech et al., 1998; Cardinaal et al., 2000a) and functionally, as reflected by a decrease in the endocochlear potential (EP), which is generated by the stria vascularis (Klis et al., 2000; Tsukasaki et al., 2000; O'Leary et al., 2001; Klis et al., 2002). One might surmise that damage to the stria vascularis impairs the maintenance of the EP, which leads to increases in the CAP and CM thresholds. The intermediate cells in the stria vascularis, which are actually melanocytes (Hilding and Ginzberg, 1977) are necessary for normal strial function (Motohashi et al., 1994). Meyer zum Gottesberge (2000) has suggested that melanocytes in the cochlea are under α–MSH control. The ameliorating effects of α–MSH on cisplatin ototoxicity might be linked to the activity of strial melanocytes. Although the possibility of direct involvement of strial melanocytes needs to be investigated further, it is nevertheless clear from our experiments that α–MSH seems to act through a cochlear target, as cisplatin was delivered directly to the cochlea. In other words, systemic effects of α–MSH on cisplatin pharmacodynamics, or other absorption- or transport-factors can be excluded by our approach.

Conclusion

The present study confirms the previously reported results with regard to ototoxic effects of cisplatin after local administration into the perilymphatic compartment. The ototoxic effects were significantly delayed when the animals were co-treated with systemically administered α–MSH. This delay (an increase of 44% in time to toxicity) might be of clinical significance. Since cisplatin was delivered directly to the cochlea, the ameliorating effect of α–MSH probably involves a cochlear target, possibly the strial melanocytes. Thus, the

α–MSH peptide remains interesting. Further research with this peptide could reveal matters relating to the mechanism of cisplatin ototoxicity and the regulation of strial activity, as well as an effective clinical strategy to attenuate cisplatin ototoxicity.

Acknowledgements

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Chapter 6

Perilymphatic application of α–melanocyte stimulating hormone ameliorates hearing loss caused by systemic administration of cisplatin

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Summary

It has previously been demonstrated that ototoxicity induced by systemic administration of cisplatin is reduced by concomitant systemic administration of α–melanocyte stimulating hormone (α–MSH). In this study we investigated the effects of cochlear, perilymphatic application of α–MSH during intraperitoneal administration of cisplatin. Guinea pigs, implanted with a round window electrode, allowing daily monitoring of the compound action potential (CAP), and a mini-osmotic pump, pumping at a rate of 0.25 μl/h either physiological saline or α–MSH solution (0.02, 2, 20 μg/ml), were treated daily with a bolus injection of cisplatin (2 mg/kg) until the electrocochleogram showed an increase in CAP threshold of 40 dB at 8 kHz. Then, cisplatin treatment was stopped, but intracochlear perfusion of α–MSH or physiological saline was continued for 10 days to evaluate possible effects of α–MSH on the expected recovery. On day 10, the animals were sacrificed and the cochleas were fixed and processed for histological analysis. All groups required an average of 6 to 7 days of cisplatin to reach the criterion CAP threshold shift. Ten days after cessation of the cisplatin treatment, recovery of the CAP was observed in all groups and at all frequencies, although it was more pronounced at the low frequencies. With respect to recovery, small statistically significant differences were found between the saline and the α–MSH co-treated groups. Histological results showed significantly less outer hair cell (OHC) loss in the group co-treated with 2 μg/ml α–MSH as compared to the group co-treated with saline. Thus, since α–MSH was delivered directly into the cochlea, the ameliorating effect of α–MSH on OHC survival is exerted by means of a cochlear target and not through interaction with a systemic factor.

Introduction

Cisplatin is a widely used antineoplastic drug, which is effective in the treatment of different types of epithelial tumors. However, in humans, chronic treatment with cisplatin may result in ototoxic side effects such as high-frequency hearing loss and tinnitus (Schweitzer, 1993). Animal studies have shown that chronic cisplatin administration leads to loss of outer hair cells (OHCs) in the cochlea, with the hair cells in the basal turn being more severely affected than those in the middle and apical turns (Tange, 1984; Hoeve et al., 1988; Saito and Aran, 1994; Cardinaal et al., 2000a). The ototoxic effects of cisplatin are not limited to the auditory hair cells; the stria vascularis (Tange and Vuzevski, 1984; Kohn et al., 1988; Zheng and Gao, 1996; Kohn et al., 1997; Meech et al., 1998; Cardinaal et al., 2000a,b) and spiral ganglion cells (Cardinaal et al., 2000b; Hamers et al., 2003) are also affected. These ototoxic limitations can be (partially) overcome with the application of compounds that are known for their neuroprotective effects in cisplatin-induced sensory neuropathies. A family of compounds that have distinct neuroprotective properties are the melanocortins (Gispen, 1990). Previous work in our laboratory has shown that systemic administration of some of these melanocortins, *e.g.* the AdrenoCorticoTropic Hormone (ACTH)-analog ORG 2766 and the naturally occurring ACTH fragment α–melanocyte stimulating hormone (α–MSH), protect the cochlea from cisplatin-induced damage (Hamers et al., 1994; De Groot et al., 1997; Stengs et al., 1998; Heijmen et al., 1999; Smoorenburg et al., 1999; Cardinaal et al., 2000c; Wolters et al., 2003; Hamers et al., 2003). However, the systemic concomitant administration of ORG 2766 or α–MSH resulted in a clear dichotomy; individual animals were either completely protected from cisplatin ototoxicity with normal auditory thresholds and no OHC loss or demonstrated increased thresholds similar to animals treated with cisplatin alone (Hamers et al., 1994; Stengs et al., 1998; Heijmen et al., 1999). Therefore, further quantitative characterization of the interaction between melanocortin effects and cisplatin-induced ototoxicity was needed. Introduction of a longitudinal animal model allowed us to monitor the cochlear sensitivity and the beneficial effects of α–MSH and ORG 2766, via a permanent round window electrode, on a day-to-day basis (Hamers et al., 2003). Neither of the co-treatments affected the mean number of cisplatin injections necessary to reach the criterion threshold shift (≥ 40 dB at 8 kHz). Nevertheless, both α–MSH and ORG 2766 enhanced recovery of CAP thresholds and CAP amplitudes at high sound pressure levels. Furthermore, hair cell loss was significantly lower in the melanocortin co-treated groups. On the one hand, the beneficial effects of α–MSH and ORG 2766 could be evoked through a target within the inner ear. On

the other hand, systemically applied melanocortins may exert their effects indirectly, *e.g.* by accelerating renal clearance of cisplatin from the body, resulting in decreased amounts of cisplatin that can reach the cochlea. This latter explanation has been found to be the basic mechanism by which the sulphur-containing amino acid D-methionine acts as an otoprotective agent (Ekborn et al., 2002).

The present study was designed to investigate the issue of the primary target for α–MSH. A way to prevent the systemic effect of the protective compound is to apply the protective agent exclusively and directly to the cochlea. To accomplish this, a new longitudinal model was used, which involved administration of α–MSH directly into the scala tympani via a mini-osmotic pump system, while cisplatin was administered intraperitoneally. Daily recordings of the compound action potentials (CAPs) as a function of frequency and level were made from a permanent round window electrode. This study complements a similar study with a mirrored experimental design: cisplatin was applied directly into the scala tympani, whereas α–MSH was administered subcutaneously, in combination with permanent recordings (Wolters et al., 2003). In that study a clear ameliorating effect of α–MSH was found. It should be kept in mind, however, that positive results in this study would not completely rule out a systemic component, but positive results from the combination of the two studies would make that possibility extremely unlikely.

The experimental approach used in the present study has the additional advantage that since cisplatin is administered systemically, treatment can be terminated easily in contrast to an approach in which cisplatin is delivered directly into the cochlea through a mini-osmotic pump system. This allows for a better analysis of the role that α–MSH plays in recovery from cisplatin ototoxicity, especially since there are indications that α–MSH accelerates recovery (Hamers et al., 2003).

Materials and Methods

Animals and experimental design

Fifty-eight female albino guinea pigs (strain: Dunkin-Hartley, Harlan Laboratories, Horst, the Netherlands; weight 275-380 g) were implanted with a permanent round window electrode and equipped with a mini-osmotic pump containing sterile solutions of either α–MSH in saline or plain saline (0.9% NaCl). The pump fed these solutions directly into the scala tympani. The animals were housed, four together, in macrolon cages and had free access to food and water. They were maintained on a 12:12 h dark/light cycle. The ani-

mals were treated with a continuous perilymphatic perfusion of α–MSH or physiological saline, starting directly after surgery, and a daily injection of cisplatin, starting 3 days after surgery, until electrocochleograms showed ≥ 40 dB shift of the threshold at 8 kHz stimulation. When the criterion threshold shift was reached, cisplatin administration was stopped, but perilymphatic perfusion with α–MSH or physiological saline was continued for 10 days to evaluate possible effects of α–MSH on the expected recovery. During this period all animals were daily monitored by means of electrocochleography using the permanent round window electrode. On day 10, the animals were sacrificed and the ears were fixed and processed for histology.
The animals were allotted at random to the different experimental groups.
The care and use of the animals reported in this study were approved by the Animal Care and Use Committee of the University of Utrecht (DEC-UMC # 91035).

Preparation of pump, cisplatin and α–MSH solutions

A stock solution of cisplatin (1 mg/ml; Platosin®; Pharmachemie B.V., Haarlem, the Netherlands) was diluted with physiological saline to a final concentration of 0.1 mg/ml. It was administered intraperitoneally at a daily dose of 2.0 mg/kg body weight. The first injection was given 3 days after surgery. The dose was chosen based upon the previous experiments of Stengs et al. (1998) and Heijmen et al. (1999). The high dilution was chosen to stimulate diuresis and, thus, to minimize renal effects.
α–MSH (Bachem, Bubendorf, Switzerland) was dissolved in HCl (1 mM) containing 0.02% BSA (bovine serum albumin; BDH Chemicals Ltd., Poole, UK) at a concentration of 2 mg/ml (stock). This stock was further diluted with physiological saline containing 0.02% BSA to obtain the final concentrations used in this study (20, 2, and 0.02 μg/ml). The Alzet mini-osmotic pump (model #2004, Alza Corp., Palo Alto, CA, USA) was filled with either one of the three α–MSH solutions or 0.02% BSA containing saline and placed in a saline bath (37°C) for 40 hours or more before surgery, using a sterile container. After this, according to the manufacturers specifications, the pump worked immediately at a constant rate of 0.25 μl/h for 4 weeks.

Surgical techniques

Surgical implantation of the mini-osmotic pump and further techniques were performed according to the method described by Prieskorn and Miller (2000). The animals were anaesthetized with a mixture of ketamine and xylazine. The cannula (length: 7.4 cm) was filled with the same α–MSH solution as the pump. A mid-line incision was made on the dorsal surface of the head starting

2.5 cm anterior of bregma and continued post-auricularly to the base of the pinna. A superficial subcutaneous pocket was made in the back between the scapulae of the animal to accommodate the pump. Under sterile conditions the bulla of the left ear was opened retro-aurically and the skull was exposed around bregma. A small hole was made at the base of the cochlea, approximately 0.5 mm below the round window. The cannula tip was placed into the hole until the silicone ball, 0.5 mm from the tip, was seated against the cochlea to prevent leakage. The round window electrode, made of insulated stainless-steel wire (diameter 0.175 mm including teflon insulation; Advent, Halesworth, UK) with an 0.5 mm diameter gold ball micro-welded (Unitek 80F; Unitek Equipment, Monrovia, CA, USA) to the exposed and flattened tip, was positioned on the round window and secured to the bulla with carboxylate cement (Durelon, ESPE dental supplies, Utrecht, The Netherlands). The wire was soldered to a Berg 22-26 gold terminal that fitted into a Berg 2x3 mini-latch housing (Farnell, Maarssen, The Netherlands). Stainless-steel screws were inserted through the skull and connected to the mini-latch housing via two silver wires connected to a gold terminal. The mini-latch housing was connected to the skull with dental acrylic cement, which also covered and insulated the stainless-steel screws and the electrodes. The pump was removed from the water-bath and attached to the cannula. Subsequently, the pump was placed in the subcutaneous pocket, the cannula was fixed to the skull, and the subcuticular layer was closed with a continuous vicryl suture; the skin was closed with vicryl using interrupted sutures.

Electrocochleography

Measurements were performed differentially with the round window electrode as the active electrode and two screws on the skull as reference and ground electrode, respectively.

Animals that did not have a normal threshold at 8 kHz (defined as a threshold at less than 25 dB SPL stimulus level) at the day after surgery or that showed a displaced cannula or signs of otitis media when sacrificed were excluded from further analysis. Trains of tone bursts of 2, 4, 8, and 16 kHz were used as stimuli. The tone bursts were constructed with cosine-shaped rise-and-fall times of 1 ms (1.5 ms at 2 kHz) and had a duration of 8 ms. The sound stimuli were produced in an open-field configuration with a Fame tweeter (Staffhorst Electronics, Utrecht, The Netherlands) positioned at 10 cm from the pinna. Consecutive tone bursts were presented with alternating polarity at 99 ms intervals in order to avoid synchronization with the mains frequency of 50 Hz. The responses were amplified (EG&G Instruments model 5113 amplifier, Te

Lintelo Systems, Zevenaar, The Netherlands), bandpass filtered between 1 Hz and 30 kHz, AD converted at 33 kHz, and stored on disk for off-line analysis. The animals were lightly restrained, but remained awake during all measurements, avoiding the disadvantages of repeatedly having to anaesthetize the animals. CAPs were obtained by adding the responses evoked by tone bursts of opposite polarity; Cochlear Microphonics (CM) by subtracting these responses. The CAP was measured relative to the positive summating potential (SP) and not relative to the baseline of the recording since, in principle, the CAP is superimposed upon the SP. The CM was measured as the peak-to-peak amplitude in the middle of the sinusoidal response. Electrocochleography was continued for 10 days following the cessation of cisplatin treatment.
ANOVA was performed on thresholds (3 μV iso-response levels) and amplitudes of CAPs and CM. Co-treatment was a between-subjects factor; time and stimulation frequency and level were within-subject factors. STATISTICA software was used.

Histological techniques

Ten days after the cessation of cisplatin administration the cochleas were fixed by intralabyrinthine perfusion with a tri-aldehyde fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% dimethylsulfoxide in 0.1 M sodium cacodylate buffer (pH 7.4) followed by immersion in the same fixative for 3 h at room temperature. The cochleas were further processed according to the routine method for guinea pigs (De Groot et al., 1987). Semithin (1 μm) midmodiolar sections were cut and stained with 1% methylene blue and 1% azur II in 1% sodium tetraborate. In the midmodiolar sections the organ of Corti was examined at seven locations, separated by a half-turn spacing, and the number of OHCs present at each location was counted. OHC loss was expressed as the percentage of remaining OHCs per cross-sectioned half turn (2 transections each for the basal and middle turns; 3 transections for the apical turn), relative to the number of OHCs found in non-treated cochleas. All OHC counts were performed by two investigators, independently of one another, in a single-blind fashion (*cf.*, De Groot et al., 1997; Cardinaal et al., 2000a; Wolters et al., 2003). ANOVA was used for statistical evaluation of the OHC counts. Co-treatment was a between-subjects factor and cochlear location a within-subject factor.

Results

General findings

Sixteen of the 58 operated animals showed an abnormal threshold at 8 kHz directly after surgery and fourteen animals demonstrated a displaced cannula or infections upon inspection of the left (operated) bulla when sacrificed. These animals were excluded from the experiment. Table 1 shows the distribution of the remaining animals (n=28) over the four experimental groups.

Table 1: Distribution of the animals over the experimental groups

Pump filling	Number of animals
α–MSH (20 μg/ml)	6
α–MSH (2 μg/ml)	8
α–MSH (0.02 μg/ml)	8
Physiological saline	6

Effects of cisplatin on CAP thresholds in relation to co-treatment

During the first days of cisplatin treatment only small random changes in CAP threshold were observed. At some moment between day 5 and day 11 after the start of cisplatin administration, the animals showed a sudden deterioration of the CAP threshold. This was reported before when a similar experimental design was applied (Klis et al., 2000; Wolters et al., 2002; Hamers et al., 2003). Figure 1 shows the time required to reach the criterion threshold shift of ≥ 40 dB at 8 kHz.

Saline co-treated animals reached this criterion threshold shift after an average of 6.8 days. Animals that received the lowest concentration of α–MSH (0.02 μg/ml in the pump) reached the criterion threshold shift almost one day earlier (6 days) and also showed significantly less variability (Levene's test, $P<0.026$). Animals receiving higher concentrations of α–MSH (2 or 20 μg/ml in the pump) reached the criterion threshold shift after 7.3 and 6.7 days of cisplatin administration, respectively. ANOVA showed that the mean number of days of cisplatin administration necessary to cause hearing loss did not differ between the four groups in a statistically significant way ($F_{(3,24)}= 0.719$, $P=0.55$).

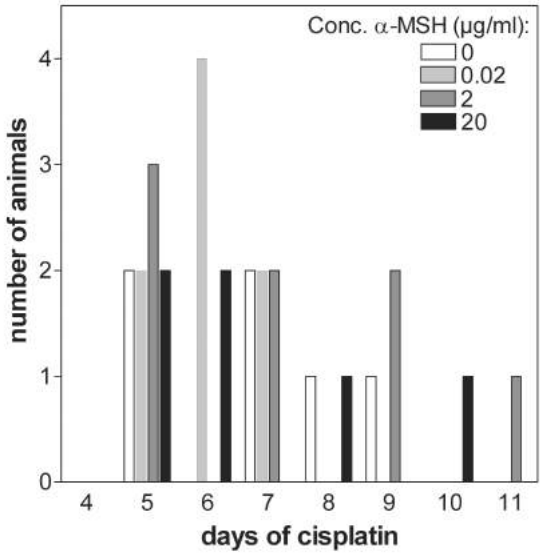

Figure 1: The time required to induce a hearing loss of ≥ 40 dB in the 3 μV CAP iso-response level (threshold) at 8 kHz for the four different groups.

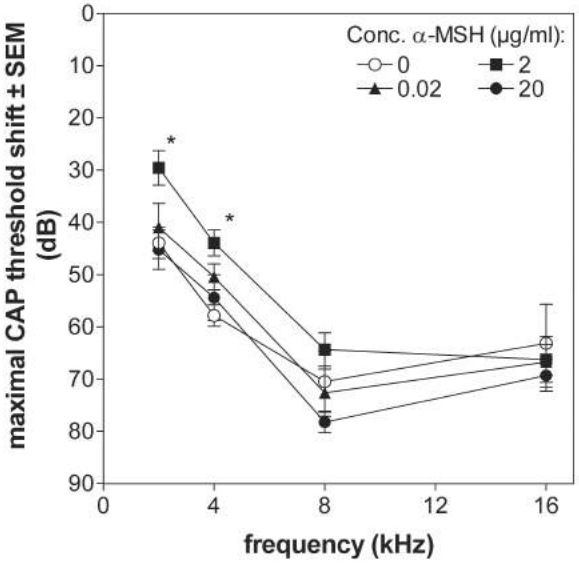

Figure 2: Maximum shift in CAP threshold (3 μV criterion) at the day cisplatin administration was terminated or shortly thereafter, as a function of stimulus frequency for the four different groups.

CAP thresholds

Maximum CAP threshold shifts occurred either at the day cisplatin administration was terminated or 1 to 2 days later. After the maximum CAP threshold shift was reached, the threshold recovered in 50% of the animals (*cf.*, Fig. 3). Maximum threshold shift in dB as a function of frequency is shown in Figure 2 for all groups.

Although the hearing loss occurred within a broad frequency range, it clearly increased as a function of frequency. ANOVA showed a significant main effect of frequency ($F_{(3,72)}=112.8$, $P<0.001$) but no main effect of co-treatment ($F_{(3,24)}=2.07$, $P=0.13$). Although only a small significant interaction between

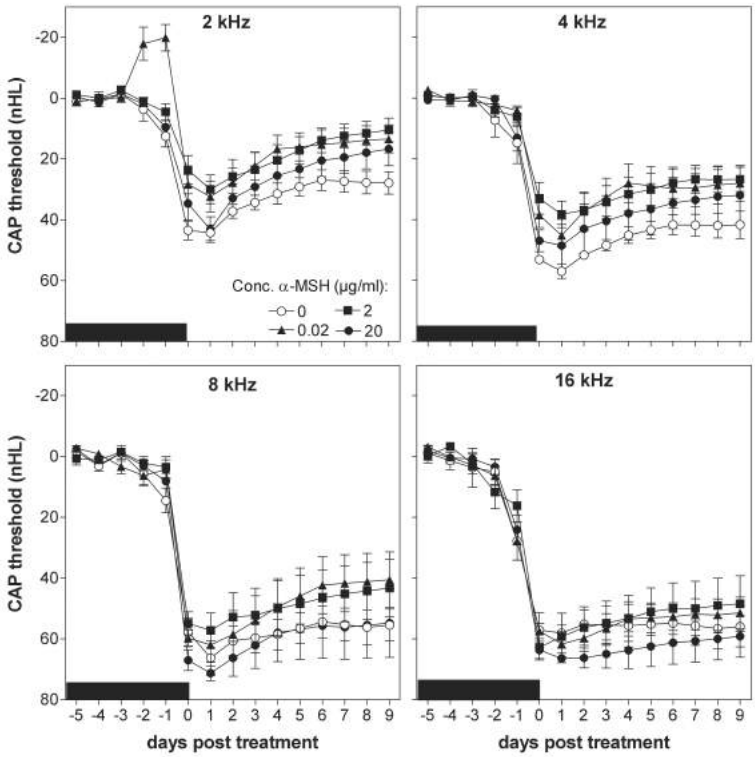

Figure 3: Build-up and recovery of the CAP threshold shift at 2, 4, 8, and 16 kHz (± S.E.M.) as a function of time for the 4 different groups. The horizontal black bar represents the last 6 days of cisplatin administration. Day 0 represents the day that cisplatin administration was discontinued. CAP threshold baseline values between day -6 and day -2 before cessation of cisplatin administration were averaged for each individual animal and set to zero. These values were taken as the reference "normal hearing level" (nHL).

both factors ($F_{(9,72)}$=1.97, P=0.055) was found, we performed an analysis of the effect of α–MSH co-treatment for each frequency separately. Animals co-treated with 2 μg/ml α–MSH showed a significantly smaller threshold shift than animals treated with saline at 2 and 4 kHz ($F_{(1,12)}$=9.60, P=0.009 and $F_{(1,12)}$=16.90, P=0.001, respectively), but not at 8 and 16 kHz ($F_{(1,12)}$=0.98, P=0.343 and $F_{(1,12)}$=0.185, P=0.67, respectively).

Figure 3 shows CAP thresholds at 2, 4, 8, and 16 kHz, based on the 3 μV response amplitude criterion as a function of time. Since baseline threshold values varied between animals, the CAP threshold baseline values between day -6 and day -2 before cessation of cisplatin administration were averaged for each individual animal and set to zero. These values were taken as the reference "normal hearing level" (nHL). As mentioned before, pronounced recovery of the CAP threshold was observed in all groups after cessation of cisplatin administration and it was observed at all frequencies, although it was more prominent at the lower frequencies. Statistical analysis (ANOVA for repeated measurements) of the data starting at day 0 showed a significant effect of time (recovery) at all frequencies (2 kHz: $F_{(9,216)}$=44.13, P<0.001; 4 kHz: $F_{(9,216)}$=20.67, P<0.001; 8 kHz: $F_{(9,216)}$=10.88, P<0.001; 16 kHz: $F_{(9,216)}$=5.59, P<0.001). Animals co-treated with either 0.02 or 2 μg/ml α–MSH showed significantly lower CAP thresholds, between day 0 and day 9, than those receiving saline co-treatment at 2 kHz (0.02 μg/ml: $F_{(1,12)}$=5.77, P=0.033; 2 μg/ml: $F_{(1,12)}$=6.82, P=0.023) and 4 kHz (0.02 μg/ml: $F_{(1,12)}$=4.22, P= 0.062; 2 μg/ml: $F_{(1,12)}$= 7.21, P=0.020), but not at 8 and 16 kHz, although a similar trend is present at these latter frequencies.

CM thresholds

Figure 4 shows the CM thresholds at 16 kHz, based on a 3 μV criterion. As the cochlear electrode was positioned at the round window, the CM generated in the basal turn contributed most to the signal, and hence only the CM elicited at 16 kHz stimulation was analyzed. CM threshold baseline values between day -6 and day -2 before cessation of cisplatin administration were averaged for each individual animal and set to zero. The CM thresholds at 16 kHz did not show significant recovery ($F_{(9,216)}$=1.34, P=0.215). Still, animals treated with 0.02 μg/ml α–MSH showed a significantly lower CM threshold shift, between day 0 and day 9, than those co-treated with saline ($F_{(1,12)}$= 7.88, P= 0.016). The higher concentrations of α–MSH did not show any significant effect at all.

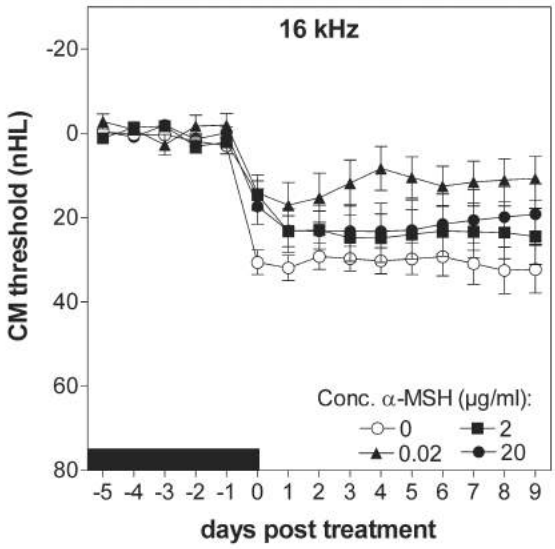

Figure 4: Build-up and recovery of the CM threshold shift at 16 kHz (± S.E.M.) for the 4 different groups. The horizontal black bar represents the last 6 days of cisplatin administration. Day 0 represents the day that cisplatin administration was discontinued.

CAP and CM amplitudes in relation to recovery and treatment

In addition to threshold measurements it is informative to study the effect at higher stimulus levels. Figure 5 shows the CAP amplitudes obtained at stimulus levels of 78 to 87 dB SPL during and after cisplatin administration. Local perfusion with α–MSH resulted in less CAP amplitude reduction, especially at the lower frequencies. ANOVA from day 0 to day 9, performed on the logarithmically transformed CAP amplitudes, demonstrated main effects of frequency ($F_{(3,72)}$=19.49, P<0.001) and time ($F_{(9,216)}$=20.39, P<0.001) and a strong interaction between both factors ($F_{(27, 648)}$=3.30, P<0.001). A separate analysis per frequency revealed a pronounced effect of co-treatment with 2 μg/ml α–MSH at 2 kHz ($F_{(1, 12)}$= 9.34, P=0.010). At the other frequencies there was only a main effect of time (recovery). Analysis of CM amplitudes at 16 kHz, 78 dB SPL (data not shown), starting at day 0, did not show an effect of co-treatment ($F_{(3,24)}$=1.03, P=0.40), nor of time ($F_{(9,216)}$=1.66, P=0.10).

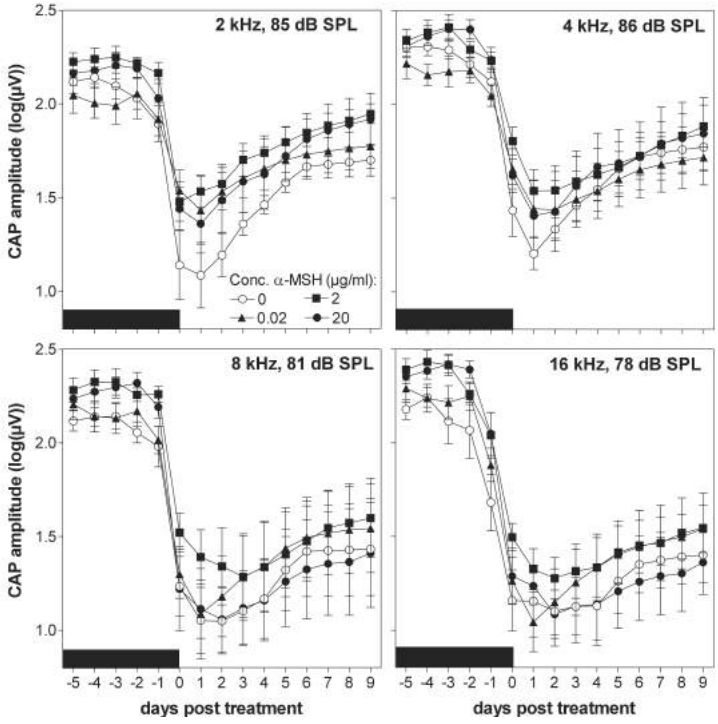

Figure 5: Collapse and recovery at 2, 4, 8, and 16 kHz (± S.E.M.) of the CAP amplitude at 78-86 dB SPL stimulus level for the four different groups. The horizontal black bar represents the last 6 days of cisplatin administration. Day 0 represents the day that cisplatin administration was terminated.

OHC loss in relation to co-treatment

OHC counts for the right and left (implanted) ears of the four groups of animals are presented in Fig. 6. OHC losses were observed in the basal and middle turns of both left and right cochleas. OHC losses in the basal turns were more severe than in the middle ones. Remarkably, OHC loss in the left ears (with saline co-treatment) was more severe (10-15% remaining OHCs) than those in the right ears without cannula (50% remaining OHCs) ($F_{(1,23)}=5.20$, $P=0.032$). This finding is in line with observations in chickens of Roberson et al. (2000). They found that cannula implantation itself may cause OHC death and can also cause potentiation of hair cell death induced by systemic gentamicin administration.

Since the cannula implantation itself seems to have a negative effect on hair cell survival, the effect of α–MSH co-treatment could only be compared within the left (implanted) ears. Statistical analysis showed a significant effect of

treatment in the basal turn of the ears co-treated with 2 μg/ml α–MSH as compared to the ears co-treated with saline ($F_{(1,11)}$=6.05, P=0.032). Co-treatment with the lowest (0.02 μg/ml) and highest (20 μg/ml) concentration of α–MSH did not reach statistical significance ($F_{(1,12)}$=3.94, P=0.071; $F_{(1,10)}$=2.65, P=0.135, respectively).

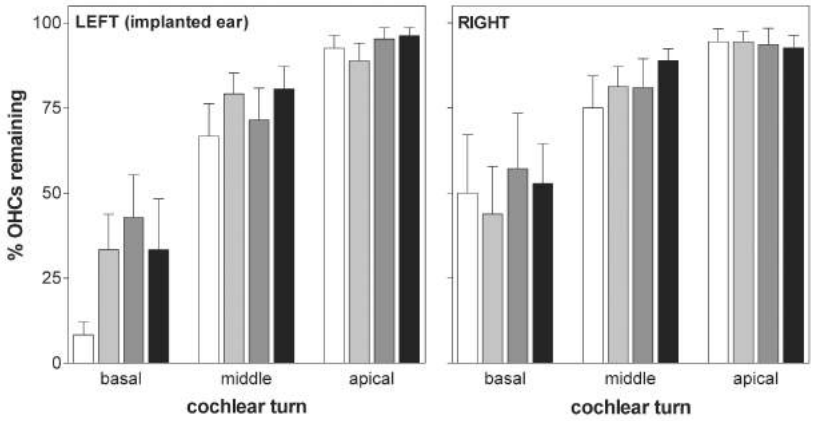

Figure 6: Outer hair cell counts (percentage remaining OHCs) for the left (implanted) and right ear, as a function of position in the cochlear turns. Co-treatments: Open bar: saline; light-gray bar: 0.02 μg/ml α–MSH; dark-gray bar: 2 μg/ml α–MSH; black bar: 20 μg/ml α–MSH. Error bars represent S.E.M.

Discussion

α–MSH has a dose-dependent ameliorating effect on cisplatin intoxication

The first issue in this study was whether ameliorating effects of α–MSH on cisplatin ototoxicity, which were seen in previous studies (Heijmen et al., 1999; Wolters et al., 2003; Hamers et al., 2003), remain when α–MSH is applied exclusively perilymphatically as opposed to systemically. This study shows that intracochlear administration of 2 μg/ml α–MSH, with a mini-osmotic pump, indeed has an ameliorating effect on cisplatin-induced threshold shifts and the recovery in time (Figs. 2, 3) and also protects against OHC loss (Fig. 6). The group that received 0.02 μg/ml α–MSH showed small effects on both CAP and CM threshold shifts (Figs. 3, 4). With 20 μg/ml α–MSH in the pump, no statistically significant effects were found. Thus, the intermediate concentration (2 μg/ml in the pump) was the most optimal concentration in this study. Surprisingly and in contrast to the mirror experiment in which cisplatin was applied locally and α–MSH systemically (Wolters et al., 2003), we did not find an increase in the number of injections (days) necessary to evoke ototoxic

effects at any α–MSH concentration. This discrepancy can probably be explained by the large inter-animal variability associated with systemic cisplatin application (Klis et al., 2002) in combination with the relatively small size of the effect.
However, how can we explain that the highest concentration α–MSH applied in this study (20 μg/ml in the pump) is not effective while lower concentrations are? De Koning et al. (1986) found that the $ACTH_{(4-9)}$ analog ORG 2766 has an ameliorating effect on peripheral nerve regeneration with an inverted U-shaped dose-effect curve, *i.e.*, low and high doses of ORG 2766 were inactive and only intermediate doses resulted in enhanced peripheral nerve recovery. We hypothesize that α–MSH, also an ACTH fragment, has a similar dose-effect curve for its ameliorating effects on cisplatin ototoxicity and that the highest dose results in a perilymphatic concentration that is well above the effective range. A point of concern with this hypothesis is that we do not know the validity of the comparison between both melanocortins, since we do not know the (sub)cellular mechanism by which both compounds exert their effects, neither with respect to enhancing peripheral nerve regeneration by ORG 2766, nor with respect to ameliorating cisplatin ototoxicity by α–MSH. Nevertheless, the present study has brought new information about the target of α–MSH.

α–MSH exerts its ameliorating effects on cisplatin-induced ototoxicity through a cochlear target

The most salient result in this study is that the positive effects we found with α–MSH, applied directly and exclusively to the cochlea, can almost only be explained by postulating a cochlear target for the compound. Especially in combination with the mirror study (Wolters et al., 2003), the possibility seems extremely unlikely that α–MSH works through interaction with a systemic factor or target. Given the strong possibility of a cochlear target, the question is of course which target? This is intimately related with the question about the target for cisplatin in the cochlea. On the basis of earlier data (Hamers et al., 1994; De Groot et al., 1997; Klis et al., 2000, 2002; O'Leary and Klis, 2002), we hypothesize that cisplatin toxicity in the cochlea first occurs in the stria vascularis. Interference with strial function leads to a drop in EP (Klis et al., 2000, 2002; O'Leary and Klis, 2002) and subsequently to a large increase of the CAP threshold. In a causally and yet unknown manner, these events might be related to OHC loss, progressing apically from the basal turn. Further, evidence is accumulating that suggests involvement of spiral ganglion cells (Zheng and Gao, 1996; Cardinaal et al., 2000b; Alam et al., 2000; Hamers et al., 2003), once again in an unknown relation to the strial and OHC effects described above. All these targets for cisplatin need to be considered as cochlear components with

which α–MSH might interfere.
α–MSH may exert its otoprotective effect by affecting the potency of the stria vascularis to counteract the effects of cisplatin. In principle, α–MSH might exert its protective action through activation of the melanocortin-1 receptor, which is commonly found in epidermal melanocytes. Intermediate cells, which are necessary for normal strial function and are involved in the generation of EP, have been identified as melanocytes (Motohashi et al., 1994). Also, it has been suggested that cochlear melanocytes are under α–MSH control (Meyer zum Gottesberge, 2000).
Since melanocortins have shown to exert neuroprotective effects in the peripheral sensory nerve system after cisplatin administration (De Koning et al., 1987; Hamers et al., 1993; Ter Laak et al., 2003), the otoprotective effect of the melanocortin α–MSH might also be related to interference with ganglion cells or the Schwann cells (Hol et al., 1994). Such an interference would explain the significantly smaller decrease in CAP amplitude at high sound levels in the 2 μg/ml α–MSH group (Fig. 5). Since the CAP at high stimulus levels is shown to depend on the viability of the ganglion cells, not so much on the viability of the OHCs (Hall, 1990; Schmiedt et al., 2002).
A direct effect of α–MSH on OHCs is, for lack of data about interference of melanocortins with hair cells, at present a purely hypothetical possibility.

Conclusion

The present study provides further evidence for the previously reported protective effect of α–MSH in cisplatin ototoxicity. The ototoxic effects of cisplatin were significantly reduced when the animals were co-treated with perilymphatically applied α–MSH. This preventive effect was dose-dependent and rather small, perhaps too small for clinical relevance. Since α–MSH was delivered directly to the cochlea, it is plausible that the ameliorating effect of α–MSH involves a cochlear target, possibly the strial melanocytes or the spiral ganglion cells.

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Chapter 7

General discussion and summary

Melanocortins, peptides derived from AdrenoCorticoTropic Hormone (ACTH), such as α–MSH, and derivatives thereof, such as ORG 2766 and melanotan-II (MT-II), have been shown to protect against cisplatin-induced peripheral neuropathy. Previous experiments performed by our group have also shown protective effects of some of these peptides against cisplatin-induced ototoxicity. Based on these results, the aim of this thesis was to further characterize the protection efficacy of co-treatment with these neurotrophic peptides in relation to cisplatin-induced ototoxicity and, subsequently, to analyze the mechanisms involved. The studies performed were based on the following questions:

1) Do ORG 2766, α–MSH and MT-II delay the action of cisplatin?
2) Do the melanocortins enhance recovery after cisplatin treatment, and how?
3) Is there a neural component in cisplatin ototoxicity or is the effect confined to hair cells and stria vascularis?
4) Is the protective effect a direct local effect of the peptides or does the effect depend upon a systemic (intermediate) effect?
5) Can these melanocortin(-like) peptides be used in the clinic as otoprotective agents?

1) Do ORG 2766, α-MSH and MT-II delay the action of cisplatin?

Previous studies, in which a fixed number of injections of 1.5 or 2 mg/kg cisplatin was administered daily in combination with 75 μg/kg ORG 2766 (Hamers et al., 1994; Stengs et al., 1998b) or 75 μg/kg α–MSH (Heijmen et al., 1999), demonstrated a considerable number of co-treated animals that displayed preserved hearing after cessation of cisplatin and melanocortin treatment. This was not found in the cisplatin/saline controls. In the longitudinal animal model used in chapters 2, 3, and 4 of this thesis all animals were treated with cisplatin until a pronounced (criterion of ≥ 40 dB loss at 8 kHz) hearing loss occurred. Following the dichotomous results in the co-treated groups with the fixed dose experiments, we hypothesized that in the experiments with the longitudinal model the animals co-treated with ORG 2766 or α–MSH would require more injections of cisplatin than the saline co-treated animals to evoke the criterion threshold loss. Unexpectedly, neither α–MSH nor ORG 2766 consistently increased the mean number of cisplatin-injections (1.5 mg/kg/day) necessary to evoke this threshold loss. Also, the melanocortin MT-II (chapter 2) did not delay cisplatin ototoxicity. This can be explained by the large variability seen with cisplatin treatment in combination with a relatively

small effect. In the experiments described in this thesis, the most susceptible animals required 5 injections of cisplatin before reaching criterion threshold loss while the most resistant animal required 18 injections (without co-treatment). In such a noisy background only large delays can be proven to be statistically significant. Nevertheless, in several co-treated animals the onset of the ototoxic reactions seemed considerably delayed; the animals that required the highest cumulative dose of cisplatin were usually the co-treated ones, which might be an explanation of the dichotomy observed by Hamers et al. (1994), Stengs et al. (1998b), and Heijmen et al. (1999). These studies showed animals that were completely protected from cisplatin ototoxicity with normal auditory thresholds and no OHC loss but also animals that demonstrated increased thresholds similar to the animals treated with cisplatin alone.
In an attempt to decrease the variability in susceptibility to cisplatin an alternative route of application was used in which we applied cisplatin directly into the cochlea (chapter 5). With this approach systemic factors that might be responsible for the large variability associated with systemic treatment could be eliminated. This experiment indeed showed a statistically significant delay in the group of animals that were systemically co-treated with α–MSH (75 μg/kg/day s.c.). However, when α–MSH was delivered directly into the cochlea via an osmotic pump system and cisplatin systemically (chapter 6) again only a trend was visible, which showed that some animals receiving the high dose of α–MSH (2 or 20 μg/ml in the pump) required more injections of cisplatin (2 mg/kg/day) to reach the criterion threshold shift than the animals with pure saline in the pump. Once again, we attribute this lack of effect of α–MSH to the higher variability associated with systemic application of cisplatin. However, we can not exclude causes associated with the different mode of application of α–MSH (local versus systemic).

2) Do the melanocortins enhance recovery after cisplatin treatment, and how?

Previous experiments by Klis et al. (2000, 2002) showed that when cisplatin treatment is stopped after reaching the criterion threshold shift, a pronounced recovery of hearing sensitivity occurs. This recovery reaches asymptotic levels after 10 days and is better at lower than at higher frequencies. Co-treatment with the melanocortin(-like) peptides ORG 2766 and α–MSH (chapter 3, 6) significantly changed the recovery after cisplatin treatment. Recovery of CAP threshold and CAP amplitude at high sound pressure levels was faster and

more complete. Furthermore, hair cell loss was significantly lower in the peptide co-treated groups. In contrast to the ameliorating effect of ORG 2766 and α–MSH, co-treatment with MT-II did not show a significant effect on recovery (chapter 2), although this compound is a more potent melanocortin-1 (MC1) and MC4-receptor agonist than α–MSH.

Thus, since not all melanocortin(-like) peptides enhance recovery after cisplatin-induced ototoxicity it is difficult to identify the cochlear target(s) and the exact cellular mechanism involved in the otoprotective effects of the melanocortin(-like) peptides. Cisplatin causes pronounced damage to and even loss of OHCs (Komune et al., 1981; De Groot et al., 1997; Cardinaal et al., 2000a, b) and spiral ganglion cells (Zheng and Gao, 1996; Cardinaal et al., 2000b), and damage to the stria vascularis (Nakai et al., 1982; Kohn et al., 1988, 1997; Meech et al., 1998; Cardinaal et al., 2000a). Thus, one or more of these cochlear components might be involved in the recovery-process. Further characterization of the melanocortin-enhanced recovery process might bring us closer to the actual target of the melanocortins (and cisplatin itself) and with that the mechanism involved in the otoprotective action of melanocortins.

Co-treatment with α–MSH, or its derivatives, might enhance recovery from cisplatin-induced ototoxicity through activation of the MC1-receptor known to be present in melanocytes, for instance in the stria vascularis (Hilding and Ginzberg, 1977). The melanocytes in the stria, the so-called intermediate cells, have been suggested to be under α–MSH control (Meyer zum Gottesberge, 2000). However, co-treatment with the more potent MC1-receptor agonist MT-II did not show an enhancement of the recovery-process (chapter 2). Furthermore, ORG 2766 has no melanotrophic or corticotrophic activity (Greven and De Wied, 1973) and is known not to activate any of the five currently known MC-receptor subtypes (Adan et al., 1994, 1996). Since both α–MSH and ORG 2766 have similar protective and/or recovery enhancing effects in models of mechanical peripheral nerve damage (Bijlsma et al., 1984; Van der Zee et al., 1991) and ototoxicity (Hamers et al., 1994; De Groot et al., 1997; Stengs et al., 1998b; Heijmen et al., 1999) the question arises whether or not there is an as yet unidentified receptor for ORG 2766 that can also be activated by α–MSH.

3) Is there a relevant neural component in cisplatin ototoxicity or is the effect confined to hair cells and stria vascularis?

On the basis of earlier data (Hamers et al., 1994; De Groot et al., 1997; Klis et al., 2000), we hypothesized that cisplatin toxicity in the inner ear first occurs in the stria vascularis, since the endocochlear potential (EP) was decreased early in the process. Possibly, interference of cisplatin with strial function impairs its electrogenic activity, leading to a precipitous drop of the EP and a strong increase in CAP threshold (Hamers et al., 1994). In a causally and yet unknown manner, these events might be related to OHC loss, progressing apically from the basal turn. Discontinuation of cisplatin treatment enables the stria vascularis and thus the EP to recover, with concomitant CAP threshold recovery, provided that a sufficient number of OHCs have survived and are functional. The preliminary data in chapter 4 of this thesis, however, showed that the relation between EP recovery and CAP recovery may not be as strong as suggested above. The EP did not show recovery after 3 days, although significant recovery of the CAP threshold at 2 kHz was observed. However, CAP amplitudes at higher frequencies and at high stimulus levels did not show significant recovery in the limited time frame of chapter 4. In other words, we may have missed an essential part of the recovery process by limiting our time window to 3 days after cessation of the cisplatin treatment. We cannot rule out that the melanocortin(-like) peptides might exert their effect through another target than the stria. Both ORG 2766 (*in vivo*; Gerritsen van der Hoop et al., 1988; Muller et al., 1990; Hamers et al., 1993a) and α–MSH (*in vitro*; Windebank et al., 1994) have shown to protect from cisplatin-induced peripheral neuropathy. Thus, we cannot exclude the possibility that these compounds (partially) induce their protective effect in cisplatin-induced ototoxicity through modulation of a neural component. To date, some evidence for the involvement of the auditory nerve in cisplatin-induced hearing loss has been published. Both animal studies (Zheng and Gao, 1996; Alam et al., 2000; Cardinaal et al., 2000b) and a human study of post-mortem temporal bone material (Hinojosa et al., 1995) described morphological damage to the spiral ganglion cells in addition to OHC and strial damage.

4) Is the protective effect a direct local effect of the melanocortin-like peptides or does the effect depend upon a systemic (intermediate) effect?

So far we suggested that the melanocortin(-like) peptides ameliorate cisplatin-induced ototoxicity through a local (cochlear) target. However, since both cisplatin and the peptides were administered systemically in several studies (chapters 3 and 4) their effect could also have been mediated through a systemic effect. Such a systemic effect has been found earlier with the anti-oxidants, a group of sulphur containing compounds that are also known to prevent cisplatin-induced ototoxicity. The sulphur-groups in the anti-oxidants are known to bind irreversibly to heavy metals such as platinum. When both cisplatin and anti-oxidants are administered systemically, inactive sulphur-platinum-complexes are formed which are quickly excreted. This may result in a lowered systemic exposure to cisplatin, reducing its side effects but probably also its anti-tumor effect. Indeed, such a reduction in the systemic cisplatin concentration has been found when cisplatin was administered together with D-methionine (Ekborn et al., 2002). Since both ORG 2766 and α–MSH have been administered in our studies in doses at least 20 times smaller than the cisplatin dose, the protective effect of these peptides cannot be due to direct chemical interaction between cisplatin and α–MSH or ORG 2766.

Another possibility might be that melanocortin(-like) peptides exert their protective effect through enhancement of cisplatin clearance. The results in both chapter 5 and chapter 6 invalidate this hypothesis. In chapter 5 it was found that, despite the fact that cisplatin was administered locally through an osmotic pump system, systemic co-treatment with α–MSH significantly altered the number of days necessary to reach the criterion threshold shift. Furthermore, in chapter 6, in which α–MSH was administered directly into the ear and cisplatin systemically, we found small but significant effects on CAP threshold and OHC loss. Therefore, systemic effects of α–MSH on cisplatin excretion, or other absorption or transport factors can be excluded.

5) Can α-MSH and ORG 2766 be used in the clinic as an otoprotective agent?

With the results from this thesis we hoped to contribute to better understanding of cisplatin ototoxicity, which in turn might provide a key to ameliorate cisplatin ototoxicity in humans. With this knowledge we also hoped, eventually, to be able to generalize our results and find medical treatment for other acute cochlear insults, like those due to carboplatin treatment, aminoglycoside antibiotic treatment or even noise-induced hearing loss. The first part of this objective was reached. We showed that α–MSH does not delay the onset of cisplatin effects when cisplatin is applied systemically, but it enhances recovery and partly prevents OHC loss. The experiments with local application of cisplatin or α–MSH showed that α–MSH does not cause its effect through direct interaction with cisplatin or through stimulation of cisplatin excretion, but that the ameliorating effect of α–MSH probably involves a cochlear target, possibly the strial melanocytes or the spiral ganglion (nerve) cells. Thus, the results from this thesis confirm that α–MSH may be used to reduce cisplatin-induced ototoxicity. Also, the fact that α–MSH and its analogs show little side effects in clinical studies (Gerritsen van der Hoop, 1990; Wessels et al., 2000) pleads for introduction of the melanocortin-like peptides as a treatment for cisplatin-induced ototoxicity. However, this thesis shows that the protective effects of these peptides are rather small and that the effect varies considerably between individuals. This seriously hampers the introduction of these peptides as an otoprotective agent in patients. More research, especially about the dose-effect relationship of the peptides, has to be performed. Furthermore, the mechanism through which cisplatin exerts its ototoxic effects is such a complicated process that further research, especially at the morphological and molecular level, is necessary to reliably identify both the cochlear targets and the exact cellular mechanism involved in the otoprotective effect of the melanocortin(-like) peptides. The research performed within this thesis brought us a step closer to the unraveling of the mechanism of cisplatin-induced ototoxicity and ways and means to protect the ear against this highly potent ototoxicant.

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Abbreviations

α–MSH	α–Melanocyte Stimulating Hormone
4-MTBA	4-Methylthiobenzoic acid
ABR	Auditory brain stem response
ACTH	AdrenoCorticoTropic Hormone
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
CAP	Compound Action Potential
Cisplatin	Cis-diamminedichloroplatinum-II
CM	Cochlear Microphonics
dB	Decibel
DDTC	Diethyldithiocarbamate
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ECochG	Electrocochleography
EP	Endocochlear potential
GDNF	Glial-derived neurotrophic factor
i.m.	Intramuscular drug administration
i.p.	Intraperitoneal drug administration
IHCs	Inner hair cells
K^+	Potassium-ion
kHz	Kilohertz
MC	Melanocortin
MT-II	Melanotan-II
Na^+	Sodium-ion
NGF	Nerve growth factor
NT	Neurotrophin
OAE	Otoacoustic emission
OHCs	Outer hair cells
ORG 2766	H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH
p75	NGF low affinity receptor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reaction oxygen species
s.c.	Subcutaneous drug administration
s.d.	Standard deviation
SEM	Standard error of the mean

SNCV	Sensory nerve conduction velocity
SP	Summating Potentials
SPL	Sound pressure level
STS	Sodium thiosulphate
Trk	Tyrosine kinase receptor
VPT	Vibration perception threshold

Nederlandse samenvatting

Cisplatine is een belangrijk geneesmiddel voor de behandeling van hoofd- en halstumoren, longkanker en kanker aan de eierstokken of teelballen. Helaas kan behandeling met cisplatine gepaard gaan met een aantal schadelijke effecten, zoals schade aan de zenuwen (neurotoxiciteit) en verslechtering van het gehoor (ototoxiciteit). Patiënten, die behandeld worden met cisplatine, kunnen een gehoorverlies ontwikkelen dat wordt gekarakteriseerd door een tweezijdig, meestal permanent verlies van de hoge tonen. Herstel van dit verlies treedt sporadisch op en is meestal slechts gedeeltelijk. Uit experimenten in proefdieren is gebleken dat een langdurige cisplatinebehandeling leidt tot verlies van de buitenste haarcellen, voornamelijk in de basale winding van het slakkenhuis (cochlea). Daarnaast kan cisplatine in het oor ook de cellen in de stria vascularis (de bloedvoorziening) en de spirale ganglioncellen (zenuwcellen) beschadigen. Deze drie structuren in de cochlea zijn betrokken bij het omzetten van de geluidsgolven in zenuwactiviteit. De buitenste haarcellen zorgen voor de versterking van de geluidsgolven. De stria vascularis is belangrijk voor de handhaving van de elektrolytische compositie en de endocochleaire potentiaal (EP) van de endolymfe (de vloeistof in de scala media), welke noodzakelijk zijn om de elektrische signalen in de haarcellen op te kunnen wekken. De spirale ganglioncellen zorgen voor de geleiding van de zenuwimpulsen naar de hersenen. Wanneer een of meer van de bovengenoemde structuren beschadigd wordt, kan verlies van (een deel van) het gehoor optreden.

Diverse pogingen zijn ondernomen om de bijwerkingen van cisplatine te verminderen, o.a. door het doseringsschema te wijzigen, door de wijze van toediening te veranderen of zelfs door cisplatine te vervangen door een minder schadelijk antitumormiddel. Helaas hebben deze methoden tot op heden de bijwerkingen slechts gering kunnen verminderen. Verschillende onderzoeksgroepen zijn zich dan ook gaan concentreren op een andere methode om gehoorschade te verminderen: namelijk de farmacologische interventie. Bij deze methode wordt tegelijkertijd met cisplatine een andere stof toegediend die de bijwerkingen moet verminderen, maar geen invloed mag hebben op de antitumorwerking. Tegenwoordig bestaan er verschillende groepen stoffen die de door cisplatine veroorzaakte gehoorschade kunnen verminderen of in sommige gevallen zelfs helemaal kunnen voorkomen. Voorbeelden daarvan zijn de anti-oxidanten, de groeifactoren en de melanocortinen. Anti-oxidanten moeten echter in grote hoeveelheden worden toegediend. Bovendien berust hun werking waarschijnlijk op het wegvangen van cisplatine. Het laatst

genoemde heeft tot gevolg dat er minder cisplatine beschikbaar is voor de antitumorwerking. Bij de groeifactoren is bewezen dat ze in celsystemen een beschermend effect hebben op de door cisplatine veroorzaakte haarcelschade. Echter, in proefdieren is een beschermende werking van deze stoffen nog niet aangetoond. Onze onderzoeksgroep heeft zich daarom op de beschermende eigenschappen van de melanocortinen gericht.

De melano-cortinen zijn de van adrenocorticotroop hormoon (ACTH) afgeleide neuropeptiden, waarvan een aantal neuroprotectieve eigenschappen heeft. Van zowel het lichaamseigen α–melanocyt stimulerend hormoon (α–MSH) als een synthetisch fragment daarvan (ORG 2766) is in het verleden aangetoond dat ze zowel zenuwen kunnen beschermen als de schadelijke effecten van cisplatine kunnen verminderen. Helaas liet bovengenoemde studie zien dat er naast dieren die volledig beschermd waren tegen de effecten van cisplatine er ook dieren waren die in het geheel niet beschermd werden. Uit deze tweedeling blijkt dat de optimale balans tussen de dosis cisplatine en melanocortine nog niet gevonden is. Daarnaast is het onderliggende mechanisme van zowel de ototoxische werking van cisplatine als de beschermende werking van de melanocortinen hierop momenteel nog niet duidelijk. Onze onderzoeksgroep vermoedt dat de neuropeptides in het binnenoor een herstelmechanisme bevorderen.

Het doel van dit promotieonderzoek was om de mogelijk beschermende werking van de melanocortinen op het binnenoor na behandeling met cisplatine nader te bestuderen. Daarbij is onderzocht of de melanocortinen ORG 2766, α–melanocyt stimulerend hormoon (α–MSH) en het cyclisch, van α–MSH afgeleide peptide melanotan-II (MT-II) in staat zijn om de ototoxische bijwerkingen van cisplatine uit te stellen en is onderzocht of ze een herstelmechanisme kunnen bevorderen. Tevens werd bestudeerd of het effect van de melanocortinen een lokaal effect in het binnenoor is of dat de melanocortinen ergens anders in het lichaam processen beïnvloeden waardoor cisplatine sneller uitgescheiden wordt.

In het onderzoek is gebruik gemaakt van een longitudinaal diermodel. In dit model werden cavia's geïmplanteerd met een permanente elektrode op het ronde venster van de cochlea. Met de electrode werd dagelijks de gevoeligheid van het gehoororgaan voor geluid (piepjes), met afnemende geluidssterkte, in het frequentiegebied van 2 tot 16 kHz bepaald. Hierbij werd gekeken naar de minimale geluidssterkte waarbij nog een reactie van de

gehoorzenuw wordt waargenomen (CAP drempel) en de grootte van de zenuwimpuls bij harde geluidsniveaus (CAP amplitude). Na het bereiken van een bepaald criterium (40 dB drempelverlies bij 8 kHz) was het mogelijk om de cisplatinebehandeling te staken en vervolgens de drempelbepalingen voort te zetten zodat een (mogelijk) herstel van de gehoordrempel vastgesteld kon worden.

In hoofdstuk 2 is aangegeven hoe met behulp van het longitudinale diermodel het beschermend effect van het cyclisch, van α–MSH afgeleide peptide, melanotan-II (MT-II) getest is. Van MT-II heeft men in het verleden laten zien dat het beter bindt aan de melanocortine-receptor dan α–MSH en dat behandeling in ratten bescherming kan bieden tegen de door cisplatine veroorzaakte zenuwschade. In ons experiment werden cavia's dagelijks geïnjecteerd met 1.5 mg/kg cisplatine en vervolgens met MT-II (3 of 30 μg/kg) of een zoutoplossing (controle). Na 5 tot 13 dagen trad, onafhankelijk van de toegediende concentratie MT-II, bij alle cavia's een gehoorverlies op. Daarna werd de behandeling met cisplatine gestaakt en werd nog een extra injectie van MT-II of zoutoplossing gegeven. De gehoordrempel werd nog gedurende 4 weken gecontroleerd op een mogelijk herstel van het gehoorverlies. Het bleek dat binnen 10 dagen nadat de cisplatinebehandeling was gestopt in alle drie de groepen en bij alle frequenties een herstel van de gehoordrempel optrad. Dit herstel was beter bij lage stimulus frequenties. Helaas werd in de dieren die behandeld waren met één van de twee MT-II concentraties geen extra toename van het herstel waargenomen. In tegenstelling tot de eerder gevonden beschermende effecten van de andere melanocortinen en de beschermende werking van MT-II bij cisplatine neurotoxiciteit laat MT-II geen beschermend effect zien op de door cisplatine veroorzaakte gehoorschade.

In hoofdstuk 3 is beschreven hoe in hetzelfde longitudinale diermodel de beschermende effecten van twee andere melanocortinen, te weten α–MSH en ORG 2766, op de door cisplatine veroorzaakte gehoorschade zijn getest. Wederom werden cavia's dagelijks behandeld met 1.5 mg/kg cisplatine en 75 μg/kg α–MSH, 75 μg/kg ORG 2766 of een zoutoplossing totdat een gehoorverlies van 40 dB bij 8 kHz optrad. Vervolgens werden twee groepen gevormd. Bij de eerste groep dieren werd 1 tot 2 dagen na het drempelverlies de endocochleaire potentiaal (EP) gemeten. Na de EP meting werden de cochleas van de cavia's bewerkt voor de histologie, zodat de effecten van cisplatine ook op cellulair niveau bekeken konden worden. Bij de overige dieren werd de EP 28 dagen na het beëindigen van de cisplatinebehandeling gemeten. Zowel de

dieren die behandeld waren met α–MSH of ORG 2766 als de dieren die behandeld werden met zoutoplossing lieten na 5 tot 14 injecties cisplatine een drempelverlies van 40 dB bij 8 kHz zien. Na beëindiging van de cisplatinebehandeling trad in alle drie de groepen een herstel op van zowel de CAP drempelwaardes als de CAP amplitude. Dit herstel bleek significant beter te zijn bij dieren die behandeld waren met α–MSH of ORG 2766. Bovendien lieten deze met melanocortine behandelde dieren ook minder haarcelverlies zien in de basale winding dan de dieren die met de zoutoplossing waren behandeld. Hieruit kan worden afgeleid dat zowel ORG 2766 als α–MSH een beschermende werking blijken te hebben op door cisplatine veroorzaakte gehoorschade.

Het bovenstaande onderzoek met α–MSH en ORG 2766 liet zien dat, alhoewel de endocochleaire potentiaal (EP) verlaagd was gedurende de eerste dagen na het beëindigen van de cisplatinebehandeling, deze potentiaal na 28 dagen weer volledig hersteld was. Dit deed vermoeden dat het herstel van de CAP en het beschermend effect van de melanocortinen mogelijk gerelateerd zou kunnen zijn aan het herstel van de schade aan de stria vascularis en aan het herstel van de EP. Om dit verder te onderzoeken werd met behulp van het longitudinale diermodel de aandacht gericht op de effecten van cisplatine, met of zonder α–MSH, op het herstel van de EP (hoofdstuk 4). Cavia's werden behandeld met 1.5 mg/kg cisplatine tot het moment dat het gehoorverlies optrad. Vervolgens werden de dieren in drie groepen verdeeld waarbij na 1, 2 of 3 dagen herstel de EP werd gemeten. Net als in de voorgaande studies hadden de dieren 4 tot 10 dagen cisplatine nodig om het gehoorverlies te bereiken. De EP bleek, bij het bereiken van een drempelverlies van 40 dB bij 8 kHz, van de normale waarde tussen 75 en 85 mV verlaagd te zijn tot een waarde van gemiddeld 58 mV. Echter, anders dan verwacht was op basis van de resultaten van hoofdstuk 3 liet de EP geen herstel zien binnen de twee dagen na de beëindiging van de cisplatinebehandeling. Behandeling met α–MSH bleek noch effect te hebben op het verlies noch op het herstel van de EP. Daar de CAP drempels toch een klein herstel lieten zien bij de lage frequenties en de met α–MSH behandelde dieren op dag 3 een significant hogere CAP amplitude lieten zien dan de controledieren, werd voorlopig geconcludeerd dat α–MSH niet alleen een effect heeft op de stria vascularis, maar dat de beschermende werking van deze stof mogelijk complexer is dan tot op heden was aangenomen.

Om meer inzicht te krijgen via welk mechanisme α–MSH zijn beschermend effect teweegbrengt, werd een nieuw diermodel geïntroduceerd, waarbij naast de elektrode op het ronde venster ook een osmotisch pompje met de cochlea van de cavia werd verbonden. Met behulp van dit osmotisch pompsysteem was het mogelijk om continu een (beschermende) stof direct in de cochlea toe te dienen. Experimenten met deze osmotische pompjes hebben in het verleden laten zien dat door deze methode door toediening de variabiliteit tussen de dieren verkleind kan worden, doordat de individuele (systemische) effecten van de dieren, zoals bijv. de zuivering van de nieren of de snelheid van uitscheiden, worden omzeild.

Vervolgens is met behulp van dit nieuwe proefdiermodel een oplossing van 15 μg/ml cisplatine door middel van een osmotisch pompje continu met een snelheid van 0.5 μl/uur in de cochlea gepompt (hoofdstuk 5). De canule tussen de pomp en de cochlea werd gevuld met een zoutoplossing waardoor de cavia's nog anderhalve dag konden herstellen van de operatie voordat de cisplatine het oor bereikte. Bovendien werden de cavia's dagelijks behandeld met een injectie van 75 μg/ml α–MSH of een zoutoplossing totdat een 40 dB gehoorverlies bij 8 kHz optrad. Dit gehoorverlies werd in de zoutgroep na 2 tot 3 dagen intracochleaire toediening van cisplatine bereikt. Bij de dieren die behandeld waren met α–MSH trad dit gehoorverlies een dag later op. De haarceltellingen, welke gedaan werden op het moment dat de dieren de 40 dB drempelverlies bereikt hadden, lieten geen verschil zien tussen de beide behandelingen. Dit duidt erop dat beide behandelde groepen hetzelfde eindpunt bereikt hadden. Met andere woorden α–MSH is in dit nieuwe diermodel in staat om de schadelijke effecten van cisplatine met 1 dag uit te stellen. Daar cisplatine direct in de cochlea werd toegediend en α–MSH via een injectie, kan uit deze studie geconcludeerd worden dat de beschermende werking van α–MSH waarschijnlijk te maken heeft met een interactie met een van de celstructuren in de cochlea.

Om de bovenstaande studie te completeren werd, zoals beschreven in hoofdstuk 6, een experiment met de omgekeerde opzet uitgevoerd. Cavia's werden geïmplanteerd met een permanente elektrode en een osmotisch pompje dat met een snelheid van 0.25 μl/uur een zout-, of een α–MSH-oplossing (0.02, 2 of 20 μg/ml) in de cochlea pompte. Daarnaast werden de dieren dagelijks via injecties behandeld met 2 mg/kg cisplatine totdat een drempelverlies van 40 dB bij 8 kHz optrad. Vervolgens werd de cisplatinebehandeling gestaakt, maar de electrocochleografie en de intracochleaire toediening van α–MSH werden

nog gedurende 10 dagen voortgezet om een eventueel herstel te kunnen meten. De norm voor het gehoorverlies werd na 5 tot 11 dagen cisplatine-behandeling bereikt, wat overeen komt met eerder gevonden resultaten. Uit dit onderzoek bleek verder dat vooral de groep dieren met 2 μg/ml α–MSH in de osmotische pomp minder verlies liet zien van de CAP drempelwaardes bij lage frequenties. Ook het haarcelverlies in de basale winding was voor deze groep dieren significant minder dan in de met zout behandelde dieren. De dieren met 0.02 μg/ml of 20 μg/ml α–MSH lieten geen significant beter herstel zien dan de met zout behandelde dieren. Kortom: de resultaten uit deze studie laten wederom zien dat α–MSH een bescherming biedt tegen cisplatine gehoorschade. Omdat α–MSH lokaal toegediend is kunnen we, overeenkomstig de eerder gevonden resultaten (hoofdstuk 5), ervan uitgaan dat de beschermende werking van α–MSH bij cisplatine gehoorschade te maken heeft met één van de cochleaire celstructuren, hoogstwaarschijnlijk de stria vascularis of de spirale ganglioncellen.

Met de resultaten van dit promotieonderzoek hopen we een bijdrage te kunnen leveren aan een beter inzicht in de beschermende effecten van α–MSH op de door cisplatine veroorzaakte gehoorschade, wat vervolgens weer een handvat zou kunnen bieden om cisplatine-ototoxiciteit in mensen te behandelen. Uiteindelijk zou deze kennis mogelijk ook van pas kunnen komen bij de behandeling van gehoorschade veroorzaakt door behandeling met carboplatine, aminoglycosides of zelfs bij de behandeling van gehoorschade veroorzaakt door lawaai. We hebben aangetoond dat α–MSH de door cisplatine veroorzaakte gehoorschade niet in alle gevallen uitstelt, maar dat behandeling wel een beter herstel van de gehoordrempel teweeg kan brengen en dat behandeling met α–MSH gedeeltelijk kan beschermen tegen buitenste haarcelverlies. Het experiment waarbij cisplatine lokaal aangeboden werd, demonstreerde dat de beschermende werking van α–MSH niet veroorzaakt wordt door een directe interactie met cisplatine of door bevordering van de uitscheiding van cisplatine, maar dat de beschermende werking van α–MSH waarschijnlijk te maken heeft met effecten op één van de celstructuren in de cochlea, mogelijk de stria vascularis of de spirale ganglioncellen. De resultaten van dit proefschrift bevestigen dat α–MSH gebruikt zou kunnen worden om de door cisplatine veroorzaakte schadelijke bijwerkingen op het gehoor te behandelen. Ook het feit dat α–MSH en analoge stoffen weinig bijwerkingen laten zien in klinische studies pleit voor de introductie van melanocortinen bij de behandeling van cisplatine ototoxiciteit. Dit proefschrift laat echter ook zien dat de beschermende effecten van de melanocortinen betrekkelijk klein zijn en dat er veel variabiliteit is tussen de verschillende individuen. Dit staat de

introductie van deze peptiden als otoprotectieve agentia bij patiënten in de weg. Meer onderzoek, met name naar de dosis-effect relatie is nodig. Bovendien blijkt het mechanisme waardoor cisplatine zijn schadelijke effecten in het oor teweegbrengt zo complex dat ook op dit punt meer onderzoek nodig is. Zowel het cochleair doelorgaan als het precieze cellulaire mechanisme dat betrokken is bij de beschermende effecten van de melanocortinen zijn tot op heden nog niet bekend. Toch heeft het onderzoek, beschreven in dit proefschrift, ons én een stap dichter bij het ontrafelen van het mechanisme van door cisplatine veroorzaakte gehoorschade gebracht, én bovendien dichter bij een middel om het oor tegen het sterk ototoxische cisplatine te beschermen.

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Gedurende de afgelopen vier jaar heb ik in Utrecht met veel plezier aan mijn promotieonderzoek gewerkt. De resultaten die ik met dit onderzoek verkregen heb, mocht ik op een aantal belangrijke internationale congressen presenteren. Zo heb ik congressen bijgewoond in Uppsala (Zweden), Florida (USA), Luik (België) en Dresden (Duitsland). Daarnaast heb ik gedurende 3 maanden extra ervaring op kunnen doen in Ann Arbor (USA) op het gebied van het implanteren van osmotische pompjes. Maar één van de hoogtepunten was de avond van Wetenschap en Maatschappij in oktober 2001. Als vertegenwoordiger van de belangenvereniging AIO's/OIO's Utrecht (BAU) mocht ik een diner in de Ridderzaal bijwonen, waar een groot aantal prominenten uit zowel de wetenschap, de politiek als de sport voor waren uitgenodigd. Gedurende deze avond heb ik met een aantal bekende Nederlanders waaronder Z.K.H. Prins Willem Alexander, Mevr. Hanja Maj-Weggen, Thom de Graaf en de directeuren van Philips en Unilever van gedachten kunnen wisselen over de voor- en nadelen van het promoveren. Een promotie heeft namelijk naast hele leuke momenten toch ook moeilijke momenten. De beruchte "man met de hamer" ben ik ook zeker tegengekomen. Maar dankzij het luisterend oor en de vele motiverende opmerkingen van familie, vrienden en collega's heb ik me gelukkig ook door deze moeilijke periodes heen kunnen slaan.

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Curriculum Vitae

Ciska Wolters werd op 29 mei 1977 geboren te Leidschendam. Aan het St. Maartenscollege te Voorburg werd in 1994 het V.W.O. diploma behaald. In datzelfde jaar werd begonnen met de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. Tijdens haar doctoraal studie werden stages gelopen bij de sectie Farmacologie van het Leiden Amsterdam Center of Drug Research (LACDR), Universiteit Leiden, onder begeleiding van drs. S.A.G. Visser, dr. P.H. van der Graaf en prof. dr. M. Danhof en bij de Medical Toxicology Unit in London, Verenigd Koninkrijk onder begeleiding van dr. M. Ruprah, dr. B. Widdop, prof. dr. F.A. de Wolff en prof. dr. G.J. Mulder. In augustus 1999 werd het doctoraal diploma in de Bio-Farmaceutische Wetenschappen behaald. Van oktober 1999 tot oktober 2003 werkte zij als assistent in opleiding bij de vakgroep Experimentele Audiologie, Universitair Medisch Centrum Utrecht (promotor prof. dr. G.F. Smoorenburg, co-promotores dr. S.F.L. Klis en dr. F.P.T. Hamers), alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd. Tijdens dit promotieonderzoek werd van mei tot augustus 2000 een werkbezoek gebracht aan het Kresge Hearing Research Institute, Universiteit van Michigan in Ann Arbor, Verenigde Staten onder begeleiding van mevr. D.M. Prieskorn en prof. dr. J.M. Miller. Sinds juli 2003 is de auteur werkzaam als scientist bij Yamanouchi Europe bv te Leiderdorp.

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