Co-treatment with melanotan-II, a potent melanocortin, does not protect against cisplatin ototoxicity
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, \(\alpha\)-melanocyte stimulating hormone (\(\alpha\)-MSH) and melanotan-II (MT-II). In albino guinea pigs, our group has found that ORG 2766 and \(\alpha\)-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 \(\mu\)g/kg/day s.c.) or saline until a 40 dB suppression of the compound action potential (CAP) threshold (3 \(\mu\)V 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]NH2; 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

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

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 $\geq 40$ dB at 8 kHz was 5 to 18 days of treatment (Fig. 1).
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 (O) 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.](image-url)
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.,


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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