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Research paper

Prevention of noise- and drug-induced hearing loss with D-methionine

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Abstract

A number of otoprotective agents are currently being investigated. Various types of agents have been found in animal studies to protect against hearing loss induced by cisplatin, carboplatin, aminoglycosides, or noise exposure. For over a decade we have been investigating D-methionine (D-met) as an otoprotective agent. Studies in our laboratory and others around the world have documented D-met's otoprotective action, in a variety of species, against a variety of ototoxic insults including cisplatin-, carboplatin-, aminoglycoside- and noise-induced auditory threshold elevations and cochlear hair cell loss. For cisplatin-induced ototoxicity, protection of the stria vascularis has also been documented. Further D-met has an excellent safety profile. D-Met may act as both a direct and indirect antioxidant. In this report, we provide the results of three experiments, expanding findings in D-met protection in three of our translational research areas: protection from platinum based chemotherapy-, aminoglycoside- and noise-induced hearing loss. These experiments demonstrate oral D-met protection against cisplatin-induced ototoxicity, D-met protection against amikacin-induced ototoxicity, and D-met rescue from permanent noise-induced hearing loss when D-met is initiated 1 h after noise exposure. These studies demonstrate some of the animal experiments needed as steps to translate a protective agent from bench to bedside.

Keywords: D-Methionine; Otoprotection; Protection; Ototoxicity; Hearing loss; Cisplatin; Noise-induced hearing loss; Aminoglycosides

1. Introduction

A number of otoprotective agents are currently under investigation for protection against hearing loss induced by platinum-based chemotherapeutics, aminoglycoside antibiotics, and noise exposure. Some protective agents are being studied largely to elucidate the mechanisms of these various etiologies of hearing loss rather than to develop a clinical treatment. Other agents are patented and sometimes licensed in anticipation of eventual clinical applicability, but are in the early pre-clinical development stages only. Others are in various translational research stages ranging from pre-clinical to in or approaching clinical trials. In this report we focus specifically on D-methionine (D-met) as an otoprotective agent, providing some of our translation research experiments with D-met for protection from cisplatin-induced and aminoglycoside-induced hearing loss and rescue from noise-induced hearing loss to support clinical trials in these areas. Translational research experiments focus on safety and efficacy, rather than mechanisms, of the protective agent to work towards FDA approval of the proposed protective agent.

For any type of medical disorder or condition, optimally a variety of pharmacologic agents is available to physicians and patients to provide treatment choices. Currently no drug is FDA approved to prevent or treat ototoxin-induced or noise-induced hearing loss. Hopefully within the next decade that situation will change and several otoprotective

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agents will be FDA approved and available for clinical use, potentially ameliorating or preventing hearing loss in thousands or perhaps millions of patients, world-wide every year. We are currently working towards the goal of developing D-met as one of those FDA approved agents in the future. We believe that D-met is one of the most promising otoprotective agents at this time.

We first reported in 1996 that D-met, the optical isomer of the essential amino acid L-methionine, significantly protected against cisplatin-induced auditory brainstem response (ABR) threshold shifts, outer hair cell loss and weight loss in rats (Campbell et al., 1996). Since that time, we, and others, have conducted a variety of studies demonstrating D-met's otoprotective action from a variety of cochlear insults, in several species, and with a variety of administration methods.

1.1. Cisplatin- and carboplatin-induced ototoxicity

D-Met protects against cisplatin-induced outer hair cell loss *in vivo* when D-met is administered systemically (Campbell et al., 1996; Reser et al., 1999) or on the round window with cisplatin delivered either systemically (Reser et al., 1999; Wimmer et al., 2004) or to the round window (Korver et al., 2002.) Systemic D-met also protects the stria vascularis against cisplatin damage in the rat (Campbell et al., 1999). Specifically, D-met reduces cisplatin-induced strial edema, protects the optical density ratios between the marginal cells and intermediate cells reflecting preservation of the marginal cell cytoplasmic organelles, and prevents bulging and compression of the marginal cells' luminal edges. For systemically administered carboplatin, injected D-met protects against the carboplatin-induced selective inner hair cell loss that occurs in the chinchilla (Lockwood et al., 2000).

Physiologically, D-met administration protects against both cisplatin-induced ABR threshold shifts (Campbell et al., 1996; Reser et al., 1999; Li et al., 2001) and distortion product otoacoustic emission amplitude (DPOAEs) decrements (Wimmer et al., 2004). Thus for platinum-based chemotherapeutics, D-met protects outer hair cell function as measured by DPOAEs, auditory thresholds as measured by ABR, and protects against damage to the outer hair cells and stria vascularis.

In addition to the D-met protection from cisplatin-induced ototoxicity, D-met also provides other systemic protections. D-Met significantly reduces cisplatin-induced weight loss in rats (Campbell et al., 1996.) *In vivo*, D-met also protects against nephrotoxicity as measured by blood urea nitrogen and serum creatinine levels, but does not significantly alter renal platinum levels (Jones and Basinger, 1989). *In vitro*, methionine reduces cisplatin cytotoxicity in renal cells as well as cisplatin uptake into the cells (Kroning et al., 2000). (Deegan et al., 1994a,b), noted that a cisplatin–methionine complex is significantly cytotoxic for tumor cells yet lacks the affiliated renal toxicity. When used in combination with brain derived neurotrophic factor, D-met also protects against cisplatin-induced loss of auditory neurons (Gabaizadeh et al., 1997).

1.2. Aminoglycoside-induced ototoxicity

Although less well studied, D-met partially protects against aminoglycoside-induced ototoxicity. Sha and Schacht (2000) administered twice daily injections of 200 mg/kg D-met 7 h apart in guinea pigs administered 120 mg/kg/d gentamicin for 19 days. Gentamicin-induced ABR threshold shifts were reduced from approximately 60 to 20 dB SPL at 18 kHz and from 50 to 30 dB SPL at 9 kHz.

Further, in studies to date, D-met does not appear to interfere with the antimicrobial efficacy of aminoglycoside antibiotics. In an *in vitro* study by Herr et al. (2001), we tested D-met in combination with amikacin and gentamicin against 10 clinical isolates each of *Escherichia coli* and *Staphylococcus aureus*. Two other antibiotics were also included; ceftriaxone and vancomycin (*S. aureus* only). The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC), defined as the minimum concentrations of the antibiotic required to inhibit growth or kill 99% of bacteria, respectively, were determined with and without D-met. Our data showed that D-met (0.01, 0.001 and 0.001 M) had no significant interference with the antimicrobial effectiveness of the tested antibiotics against the tested isolates. Further, Sha and Schacht (2000) documented that D-met does not alter serum levels of gentamicin. Therefore treatment efficacy should be retained even with D-met protection from ototoxicity and possibly nephrotoxicity. However further studies are planned.

D-Met may itself have antimicrobial activity and enhance the antimicrobial activity of other antibiotics. D-Met, at high concentration, had lethal effects against bacteria when used alone due to its incorporation into the peptidoglycan layer of the cell wall (Caparros et al., 1992). Because of the bacterial cell wall site of action, many studies concentrated more on the possible combinations of D-met with β-lactam antibiotics. D-Met increased the antimicrobial efficacy of imipenem and ampicillin against the ATCC 8739 strain of *E. coli* (Gillissen et al., 1991) and enhanced penicillin induced bacteriolysis against staphylococci (Wecke et al., 1992) *in vitro*. Because of the different mechanisms of actions of D-met and aminoglycosides, we anticipate that D-met will not interfere with, and may augment, the antimicrobial action of aminoglycosides.

1.3. Noise-induced hearing loss

We have also established that D-met significantly protected against permanent noise-induced hearing loss and both inner and outer hair cell loss when D-met was administered twice per day at 12 h intervals for two days prior to a 105 dB SPL 6 h 4 kHz octave band noise-exposure (Kopke et al., 2002). Although protection from temporary threshold shift was not obtained, significant D-met protec-

tion from ABR threshold shift for all test frequencies (2, 4, 6, and 8 kHz) was noted 7 days after noise exposure, with the difference between the D-met protected and noise-exposed control groups even greater at 14 and 21 days after noise exposure. We have been conducting additional experiments with D-met protection and rescue from noise exposure one of which is presented in this paper.

1.4. Species comparisons

D-Met's otoprotective action has been documented in a variety of species. For *in vivo* studies, in the Wistar rat, both systemic and round window D-met administration protects against cisplatin-induced ototoxicity (Campbell et al., 1996, 1999; Reser et al., 1999). In pigmented guinea pigs, D-met protects against cisplatin-induced (Wimmer et al., 2004) and gentamicin-induced ototoxicity (Sha and Schacht, 2000). In the chinchilla, D-met protects against topical cisplatin-induced ototoxicity (Korver et al., 2002), systemic carboplatin-induced ototoxicity (Lockwood et al., 2000) and noise-induced hearing loss (Coleman et al., 2002; Kopke et al., 2002). For *in vitro* studies, D-met protects against; cisplatin-induced outer hair cell loss, and in conjunction with brain derived neurotrophic factor, protects against both cisplatin-induced spiral ganglion cell and outer hair cell loss in organ of Corti explants harvested from 3 or 4 day post-partum Wistar rats (Kopke et al., 1997; Gabaizadeh et al., 1997). Different animal species are frequently used for experiments using different types of ototoxins or for different methods of delivery. For systemic cisplatin administration, the Wistar rat is a well established model of ototoxicity for studies of D-met protection (Campbell et al., 1996, 1999; Reser et al., 1999). For over two decades rats, usually either Wistar or Fischer strains, have been used for a variety of systemic cisplatin ototoxicity studies (Ohtani et al., 1984a,b; Amsallen and Andrieu-Guitrancourt, 1985; Rybak et al., 1995). Unlike the chinchilla, a desert animal, rats have good renal clearance and cisplatin ototoxicity can be induced without high mortality. However, when cisplatin is applied topically to the round window, so that renal clearance is not an issue, the chinchilla can serve as an excellent model of topical cisplatin ototoxicity (Korver et al., 2002). For studies using round window application, the chinchilla has the advantage of a round window that is more easily accessible through the bulla than in the rat. Additionally the chinchilla has the same frequency range of hearing as humans, unlike other rodents such as the rat and guinea pig which have higher frequency ranges. For aminoglycosides, the most well established animal models are in guinea pigs including pigmented guinea pigs for gentamicin (Sha and Schacht, 2000) and kanamycin (Song et al., 1998), and albino guinea pigs for amikacin (Klemens et al., 2003) and tobramycin (Akiyoshi, 1978; Brummett and Fox, 1977; Brummett, 1978; Sato, 1983). For several decades the chinchilla has been a well established animal model for the study of noise-induced hearing loss (see Bohne, 1976; Bohne and Rabbitt, 1983; Kopke et al., 2002), partially because both auditory sensitivity and frequency range of hearing are very similar to humans. Using well established animal models for cisplatin-, aminoglycoside- and noise-induced hearing loss to test putative otoprotective agents allows comparison to earlier and minimizes animal use to develop new models.

1.5. Direct comparison to other agents

In studies comparing D-met with other otoprotective agents, D-met provides equal or better protection than the other studied agents. D-Met provided better protection from cisplatin-ototoxicity than glutathione (GSH), glutathione ester (GSHe), ebselen and 4-methylthiobenzoic acid *in vitro* (Kopke et al., 1997) and GSH and GSHe *in vivo* (Campbell et al., 1996, 2003a). L-Methionine also protects against cisplatin-induced ototoxicity *in vivo* (Reser et al., 1999; Li et al., 2001) and *in vitro* (Li et al., 2001) but D-met has a preferable safety profile as reviewed in Section 5. Wimmer et al., 2004 demonstrated that D-met provided better protection than sodium thiosulfate, fibroblast growth factor-2, brain derived neurotrophic factor or saline against cisplatin-induced ototoxicity, as measured by otoacoustic emissions, using a round window administration model (Wimmer et al., 2004). D-Met also provided better protection from aminoglycoside-induced ototoxicity than histidine (Sha and Schacht, 2000). For noise protection, D-met and acetyl-L carnitine (ALCAR) both provided virtually complete protection from permanent threshold shift (less than 10 dB) and OHC loss (less than 10%) secondary to a 6 h 105 dB HL 4 kHz octave band of noise. However, carbamathione provided poorer protection (than D-met or ALCAR) with OHC preservation, measuring only 30–40% vs. 60% in non-protected controls (Kopke et al., 2002). L-*N*-acetylcysteine (NAC), in combination with salicylate showed substantially less OHC preservation (50-60%) (Kopke et al., 2000) for the same noise-exposure protocol, when compared to D-met and ALCAR which both provided over 90% OHC preservation (Kopke et al., 2002). Further, Coleman et al. (2002) reported that pre-administration of a combination of low-dose D-met and NAC markedly reduced noise-induced permanent threshold shift in chinchillas, and that the protection afforded by the D-met component alone was similar to the combined administration of D-met and NAC. However when the NAC component was delivered alone, no protection was provided. Kopke et al., (2002) reported that both D-met and NAC pre-noise administration could protect against noise-induced hearing loss (NIHL) in the chinchilla. A number of other otoprotective agents have been investigated but have not yet been directly compared to D-met.

Study results from our laboratory and others support the view that D-met is one of the most promising otoprotective agents for potential clinical use. Hopefully within the next decade we will see several otoprotective agents approved by the FDA for clinical use.

1.6. Mechanisms of D-met otoprotection

D-Met may have multiple protective actions, but probably works primarily as a direct and indirect anti-oxidant. In 1995, Vogt established that, unlike many amino acids, methionine is reversibly oxidized and can serve as a free radical scavenger. Because free radical formation appears to be a primary mechanism in cisplatin-induced, aminoglycoside-induced, and noise-induced hearing loss (Yamane et al., 1995; Kopke et al., 1997; Ohlemiller et al., 1999) D-met's antioxidant actions could account for why D-met protects against all three of these types of cochlear insults. For cisplatin-induced hearing loss, D-met may also act as a sulfur containing nucleophile and thus protect sulfur containing enzymes and proteins (Melvik and Pettersen, 1987; Jones and Basinger, 1989; Miller and House, 1990; Jones et al., 1991a,b). The bound methionine–cisplatin complex retains most of its anti-tumor activity (Deegan et al., 1994a,b).

D-Met's action as a sulfur containing nucleophile could not explain its protective action against aminoglycoside-induced and noise-induced hearing loss. Thus D-met's primary protective action is probably through direct and indirect antioxidant action. However, D-met appears to have multiple antioxidant actions. At least part of the protection may be through protection of critical enzymes. D-Met significantly protects against cisplatin-induced decrements in cochlear superoxide dismutase, catalase, and glutathione reductase levels but not glutathione peroxidase levels (Campbell et al., 2003c). D-Met also significantly protects against malondialdehyde elevation (Campbell et al., 2003c). In the guinea pig vestibular labyrinth, D-met reduces cisplatin-induced decrements in Na+K+-ATPase and Ca+-ATPase activities while decreasing nitric oxide (NO) concentrations and lipid peroxidation while preserving the vestibulo-ocular reflex (Cheng et al., 2006). Similarly D-met reduces cisplatin-induced decrements in Na+K+-ATPase and Ca+-ATPase activities in the cochlear lateral wall, while preserving ABR thresholds (Cheng et al., 2005).

Methionine has also been reported to increase intracellular glutathione levels (Lu, 1998) and specifically mitochondrial glutathione (Fernandez-Checa et al., 1998) Further methionine may prevent the usual efflux of glutathione from the cell that can occur as a consequence of cellular injury (Ghibelli et al., 1998) thus providing another mechanism by which methionine may increase and protect intracellular glutathione levels. For noise-induced hearing loss, the glutathione pathway may play a particularly important role in D-met's otoprotective action. The probability of cochlear hair cell survival following noise exposure is related to mitochondrial function (Hyde and Rubel, 1995). Thus methionine's ability to increase mitochondrial glutathione levels may be directly related to its otoprotective action against noise-induced hearing loss. Further noise exposure alters the ratio between reduced and oxidized glutathione in the cochlea (Bobbin et al., 1995; Yamasoba et al., 1998a; Campbell et al., 2003b) and inhibiting glutathione increases noise-induced hearing loss (Yamasoba et al., 1998b). Thus methionine may act as an indirect antioxidant in by increasing intracellular glutathione levels, particularly mitochondrial glutathione levels, preventing the egress of glutathione from the injured cell, preserving or improving the ratio of reduced to oxidized glutathione in the cochlea or as a direct antioxidant as it is reversibly oxidized.

Aminoglycosides do not alter cochlear glutathione levels (Lautermann et al., 1997) but nonetheless if D-met augmented intracellular and particularly mitochondrial glutathione levels, that action could be protective. However additional mechanisms may be involved. Gentamicin induces reactive oxygen species and nitric oxide (NO) in the guinea pig vestibular sensory cells (Takumida and Anniko, 2001, 2002; Takumida et al., 2003). D-Met suppresses reactive oxygen species production in turn stimulating NO production and significantly protecting sensory cells (Takumida and Anniko, 2002; Takumida et al., 2003). Further research regarding the mechanisms of D-met protection from aminoglycoside-induced hearing loss is warranted.

As a part of the translational research process, including patenting and licensing D-met for clinical protection against cisplatin-induced, carboplatin-induced, aminoglycoside-induced and noise-induced hearing loss, we have conducted a variety of experiments for over a decade to support translational research development. Three studies demonstrating various aspects of D-met protection from cisplatin-induced, aminoglycoside-induced, and noise-induced hearing loss are provided as follows. Southern Illinois University institutional guidelines regarding animal experimentation were followed for all experiments.

2. Experiment #1: comparing oral and injected D-met in protecting against cisplatin-induced ototoxicity and weight loss

In this experiment we compared the proprietary oral D-met formulation (MRX 1024) to injected D-met in protecting against cisplatin-induced auditory brainstem response (ABR) threshold shifts. Oral D-met, if equally efficacious, would be easier and less expensive to administer than injected D-met in the clinical setting. Reducing cost and administration time of a protective agent can increase acceptance of its use by medical staff and patients. The purpose of this study was to determine if oral D-met can provide equivalent protection to injected D-met in preventing cisplatin-induced ABR threshold shift. The long-term objective is to prevent cisplatin-induced ototoxic hearing loss in patients receiving platinum chemotherapy.

2.1. Subjects

Six groups of five male Wistar rats each served as subjects. Group 1 comprised a normal control group receiving

saline injection only in equal volume to the injected D-met of groups 3 and 5. Group 2 comprised a treated control group given a 30 min infusion of 16 mg/kg cisplatin. Group 3 received 300 mg/kg D-met delivered i.p. 30 min before cisplatin infusion. This i.p. D-Met dose is known to fully protect against cisplatin-induced ABR threshold elevations (Campbell et al., 1996). Group 4 received 1000 mg/kg (200 mg/ml concentration) oral D-met delivered by gavage 2 h before infusion of 16 mg/kg cisplatin. Group 5 received only 300 mg/kg i.p D-met only. Group 6 received 1000 mg/kg (200 mg/ml concentration) D-met only delivered orally by gavage.

2.2. Electrophysiologic measures

ABR data collection was obtained at baseline just prior to drug delivery and again 3 days after drug administration, with an Intelligent Hearing Systems evoked potential unit. ABR thresholds were measured in response to tone-burst stimuli with 1 ms rise/fall and 0 ms plateaus gated by a Blackman envelope and centered at frequencies of 4, 8, 14, 20, and 30 kHz. An intensity series was obtained for each animal from 100 to 0 dB sound pressure levels (SPL) for tone-bursts in 10 dB decrements.

Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable ABR. Five hundred sweeps constituted each average. The recording epochs were 15 ms following stimulus onset. Responses were analog filtered with a 30–3000 Hz bandpass. Rectal temperatures were monitored throughout recordings with animal temperature maintained by a warming pad. All animals were fully anesthetized throughout all ABR procedures and prior to sacrifice with 1 ml/mg IM of Rompun cocktail (a solution containing 86.21 mg/ml ketamine and 2.76 mg/ml xylazine) which was supplemented as needed with half doses.

2.3. Statistics

The electrophysiologic data were analyzed with a repeated measures ANOVA. Overall error rate was held at 0.05 using a Bonferroni procedure. Weight data were analyzed using a one way univariate ANOVA.

2.4. Results

Both the injected and oral D-met completely protected against the cisplatin-induced ABR threshold elevations at all frequencies tested. ABR threshold shifts for all six groups of animals are presented in Fig. 1 for ABR stimuli of 4, 8, 14, 20 and 30 kHz. ABR threshold elevations for the oral and injected D-met alone groups and for the oral and injected D-met plus cisplatin groups were not significantly different from the saline control group at all frequencies tested but were significantly different from the cisplatin alone group ($p \leqslant 0.001$). The cisplatin alone group showed

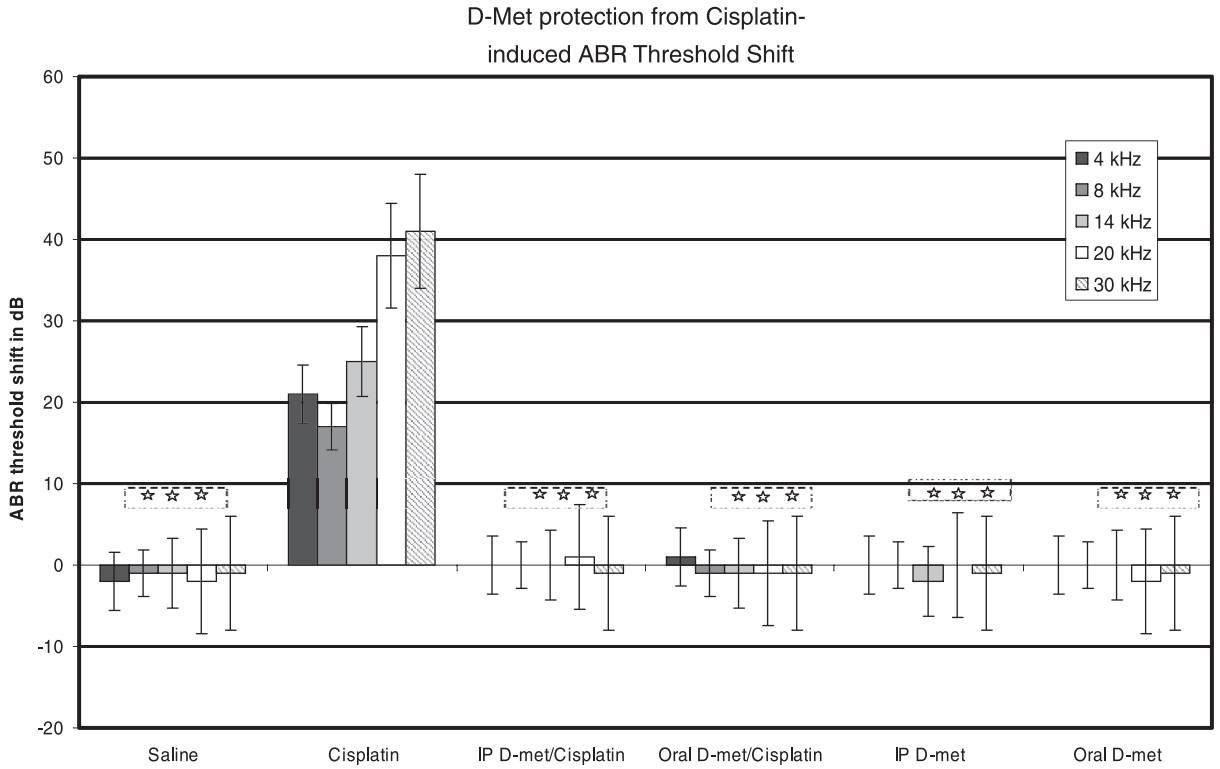

Fig. 1. ABR threshold shifts in response to 4, 8, 14, 20 and 30 kHz tone-bursts for five male Wistar rats per group receiving either: (a) saline injections only, (b) 16 mg/kg cisplatin alone, (c) 300 mg/kg ip D-met 30 min prior to 16 mg/kg D-met, (d) 1000 mg/kg oral D-met suspension 2 h prior to 16 mg/kg cisplatin, (e) 300 mg/kg D-met alone or (f) 1000 mg/kg alone. Three stars indicate that the results for all frequencies for that group were significantly different from the cisplatin alone group at the 0.001 level.

significantly greater ABR threshold shift when compared with every other group at all frequencies tested.

Injected D-met partially but significantly protected against cisplatin-induced weight loss. Oral D-met did not significantly protect against cisplatin-induced weight loss although the amount of weight loss was slightly less in the oral D-met plus cisplatin group as opposed to the cisplatin only group. Both the oral D-met alone and injected D-met alone groups showed results no different from the saline alone group although the oral D-met alone group gained slightly more weight than the saline alone group (Fig. 2).

2.5. Conclusions

Oral D-met is equally effective as injected D-met in protecting against cisplatin-induced ABR threshold shift. Both oral D-met and injected D-met completely protected against ABR threshold shift at all test frequencies of 4, 8, 14, 20, and 30 kHz. Thus the use of oral D-met in clinical trials and potentially in clinical treatment appears promising in protecting against cisplatin-induced hearing loss.

Injected D-met provided greater protection than oral D-met from cisplatin-induced weight loss. Oral D-met's lack of significant protection from cisplatin-induced weight loss does not appear to be secondary to nausea or anorexia secondary to the fluid volume administered. The group receiving oral D-met alone, which received the same oral D-met dose as the oral D-met plus cisplatin group, gained slightly more weight than the saline alone group. Thus oral D-met alone seems to augment rather than inhibit weight gain. Oral D-met's lack of significant protection from cisplatin-induced weight loss in this study could be attributed to several factors; but it is likely secondary to the differences in the timing of administering the oral vs. injected D-met in this study. We had previously established that injected D-met administered 30 min prior to 16 mg/kg cisplatin significantly protected against weight loss (Campbell et al., 1996). For the oral D-met we allowed 2 h prior to the cisplatin to allow for distribution through the gastrointestinal system and to prevent aspiration when the animal was under sedation for the 30 min cisplatin infusion. However the 2 h epoch between oral D-met administration and cisplatin infusion may have allowed the D-met to clear from the gastrointestinal system, but not the cochlea, prior to the cisplatin infusion thus reducing its protection of the gastrointestinal system for that timing of administration. Further research regarding whether the timing of oral D-met administration can increase gastrointestinal protection from cisplatin exposure is warranted. However the complete protection from cisplatin-induced ABR threshold elevation with either injected or oral D-met administration is encouraging.

3. Experiment # 2: D-met protection from amikacin-induced ototoxicity

In our early proof of concept work on D-met as an otoprotective agent we tested to determine if D-met could protect against aminoglycoside-induced hearing loss. We used amikacin as our model because it is one of the aminoglycosides that is commonly used clinically for long term administration that is primarily cochleotoxic as opposed to vestibulotoxic.

3.1. Subjects

Three groups of five male Hartley white guinea pigs (250–300 g) were included in the study. Group 1 was a control group receiving equivalent volume saline injection. Group 2 was a treated control group receiving 200 mg/kg/day amikacin for 28 days which has been previously shown to cause ototoxicity (Nishida and Takumida, 1996). Group 3 was the experimental group receiving 300 mg/kg ip D-met 30 min prior each dose of 200 mg/kg/day amikacin for 28 days.

ABR was performed immediately prior to any injections and again at 28 days, just prior to sacrifice. Subcutaneous

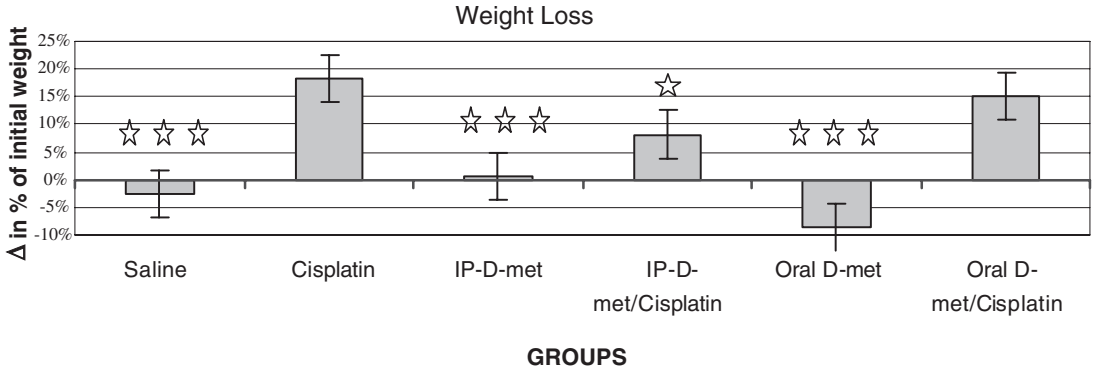

Fig. 2. Weight loss relative to initial weight for five male Wistar rats per group receiving either: (a) saline injections only, (b) 16 mg/kg cisplatin alone, (c) 300 mg/kg ip D-met 30 min prior to 16 mg/kg D-met, (d) 1000 mg/kg oral D-met suspension 2 h prior to 16 mg/kg cisplatin, (e) 300 mg/kg D-met alone or (f) 1000 mg/kg alone. Three stars indicate that the results for that group were significantly different from the cisplatin alone group at the 0.001 level. A single star indicates that results were different from the cisplatin alone group at the 0.05 level.

electrodes were placed at the vertex (non-inverting), to a point directly below the ipsilateral pinna (inverting) with a ground electrode in the hind leg.

ABR data collection was obtained with a customized Biologic Navigator system with an additional custom-made high frequency stimulator for 14 kHz. ABR thresholds were measured in response to tone-bursts centered at the frequencies of 1, 4, 8, and 14 kHz presented at 10/s. Tone-burst stimuli were gated by a Blackman envelope with 2 ms rise/fall for 1 kHz, and 1 ms rise/fall for 4, 8, and 14 kHz and 0 ms plateau. An intensity series was obtained for each animal from 100 dB sound pressure level (SPL) for tone-bursts, proceeding in 10 dB decrements to below threshold. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response.

A total of 512 sweeps constituted each average. The recording epochs were 15 ms following stimulus onset. Responses were analog filtered with a 30–3000 Hz bandpass. Rectal temperatures were monitored throughout recordings with animal temperature maintained by a warming pad. All animals were fully anesthetized throughout all ABR testing and prior to sacrifice with a 1 ml/kg IM injection of Rompun cocktail (a solution containing 86.21 mg/ml ketamine and 2.76 mg/ml xylazine) which was supplemented as needed with half doses.

3.2. Statistical analyses

Electrophysiologic measures (ABR thresholds for the various stimulus frequencies) were analyzed using a repeated measures ANOVA using the Bonferroni procedure to control overall significance level at the 0.05 level.

3.3. Results

ABR thresholds: ABR threshold shifts are presented in Fig. 3. As expected, there was no threshold shift in the untreated control group, and significant ($p \leqslant 0.05$) threshold shift occurred in the treated control group for all stimuli. For 1, and 4 kHz stimuli, ABR threshold shift was reduced in the experimental group with values not significantly different from either the treated or untreated control group, suggesting only partial protection. For the 8 kHz stimulus, the average ABR threshold shift was somewhat less than for the treated control group but was significantly greater than for the untreated control group. For the 14 kHz stimulus, the experimental group did show significantly less threshold shift than the treated control group but protection was still only partial. Although a significant difference between the treated control and experimental group was found only at 14 kHz, there was a definite trend toward otoprotection for all tone-burst stimuli. Average ABR threshold shift from the experimental group was consistently lower than for the treated control for all stimuli except clicks. The untreated control group had lower ABR thresholds than the treated control for all stimuli.

3.4. Conclusions

Our early work demonstrated that D-met can provide protection against aminoglycoside-induced ototoxicity but the ABR threshold protection was less than we and others have observed for cisplatin-induced ABR threshold shift. Sha and Schacht (2000) obtained similar levels of D-met otoprotection for gentamicin-induced ototoxicity in pigmented guinea pigs when using the same single D-met

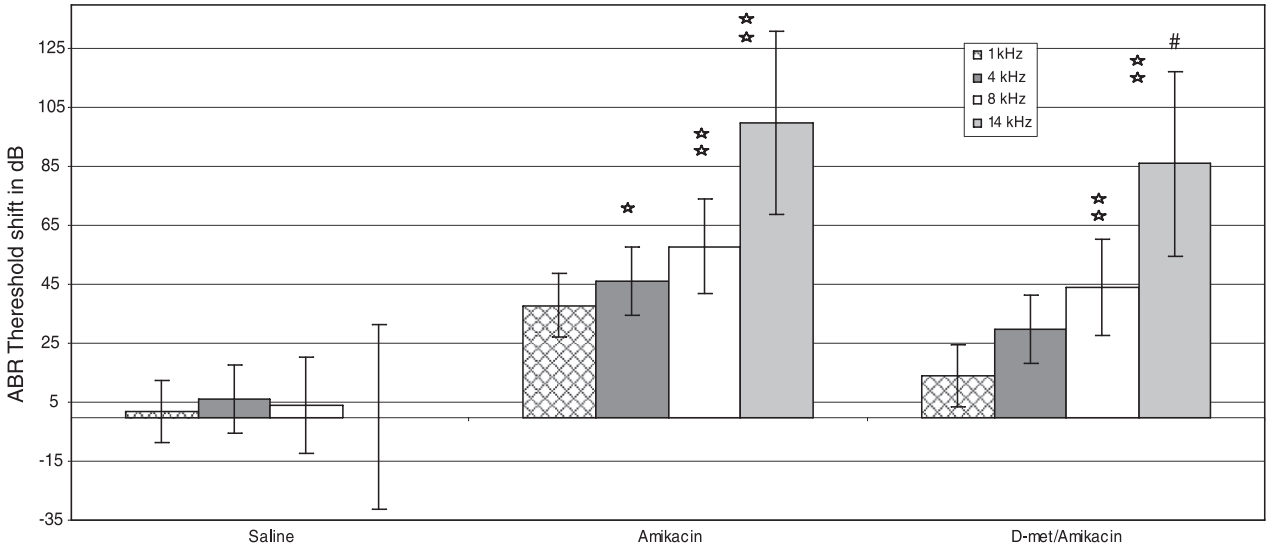

Fig. 3. ABR threshold shift from baseline, for tone-burst stimulus frequencies of 1, 4, 8, and 14 kHz. Three groups of five Harlan white guinea pigs each included a control group which received equivalent volume saline injection, a treated control group which received 200 mg/kg/day amikacin for 28 days and the experimental group which received 300 mg/kg ip D-met 30 min prior each dose of 200 mg/kg/day amikacin for 28 days. Results that are significantly different from the control group at the 0.01 or 0.001 level are indicated by two or three stars, respectively. Results significantly different from the amikacin alone group at the 0.05 level are indicated by a pound sign.

dosing paradigm. However, Sha and Schacht (2000) then conducted a further study using an additional dose of 300 mg/kg D-met 7 h after the first dose which significantly increased D-met's otoprotection against gentamicin-induced ototoxicity. From those data it cannot be determined if the additional ABR threshold protection was secondary to the timing of two doses per day of D-met or the increase in the overall daily dose. Further studies are needed to optimize D-met dosing for aminoglycoside otoprotection. Further research is also needed to determine if D-met protects against the ototoxicity of all aminoglycosides or only a select few such as amikacin and gentamicin. Theoretically it would seem that D-met could protect against the ototoxicity of all aminoglycosides but clearly differences exist in the ototoxicity of the various aminoglycosides in regard to relative risk of cochleotoxic vs. vestibulotoxic effects, the relationship of dosing to risk of ototoxicity, and the degree of risk in terms of both ototoxicity and nephrotoxicity.

Because evidence thus far suggests that D-met does not interfere with the serum levels or antimicrobial action of aminoglycosides (Sha and Schacht, 2000; Herr et al., 2001) and may in fact enhance antimicrobial action (Gillissen et al., 1991; Caparros et al., 1992; Wecke et al., 1992), the potential for developing D-met as a safe and effective otoprotective agent for aminoglycoside use appears promising.

4. Experiment # 3: D-met rescue from noise-induced hearing loss

We have previously documented that D-met protects against permanent noise-induced hearing loss when delivered both 48 h before and 48 h after a 6 h 105 dB SPL 4 kHz narrow band of noise exposure (Kopke et al., 2002). In this experiment we tested to determine if D-met could rescue from noise-induced hearing loss when D-met was first initiated 1 h after the same noise exposure. Noise exposure cannot always be anticipated particularly among military personnel but also in civilian individuals in a number of situations such at attending concerts, or motor vehicle air bag detonation. Other individuals may not wish to ingest a protective agent chronically or in anticipation of noise exposure but may seek assistance if they notice tinnitus or a temporary threshold shift following noise exposure.

4.1. Subjects

Two groups of 10 male 3 year old chinchillas Laniger served as subjects for this study. In the experimental group, D-met (200 mg/kg per dose) was administered starting 1 h post noise exposure plus four additional doses BID (five doses) at 12 h intervals. The control group received equivalent volume saline injections at the same time intervals as the D-met.

4.2. Noise exposure

Prior to being exposed to noise, the animals were housed in the SIU Laboratory Animal Care (LAM) Facility for a minimum of 5 days. The animals were acclimated to the wire cages and sound exposure booth prior to noise exposure. The noise exposure was administered inside a sound booth housed in LAM. The sound booth isolated the noise exposures from the outside environment with no exposure to individuals outside the booth. Only the animal was in the booth during the exposures.

The sound cage did not require restraints. The noise exposure comprised a 6 h duration octave band noise centered at 4 kHz, generated by a TDT GNS 40X white noise generator, and routed through an attenuator (TDT PA3), a filter (Krohn-Hite 3384) and a power amplifier (Sony 55ES) to a custom-built acoustic exponential horn with a maximum output at 4 kHz using an Altec 290E driver. The loudspeaker was suspended directly above the cage. A water bottle was placed on the outside of the cage with the nozzle feeding into the cage, giving the animals free access to water during the noise exposure period. Each animal was exposed to the 4 kHz narrow band noise at a level of 105 dB SPL for 6 h.

4.3. Electrophysiologic studies

ABR was performed just prior to the noise exposure and again on post-exposure day 21. Subcutaneous electrodes were placed at the vertex (non-inverting), to a point directly below the ipsilateral pinna (inverting) with a ground electrode in the hind leg.

ABR data collection was obtained with an Intelligent Hearing Systems evoked potential unit. ABR thresholds were measured in response to tone-bursts with 1 ms rise/fall and 0 ms plateau gated by a Blackman envelope and centered at the frequencies of 2, 4, 6, and 8 kHz. An intensity series was obtained for each animal from 100 to 0 dB peak sound pressure level (SPL) for tone-bursts in 10 dB decrements. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response.

Five hundred sweeps constituted each average. The recording epochs were 15 ms following stimulus onset. Responses were analog filtered with a 30–3000 Hz bandpass. Rectal temperatures were monitored throughout recordings with animal temperature maintained by a warming pad. All animals were fully anesthetized throughout all ABR procedures and prior to sacrifice with 1 ml/mg IM of Rompun cocktail (a solution containing 86.21 mg/ml ketamine and 2.76 mg/ml xylazine) which was supplemented as needed with half doses.

4.4. Statistical analyses

The statistical analysis consisted of Wilcoxon's rank sum test on the difference scores between the two time

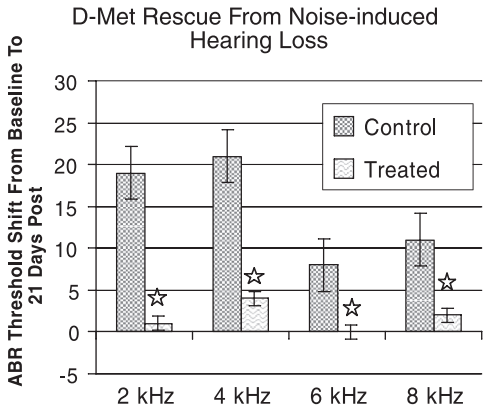

Fig. 4. ABR threshold shift from baseline to 21 days after a 105 dB SPL 6 h narrow band noise exposure centered at 4 kHz for two groups of 10 chinchillas each receiving either saline injections or 200 mg/kg D-met injections starting 1 h after cessation of the noise exposure and continuing for another four injections at 12 h intervals. Results significantly different from the control group at the 0.05 level are indicated by a star symbol.

points, for each of the four frequencies separately. Statistical significance was set at the 0.05 level.

4.5. Results

Initiating D-met treatment 1 h after a 6 h 105 dB SPL 4 kHz narrow band of noise significantly protected against permanent noise-induced ABR threshold shift at 2, 4, 6 and 8 kHz (Fig. 4). This administration protocol provided protection similar to that obtained in our earlier study starting the same dose of D-met 48 h prior to the noise exposure and then continuing for another 48 h after the noise exposure (Kopke et al., 2002).

4.6. Conclusions

When D-met administration is initiated within 1 h after noise exposure complete protection from permanent noise-induced hearing loss in the chinchilla at 21 days can be obtained, at least for the noise exposure paradigm in this study. We are currently conducting further studies to determine how long D-met treatment can be delayed and still provide protection from permanent noise-induced hearing loss. Further research will also be needed to determine if D-met rescue can be obtained for other forms or levels of noise exposure such as blast injury.

5. Discussion

For FDA approval and clinical use, agents must be both safe and effective. Thus far in animal studies, as reviewed in this paper, D-met has shown efficacy in protecting against cisplatin-induced, carboplatin-induced, aminoglycoside-induced ototoxicity and permanent noise-induced hearing loss. The additional experiments presented in this paper provide further support for future clinical trials. At this point in time, D-met appears promising as an otoprotective agent but the clinical trials process will clarify whether or not D-met is equally effective in humans as it has been in animal studies to date. Currently we are in clinical trials using oral D-met to protect against radiation-induced oral mucositis. The Investigational New Drug Application (IND) was approved by the FDA for that application in January 2005.

In the first experiment in this report, we documented that oral D-met can provide equivalent otoprotection to injected D-met. Oral D-met has been studied in animals and humans for decades for both dietary and therapeutic considerations (Kies et al., 1975; Meyer et al., 1985; Stegink et al., 1986; Kaji et al., 1987); thus it has long been known that D-met can be absorbed through oral ingestion. D-Met is commonly found in fermented proteins such as cheese and yogurt, and thus is not foreign to the human system. However confirming in animal studies that oral D-met can also adequately protect the cochlea from cisplatin-induced ototoxicity is useful by the providing proof of concept documentation in preparing for clinical trials. An oral preparation could reduce costs, and increase both patient and physician acceptance. Further oral administration can enhance safety by eliminating additional parenteral administration and the inherent considerations accompanying that route. Because in animal studies D-met can provide virtually complete protection from cisplatin-induced ABR threshold elevation and cochlear hair cell loss (Campbell et al., 1996; Reser et al., 1999; Korver et al., 2002; Wimmer et al., 2004) potential clinical utility appears promising. However, only the clinical trials process can verify safety and efficacy of a drug for any clinical treatment.

In the second experiment we presented some of our earlier proof of concept work documenting that D-met can ameliorate amikacin-induced ototoxicity. The protection from amikacin-induced ototoxicity was less than that observed for D-met's protection against cisplatin-induced ABR threshold shift as in Experiment 1, but did document that protection from aminoglycoside otoprotection could occur. Sha and Schacht (2000) documented that twice daily, as opposed to once daily, administration of the same dose of D-met significantly improved protection from gentamicin-induced ototoxicity. Hopefully more work on D-met dosing paradigms will not only enhance the degree of protection from gentamicin- and amikacin-induced ototoxicity but also allow us to protect against the ototoxicity of other aminoglycosides such as tobramycin, kanamycin and neomycin. Sometimes the utilization of these aminoglycosides is curtailed specifically because of ototoxicity.

In the third experiment, we demonstrated that in chinchillas, D-met, even when first administered 1 h after noise exposure can significantly protect against permanent noise-induced hearing loss for a 6 h 105 dB SPL 4 kHz narrow band of noise. Previously, we have documented that administering D-met for 48 h before and 48 h after the same noise exposure paradigm could significantly protect against permanent noise-induced ABR threshold shift and cochlear hair cell loss (Kopke et al., 2002). However not all noise

exposures can be anticipated 48 h in advance. Many occupations such as firefighters, police, emergency medical personnel, military personnel, and miners may be exposed to hazardous noise with little advance warning. Other individuals may be exposed to unexpectedly high noise levels at concerts, in or around some aircraft, or in the case of car airbag deployment. Thus a drug that can be used after noise exposure may be clinically useful.

D-Met may be particularly useful because, unlike some of the other otoprotective agents, it has been shown to provide protection against cisplatin-, carboplatin-, aminoglycoside-, and noise-induced hearing loss. Some patients may be exposed to a combination of these risk factors for hearing loss. Thus having an agent that can be safely and effectively used even with a combination of exposures could be helpful. Further methionine, including the d isomer D-met, has been studied for decades as an amino acid, particularly in nutrition studies. Therefore the safety and pharmacokinetics are well characterized.

5.1. D-Met safety factors

One of the primary factors in selecting an otoprotective agent for potential clinical use is its safety. Naturally occurring compounds that are already part of foodstuffs have the advantage of frequently being well studied, and not being alien to the human system. Methionine has been well studied for decades. Dietary high quality protein contains 26 mg/g methionine (National Research Council, National Academy of Sciences 1980). D-Met occurs in particularly high levels of fermented proteins such as cheese and yogurt because the fermentation process transaminates the *L* to the *D* isomer.

Methionine has been well studied as part of the diet of various species. However it has also been studied for high level administration in humans for non-dietary purposes. Therapeutically, methionine has been used at relatively high doses for several clinical applications. Both parenteral and oral D-met have been safely used in humans for a variety of applications such as radiographic imaging (Meyer et al., 1985) and many nutritional studies (Kies et al., 1975; Stegink et al., 1986; Kaji et al., 1987). Further methionine has been recommended for routine use for other applications. For example the World Health Organization (WHO, 1997) recommends methionine as an essential drug for treating acetaminophen toxicity secondary to overdose, although in the United States *N*-acetylcysteine is more commonly used. The recommended oral dosing of methionine, to counteract acetaminophen overdose, is an initial 2.5 g with three subsequent 2.5 g doses at 4 h intervals for a total dose of 10 g within 12 h. Additionally, for decades oral methionine has been available for over the counter use to reduce urinary odor and dermatitis. Recommended adult dosing is 200–400 mg 3–4 times per day (Drug Facts and Comparisons, 1991). In 1998, DiRocco et al. reported that they administered 3 g L-methionine twice per day for 6 months in 12 HIV-infected adults to treat vacuolar myelopathy with no side effects other than one complaint of nausea which may or may not have been related to the methionine. They further reported that even chronic administration of 20 g methionine per day per adult was safe. Monteagudo et al., 1986 reported that methionine was ''remarkably free of side effects'' including nausea and vomiting. Other human studies using methionine have also reported no side effects (Kaji et al., 1987; Kies et al., 1975; Stegink et al., 1986). Nonetheless, specifically in the presence of a low protein diet or in developing animals as opposed to adults, high dose racemic or L-methionine can cause toxicities (Cohen et al., 1958; Klavins et al., 1963; Klavins and Johansen, 1965; Daniel and Waisman, 1969; Benevenga, 1974; Muramatsu et al., 1971). However those dosing levels are well above the dosing anticipated for human therapeutic levels for otoprotection. Dosing levels will also be reviewed through the FDA process.

It should be noted that the studies demonstrating toxicity used either the racemate or L-methionine. Data suggest that D-met is substantially safer than the L isomer or racemate and that D-met in itself may not be toxic unless converted to the L-isomer (Stekol and Szaran, 1962; Walser et al., 1973; Blom et al., 1989; Friedman, 1999). Further, humans excrete 60–70% of D-met without transamination to the L-isomer (Printen et al., 1979; Baker, 1994) unless the subjects are L-methionine deprived (Rose et al., 1955). In humans, D-met also results in higher plasma levels than L-methionine which could may be advantageous for an otoprotective agent.

5.2. Conclusion

Based on research from our lab and many other labs, D-met appears to be one of the most promising otoprotective agents for platinum-based chemotherapeutic agents, aminoglycoside antibiotics, and noise exposures. Studies to date demonstrate efficacy in several animal species and safety factors of this amino acid have been studied for decades. However, only clinical trials will be able to truly establish its potential safety and efficacy in humans for these specific applications. Further both patients and physicians generally need a variety of treatment and or prophylaxis regimens for any medical disorder. Certainly, for the prevention and treatment of ototoxic and noise-induced hearing loss, it would seem advisable that a variety of otoprotective agents be developed through the clinical trials process for FDA approval.

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