

Combined oral delivery of ebselen and allopurinol reduces multiple cisplatin toxicities in rat breast and ovarian cancer models while enhancing anti-tumor activity

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The chemoprotective effects of combined ebselen and allopurinol in breast (MTLn3) and ovarian (NuTu-19) cancer models using a repeated cisplatin dosing schedule (6 mg/kg i.p. Z3 weeks) were studied. Otoprotection was evaluated using auditory evoked brainstem response (ABR) to determine threshold and latency shifts, and outer hair cell counts. Nephroprotection was analyzed

by serological markers [blood urea nitrogen (BUN) and creatinine] and histological evaluation. Myelotoxicity was quantified using cytological counts for platelets and changes in hematocrit. Hepatotoxicity was determined by changes in the serological markers amino alanine transferase (ALT) and aspartate amino transferase. Significant chemoprotective effects were observed for multiple organ systems including oto- (ABR threshold shifts for click and 24-kHz stimuli, $p < 0.05$, 8 and 16 kHz, $p < 0.01$, MTLn3 group; hair cell counts, $p < 0.05$ both groups), nephro- (BUN and creatinine, $p < 0.01$), myelo-(platelet $p < 0.05$, hematocrit $p < 0.05$) and hepatotoxicity (ALT $p < 0.05$) in rats receiving oral ebselen and allopurinol. Importantly, the anti-tumor activity of cisplatin was not

compromised. On the contrary, improved mortality, morbidity and outcome were observed in the ovarian cancer model. This combined oral formulation of ebselen and allopurinol is an attractive candidate for clinical evaluation. *Anti-Cancer Drugs* 16:569–579^C 2005 Lippincott Williams & Wilkins.

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Introduction

While cisplatin [cis-diamminedichloroplatinum(II)] has proven itself as a useful drug for a number of different solid tumors such as ovarian, breast and prostate cancer, its side-effects can lead to unacceptable toxicities including permanent sensorineural hearing loss, neuro-toxicity, renal damage and myelosuppression. Early audiologic studies in patients receiving cisplatin indicated that up to 90% will experience significant hearing loss, especially at high frequencies, and that these changes are irreversible and cumulative [1]. Long-term follow-up in pediatric patients shows that hearing loss associated with cisplatin exposure continues to worsen over time [2], is dose and age dependent, and risks potential impact on speech/language development. In adults, the interaction of chemotherapeutics with other auditory degenerative processes such as noise-induced hearing loss or aging can exacerbate the level of permanent disability in the speech range of hearing. Although we see an increase in audiologic monitoring programs for patients, the lack of effective chemoprotectants to treat cisplatin ototoxicity underscores the importance of addressing this unmet medical need. At present, no products are approved for the reduction

of cisplatin-related ototoxicity, neurotoxicity and myelotoxicity [3].

In the decades that have followed since the identification and characterization of cisplatin-related ototoxicity, enormous progress has been made in determining its biochemical mechanisms. Biochemical changes linked to cochlear and renal injury include an increase in free radicals or reactive oxygen and nitrogen species such as the superoxide anion (O_2^-) and nitric oxide (NO). In particular, peroxynitrite ($OONO^-$), a super radical formed from the combination of the superoxide anion and nitric oxide, causes lipid peroxidation, a process that severely injures hair cell membranes [4]. A change in the level of reduced glutathione and the activity of glutathione utilizing enzymes has been correlated with outer hair cell (OHC) loss due to cisplatin exposure [5– 7]. Cisplatin has been shown to increase xanthine oxidase (XO) activity in the kidney [8], while studies involving carboplatin exposure show a similar increase in XO activity in the cochlea [9].

Based on these biochemical findings we hypothesized that a combination of a glutathione peroxidase (GPx)

mimic and a XO inhibitor might be an effective cisplatin chemoprevention strategy. In a recent study, we demonstrated that the combined oral formulation of allopurinol, a XO inhibitor, and ebselen, a GPx mimic, was effective at preventing oto- and nephrotoxicity in an acute cisplatin dosing model [10]. Here we evaluated the efficacy of this chemoprotection strategy in a repeated (multiple-dose) cisplatin toxicity model. Fischer 344 (F-344) rats were injected with MTLn3 cells to develop breast tumors [11,12] or NuTu-19 cells to develop ovarian tumors [13,14]. We found no inhibition of cisplatin anti-neoplastic activity in the MTLn3 breast tumor model. Interestingly, we observed an enhanced anti-tumor activity of cisplatin in the NuTu-19 ovarian cancer model for rats treated with a combined oral formulation of ebselen and allopurinol. In addition, formulations containing ebselen and allopurinol afforded significant protection from cisplatin oto- and nephrotoxicity. Allopurinol is indicated for use in chemotherapy patients with hyperuricemia and has been shown to be otoprotective against noise [15,16]-, but not gentamicin [17]-induced hearing loss in animal models. Clinical experiences with cisplatin and allopurinol have involved the concurrent administration of 5-fluorouracil [18–20] with no reports of interference in anti-tumor activity of the chemotherapy.

Unfortunately, no studies evaluating the ototoxicity of cisplatin and allopurinol co-administration have been published. In previous studies with ebselen, a significant reduction of hair cell loss was achieved in cisplatin-treated neonatal rat cochlear cultures [21]. In live animal studies, ebselen significantly reduced cisplatin associated oto- [7] and nephrotoxicity [22,23], and did not interfere with cisplatin anti-tumor activity [24]. Ebselen has excellent oral availability [25] and has been evaluated in human clinical testing for the treatment of acute ischemic stroke where no adverse events were identified [26,27]. In our preclinical studies, a combined formulation of ebselen and allopurinol was developed that is well tolerated and effective at reducing cisplatin-associated oto- and nephrotoxicity. Our current studies were designed to evaluate the ebselen and allopurinol formulation on breast and ovarian tumors, and to test the chemoprotective effects in a repeated cisplatin dosing model.

Methods

Drug formulation and dosing

Solutions containing ebselen (SPI-3005): stock ebselen powder (> 98% pure; Sigma, St Louis, MO; cat. no. E-3520) was dissolved in pure dimethylsulfoxide at 20 mg/ml and stored at – 20°C for up to 1 week. The 20-mg/ml ebselen/DMSO stock solution is diluted in sterile normal saline to the appropriate concentration for oral gavage immediately prior to use. Solutions containing

allopurinol (SPI-3006): stock allopurinol powder (> 99% pure; Sigma; cat. no. A-8003) was dissolved in sterile 0.9% NaCl solution, pH 10, on the day of use. Typical volumes for oral dosing are around 0.5–0.6 ml of solution in a 150-g rat. Solutions containing cisplatin: stock cisplatin for injection at 1 mg/ml in sterile 0.9% NaCl solution, pH 3.8–5.9, was purchased from American Pharmaceuticals Partners (Schaumburg, IL). Cisplatin was dosed at 6 mg/kg over the course of 10 min to the i.p. cavity of isoflurane-anesthetized female F-344 rats on a weekly schedule for 3 continuous weeks. For oral administration of combined ebselen and allopurinol (SPI-3005/6), animals were gavaged 1 h prior to cisplatin administration at 8 mg/kg each and then twice daily for 2 days following cisplatin delivery at 4 mg/kg each. Vehicle-treated control rats for all studies were dosed on an identical schedule with similar volumes of vehicle on a per weight basis. All animals received 5 ml normal saline injected s.c. once per day for the 3 days following each cisplatin injection to improve hydration and animal health.

Animals

Female F-344 rats, 8 weeks of age, were purchased from Charles River (Wilmington, MA). Following 1 week of acclimation in our vivarium, isoflurane-anesthetized animals were implanted with a s.c. transponder (AllFlex, Dallas, TX) below the scapula for identification and tracking purposes. All rats used in this study were obtained from a specific pathogen-free (SPF) facility at Charles River and were maintained under SPF conditions in our vivarium. Routine monitoring of serological samples from sentinel rats confirmed the absence of pathogens known to be associated with hearing loss. Animals were fed a diet of irradiated rodent chow (Lab Diet Formula 5053) and water ad libitum. All procedures used on test animals were performed in accordance with the Animal Welfare Act of 1986 and following approval of the IACUC at the Institute for Systems Biology in Seattle, WA, where the animals were maintained and treated.

A total number of 55 F-344 rats were divided as follows: 23 were placed in the MTLn3 tumor group and 32 were used in the NuTu-19 tumor group. The MTLn3 animals were divided into a vehicle-treated control group (n = 8) and SPI-3005/6-treated group (n = 15). The NuTu-19 animals were divided into a vehicle-treated control group (n = 16) and SPI-3005/6-treated group (n = 16). Survivors from both MTLn3 and NuTu-19 tumor control groups were combined into one control group for analyses. Baseline weights were recorded for each animal entered into the study just prior to the first vehicle or SPI-3005/6 dose and monitored 5 times each week during subsequent doses. The final weight for each animal was measured just prior to sacrifice. Weight loss in the range of 20–30%, anticipated for F-344 rats undergoing chronic cisplatin treatment, was justified by exception in this special circumstance.

Tumor models

A breast cancer tumor model was established by injection of 10^6 MTLn3 cells into the fat pad of the lower left nipple of 8- to 10-week-old female F-344 rats. Prior to injection, MTLn3 cells were maintained in culture at 20–40% confluency. Rats injected with MTLn3 cells were allowed to develop tumor burden for 2 weeks prior to initiation of cisplatin treatment. Rats bearing MTLn3 tumor nodules that were local to the site of injection and measured greater than 0.5 cm (typically 0.6–1.0 cm) in diameter were entered into study. Generally, greater than 90% of the rats injected with MTLn3 met this criteria.

An ovarian cancer tumor model was established by injection of 10^7 NuTu-19 cells into the peritoneal cavity of 8- to 10-week-old female F-344 rats. Rats with NuTu-19 cells injected were allowed to develop tumor burden for 2 weeks prior to cisplatin treatment. A control series of 10 rats was evaluated separately for the development of ovarian tumor burden under the described conditions. All rats in the control series were sacrificed 5 weeks after NuTu-19 tumor cell injection and tumor burden was evaluated. In this series, all animals exhibited significant tumor burden exemplified by omental caking of multiple tumor nodules and large volumes of ascites (10–30 ml) in the peritoneal cavity (data not shown), paralleling previous published results with this rat tumor model [13]. A similar series of experiments was performed using 10^6 NuTu-19 cells injected i.p., resulting in only 70% of the animals exhibiting significant tumor burden at 5 weeks post-tumor inoculation (data not shown). To improve consistency in tumor formation and to create a more aggressive tumor burden, we chose to utilize the higher inoculation dose for NuTu-19 cells.

Tumor response to cisplatin treatment was analyzed in MTLn3 and NuTu-19 control and SPI-3005/6-treated groups upon necropsy of each animal. For the NuTu-19 tumor model: presence of ascites and omental tumor caking was considered non-responsive; absence of ascites but presence of more than five visible tumor nodules (each more than 0.5 mm) in the peritoneal cavity was considered a partial response; absence of ascites and presence of less than five visible tumor nodules (each more than 0.5 mm) was considered a complete response. For the MTLn3 tumor model: presence of a tumor in the injected fat pad with a diameter greater than 9 mm was considered a non-response; presence of a tumor at the site of injection with a diameter of 3–8 mm was considered a partial response; presence of a tumor less than 2 mm in diameter or absence of tumor was considered a complete response.

Cell lines and tissue culture

F-344 rat mammary adenocarcinoma clone MTLn3 cells were obtained from Dr Jeffery Segall (Albert Einstein

College of Medicine, NY). Cells were cultured in a-MEM with 5% FBS in the presence of penicillin and streptomycin. Cultures were subcultured when density reached 70–80% using 0.25% trypsin in Ca^{2+} / Mg^{2+} -free PBS. Cells were harvested using trypsin. Cell viability was determined by Trypan blue exclusion. Cells were brought to a density of 10^7 cells/ml for injection. Harvested cells with viability greater than 95% by Trypan blue exclusion were considered acceptable for injection. Cells were kept on ice for a maximum of 1 h prior to injection.

F-344 rat epithelial ovarian tumor clone NuTu-19 cells were obtained from Dr Thomas Hamilton under license from the Fox Chase Cancer Center. Initial attempts to replicate previously published tumor response with i.p. injection of these cells met with variable results. Consequently, the NuTu-19 cell line was re-derived from a metastatic clone obtained from the site of injection in one rat. This subclone closely resembled the characteristics of the NuTu-19 cell line in terms of aggressiveness, metastasis and ascites production, as previously described [13]. Cells were harvested using trypsin. Viable cells were counted using Trypan blue exclusion and brought to a density of 10^7 cells/ml for injection. Harvested cells with viability greater than 90% by Trypan blue exclusion were considered acceptable for injection. Cells were kept on ice for a maximum of 1 h prior to injection.

Auditory assessment

F-344 rats were anesthetized with inhaled isoflurane for auditory brainstem response (ABR) evaluation. Basal body temperature was maintained using a Gaymar T-pump warming pad set to 37°C, and the animals' health was monitored by observation of heart rate, respiration and circulation (e.g. skin color). Each ear was otoscopically inspected prior to placement of bilateral 3.5-mm insert eartips (Nicolet Biomedical, Madison, WI) for sound delivery. ABR was performed using the Smart EP high-frequency software/hardware package (Intelligent Hearing Systems, Miami, FL) with calibrated high-frequency transducers. Subdermal platinum needle electrodes (Grass Telefactor, West Warwick, RI; F-E2, 48 in) were placed with the active electrode at the vertex, the reference electrode ipsilateral to the test ear and the ground electrode contralateral to the test ear. Auditory stimuli included 100-ms clicks and rapidly gated 5-ms pure tone stimuli at 8, 16 and 24 kHz. Stimuli were presented at a rate of 19.3/s, and 800 sweeps were recorded and averaged at each intensity level. Thresholds were measured in 5-dB increments and defined visually by the presence of the most robust peak (III) that was replicable within 0.1 ms. Latency (III) values at threshold were also recorded for pre- and post-treatment comparisons. Only rats with normal ABR thresholds [9.2 ± 5 dB (2 SDs) click stimulus, –26.6 ± 5 dB at 8 kHz, 4.2 ± 5 dB at 16 kHz and 10.3 ± 5 dB at 24 kHz] were entered into

chemoprotection studies. ABR data were collected and analyzed by an ASHA-certified audiologist blinded to the treatment of each group.

Cytocochleogram analysis

Following animal sacrifice, the temporal bones were removed and a 1-mm hole was made at the apex of the cochlea with fine forceps. The cochleae were gently perfused with 4% paraformaldehyde (PFA) through the round window delivered by a 30-gauge needle on a 1-ml syringe. Samples were then immersed in 4% PFA for 1 h, rinsed in PBS and stored at 4°C in PBS for 1–4 weeks. The bony wall of the cochlea was removed with fine forceps in PBS under a dissection microscope. After the tectorial membrane was removed, the cochlea was carefully separated from the modiolus and decalcified with 0.5 M EDTA for 30 min. Cochlear tissue was then stained with 0.5 mg/ml FITC-phalloidin in PBS for 30 min. Microdissected turns of sensory epithelia from the whole cochlea were cut into three pieces (apical turn, basal turn and hook region) on a microscope slide in mounting media containing diamidino-2-phenylindole (DAPI). Digital images of cochleae were captured via a CCD camera (Princeton Instruments, Trenton, NJ; 1300-Y) mounted on an epifluorescent microscope (Nikon, Melville, NY; E800). Whole cochlear turn images were assembled from a series of digital images using Adobe Photoshop software. Missing OHCs were defined as the absence of a DAPI-stained nucleus and phalloidin-FITC-stained stereocilia in a region of Corti's organ where one would normally expect an OHC to be present. The number of missing OHCs along each 100 mm was counted and the percentage of OHC loss was calculated for each region. Typically, a total of 37–39 OHCs were present in a 100 mm of a normal rat cochlea.

Blood chemistry and cytology analysis

One day prior to cisplatin treatment, blood samples were collected from the tail vein of isoflurane-anesthetized rats for the analysis of serum creatinine and blood urea nitrogen (BUN), amino alanine transferase (ALT), aspartate amino transferase (AST), hematocrit and platelet counts. Following completion of post-treatment ABR data collection, animals were asphyxiated with CO₂ to cardiac arrest, and blood samples were collected by cardiac puncture into serum separation tubes (500 ml) and EDTA tubes (300 ml) for analysis. Blood chemistry analysis was performed using standard protocols on blinded samples sent to Phoenix Central Laboratory for Veterinarians (www.pclv.net; Everett, WA).

Kidney histological analysis

Kidneys were collected following CO₂ asphyxiation of rats and then fixed by immersion in PBS with 4% PFA for 1 h at room temperature. Following fixation, kidneys were stored at 4°C in PBS until paraffin embedding and sectioning. Seven-micron thick kidney sections were cut

from paraffin blocks and processed using standard histological techniques for hematoxylin & eosin staining. Kidney sections were evaluated under light microscopy to determine the extent of damage to the proximal tubule cells. Degradation of the luminal brush border membrane, cell swelling and lysis, and the presence of pyknotic nuclei were criteria used to identify the extent of injury associated with each treatment condition. The evaluation of kidney sections was performed by two independent operators blinded to the treatment of each sample.

Statistical analysis

ABR threshold and latency changes were evaluated for statistical significance using the ANOVA function in Statview V5.0 software. Analysis was performed at each individual acoustic stimulus using one-way ANOVAs with significance at $p < 0.05$. Data from area measurements for stria vascularis analysis and blood chemistry values were evaluated using one-way ANOVAs with significance at $p < 0.05$.

Results

Auditory analysis

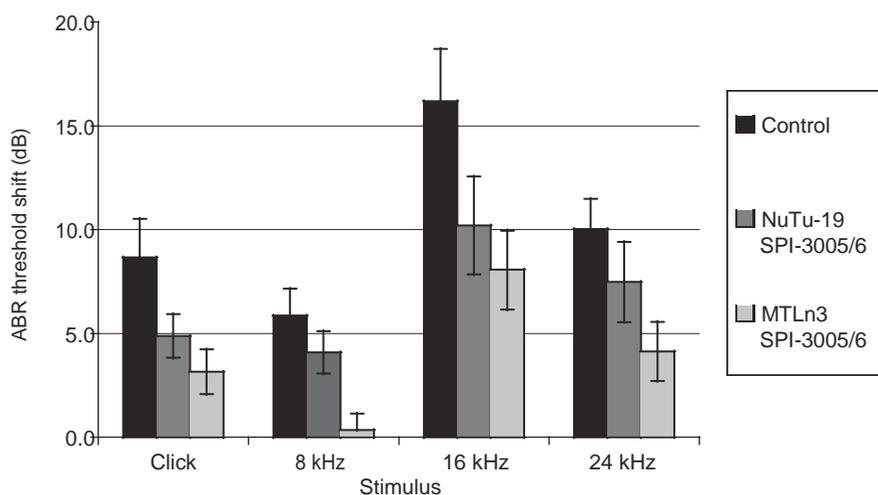
ABR testing in control rats treated with vehicle and cisplatin indicated significant threshold shifts at all tested stimuli (Fig. 1). Threshold shifts (mean dB shift \pm SEM) at each stimulus for tumor-bearing cisplatin-treated rats ($n = 22$ ears) were: click = 8.7 ± 1.9 ; 8 kHz = 5.9 ± 1.3 ; 16 kHz = 16.2 ± 2.5 ; 24 kHz = 10.0 ± 1.5 . Threshold shifts for MTLn3 breast tumor-bearing rats treated with cisplatin and SPI-3005/6 ($n = 18$ ears)

were: click = 3.2 ± 1.1 ($p = 0.021$); 8 kHz = 0.4 ± 0.8 ($p = 0.002$); 16 kHz = 8.1 ± 1.9 ($p = 0.019$); 24 kHz = 4.1 ± 1.4 ($p = 0.007$). Threshold shifts for NuTu-19 ovarian tumor-bearing rats treated with cisplatin and SPI-3005/6 ($n = 26$ ears) were: click = 4.9 ± 1.1 ($p = 0.072$); 8 kHz = 4.1 ± 1.0 ($p = 0.284$); 16 kHz = 10.2 ± 2.4 ($p = 0.092$); 24 kHz = 7.5 ± 1.9 ($p = 0.311$).

The significance of each comparison (p values) shown was derived from pairwise analysis of threshold shift at each stimulus for the cisplatin plus vehicle group versus a cisplatin plus chemoprotectant group. Significant protection at all tested frequencies was observed for the MTLn3 breast tumor group versus controls. The NuTu-19 ovarian tumor group did not exhibit significant protection from threshold shift at any tested stimulus, although the trend towards protection was evident.

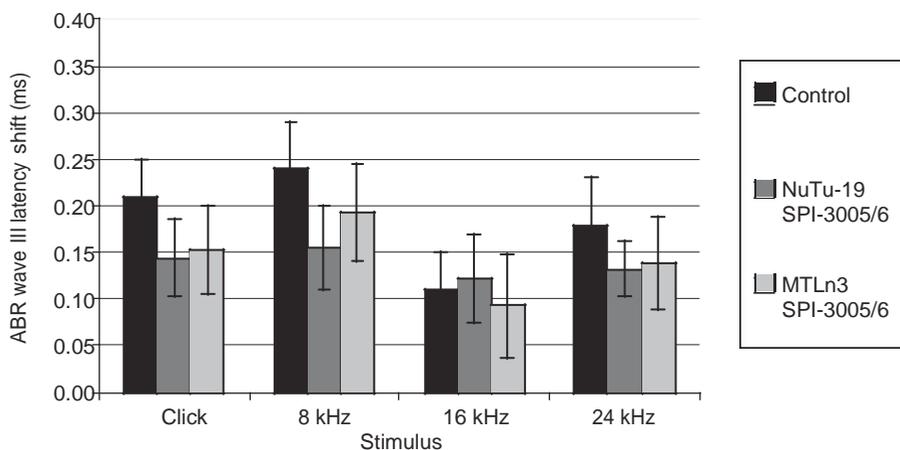
Differences in latency shift at threshold were quantified for the cisplatin plus vehicle tumor-bearing control groups versus the cisplatin plus SPI-3005/6-treated tumor groups (Fig. 2). Increases in latency were observed to be greater in animals treated with cisplatin plus vehicle versus cisplatin plus SPI-3005/6 treatment, although these differences failed to reach significance. Latency shifts (mean millisecond shift \pm SEM) for cisplatin

Fig. 1



Changes in ABR at threshold were decreased in SPI-3005/6 plus cisplatin-treated rats versus vehicle plus cisplatin-treated rats. Reduction in ABR threshold shift for the NuTu-19 tumor groups was not significant relative to controls, although trends were consistently favorable. Reduction in ABR threshold shift for MTLn3 tumor group versus controls was significant at all tested stimuli. Statistical significance between groups was determined by ANOVA (* $p < 0.05$, ** $p < 0.01$). The y-axis shows threshold shift in decibels for each stimulus listed along the x-axis. Error bars shown are \pm SEM. Control group treated with cisplatin plus vehicle: $n = 22$ ears. MTLn3 tumor group treated with cisplatin plus SPI-3005/6: $n = 18$ ears. NuTu-19 tumor group treated with cisplatin plus SPI-3005/6: $n = 26$ ears.

Fig. 2



Changes in latency at threshold were reduced in SPI-3005/6 plus cisplatin-treated tumor-bearing rats versus vehicle plus cisplatin-treated tumor-bearing control rats, although neither group achieved significance. Statistical significance between groups was determined by ANOVA. The y-axis shows latency shift at threshold in milliseconds for each stimulus listed along the x-axis. Error bars shown are \pm SEM. Control group treated with cisplatin plus vehicle: $n = 22$ ears. MTLn3 tumor group treated with cisplatin plus SPI-3005/6: $n = 18$ ears. NuTu-19 tumor group treated with cisplatin plus SPI-3005/6: $n = 26$ ears.

plus vehicle-treated tumor-bearing rats ($n = 22$ ears) were: click = 0.21 ± 0.04 ; 8 kHz = 0.24 ± 0.05 ; 16 kHz = 0.11 ± 0.04 ; 24 kHz = 0.18 ± 0.05 . Latency shifts for MTLn3 breast tumor-bearing rats treated with cisplatin plus SPI-3005/6 ($n = 18$ ears) were: click = 0.15 ± 0.05 ($p = 0.336$); 8 kHz = 0.19 ± 0.05 ($p = 0.506$); 16 kHz = 0.09 ± 0.06 ($p = 0.819$); 24 kHz = 0.14 ± 0.05

($p = 0.602$). Latency shifts for NuTu-19 ovarian tumor-bearing rats treated with cisplatin plus SPI-3005/6 ($n = 26$ ears) were: click = 0.14 ± 0.04 ($p = 0.241$); 8 kHz = 0.16 ± 0.05 ($p = 0.207$); 16 kHz = 0.12 ± 0.05 ($p = 0.846$); 24 kHz = 0.13 ± 0.03 ($p = 0.429$). No significant decreases in latencies were noted in either of the SPI-3005/6-treated tumor groups relative to the control group.

Cytocochleogram analysis

Evaluation of OHC loss in chemoprotected (SPI-3005/6 p.o., n = 8) versus cisplatin plus vehicle-treated controls (n = 8) showed little OHC loss in either group in the apical 50% of the cochleae examined. In a stereotypic manner, both groups exhibited OHC loss beginning at around 50% of the length of the cochlea from the apex. OHC loss in the SPI-3005/6-treated animals was significantly less than OHC loss in the cisplatin plus vehicle-treated group at almost every interval (Fig. 3). The reduction in OHC loss for the cisplatin plus SPI-3005/6 chemoprotected animals is consistent with lower ABR threshold shifts relative to cisplatin plus vehicle-treated control animals.

Blood chemistry and serology

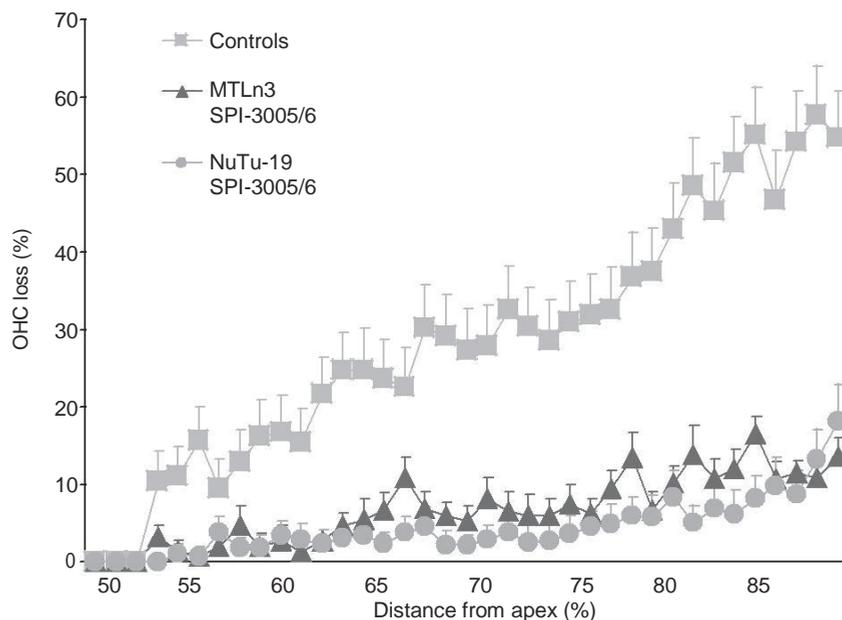
At 7 days after i.p. injection of the third weekly 6 mg/kg cisplatin dose, significant improvements in BUN serum creatinine ($p < 0.01$, Fig. 4A) and creatinine ($p < 0.05$, Fig. 4B) levels were evident in SPI-3005/6-treated animals compared to vehicle-treated controls. Improvements in liver function for SPI-3005/6-treated animals versus controls were not significant for AST ($p = 0.67$, Fig. 4C), but were significant for ALT ($p < 0.05$, Fig. 4D). The reduction in platelet counts for cisplatin-treated animals was consistently improved in SPI-3005/6-treated animals relative to controls ($p < 0.05$, Fig. 4E), indicating

less thrombocytopenia. Increases in hematocrit were observed in both groups, but were significantly less in SPI-3005/6-treated animals ($p < 0.05$, Fig. 4F). Consistent with this finding, increases in red blood cells were observed for both groups although the differences were not significant (data not shown). It should be noted that in the treatment of study animals with cisplatin, both groups exhibited significant dehydration, probably due to diarrhea associated with cisplatin activity on rapidly dividing cells lining the gastrointestinal tract. Dehydration was evident in cisplatin-treated animals despite the supplementation of 5 ml saline s.c. daily. The dehydration of our test animals may explain the observed decrease in blood volumes as evidenced by the increases in hematocrit and red blood cells.

Kidney histology

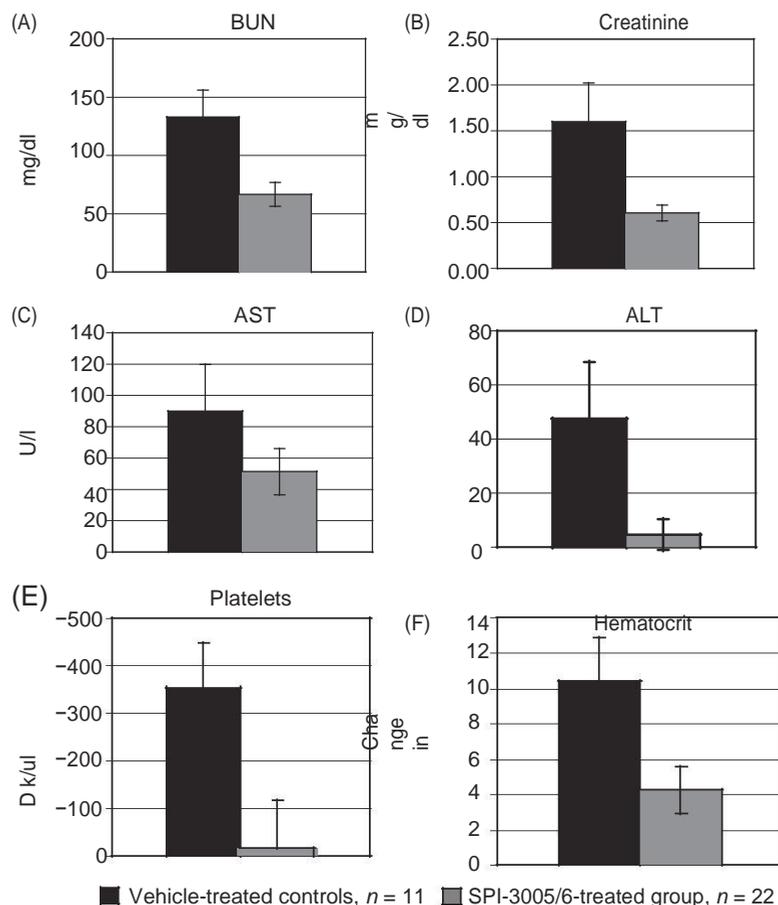
At 7 days after i.p. injection of the third weekly 6 mg/kg cisplatin dose, kidneys from cisplatin plus vehicle-treated rats exhibited severe necrosis of the proximal tubules in both the MTLn3 tumor model (Fig. 5A) and NuTu-19 tumor model (Fig. 5B), and appeared similar to kidney sections from non-tumor-bearing control rats treated identically with cisplatin and vehicle (Fig. 5C). Kidney sections from rats treated with cisplatin and SPI-3005/6 exhibit less severe necrosis and fewer pyknotic nuclei in the MTLn3 breast tumor model (Fig. 5D) and the

Fig. 3



OHC loss was significantly prevented in cisplatin-treated animals receiving combined chemoprotection with SPI-3005/6 p.o. compared to vehicle-treated animals. Increasing OHC loss was identified in an apex to base gradient in cochleae from both groups. Control group = 14 cochlea (MTLn3 n = 6, NuTu-19 n = 8), SPI-3005/6-treated group = 12 cochlea (MTLn3 n = 4, NuTu-19 n = 8). OHC loss in controls was significantly greater ($p < 0.05$) along the basal 50% of the cochlea compared to both the MTLn3 and the NuTu-19 groups treated with SPI-3005/6. No OHC loss was evident in the apical 50% of the cochlea evaluated in either group. The y-axis shows percent OHC loss average within a treatment group. Error bars shown are \pm SEM.

Fig. 4



Blood chemistry and serology were evaluated by BUN (A), creatinine (B), AST (C), ALT (D), platelet counts (E) and hematocrit levels (F). Significance at ** $p < 0.01$, * $p < 0.05$. Nephrotoxicity as evaluated by both BUN and serum creatinine changes was significantly improved ($p < 0.01$) for SPI-3005/6-treated rats versus controls. Hepatotoxicity was improved for SPI-3005/6-treated animals, improvements in AST changes were not significant ($p = 0.20$), while improvements in ALT were significant ($p < 0.05$). Changes in platelet counts and RBC were both significantly improved in animals treated with SPI-3005/6 ($p < 0.05$).

NuTu-19 (Fig. 5E) ovarian tumor model. Kidney sections from untreated control rats had normal cellular architecture and lack of pyknotic nuclei (Fig. 5F).

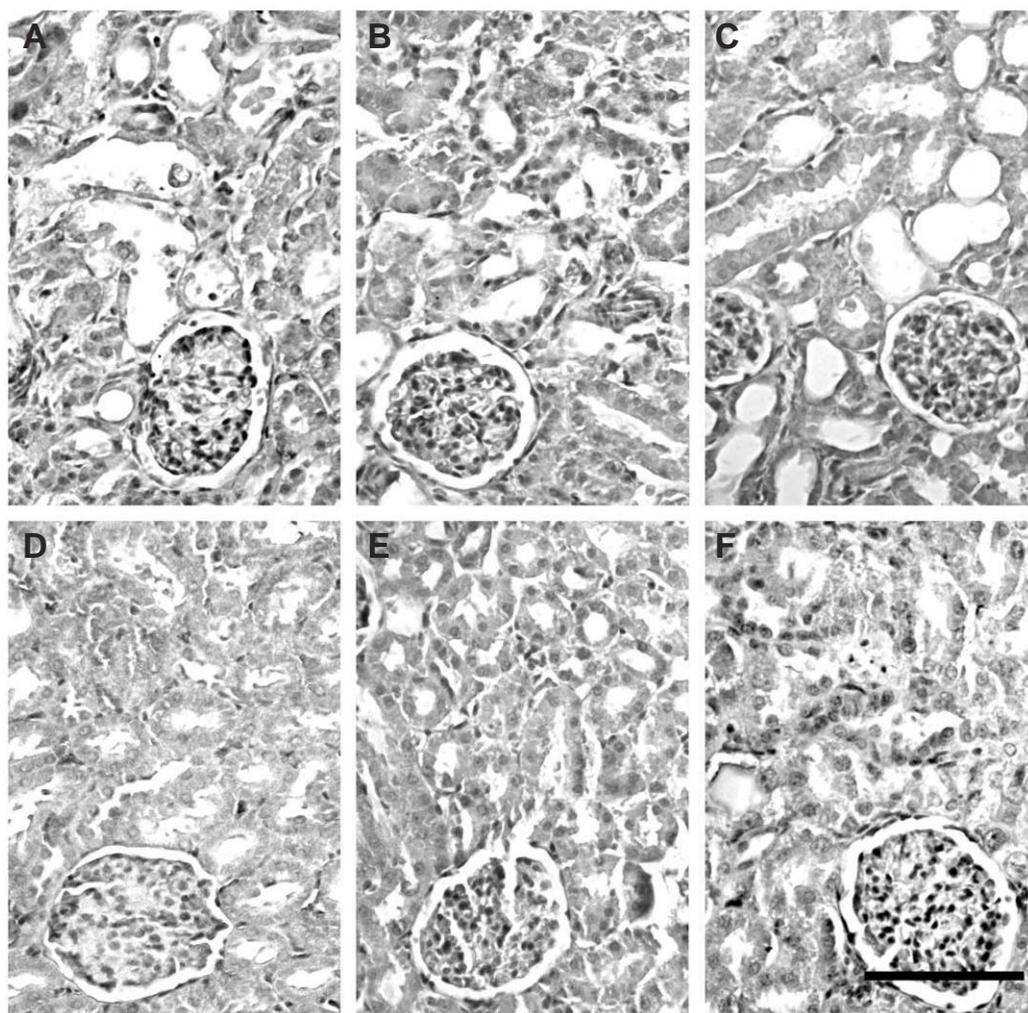
Tumor burden and animal survivability

Tumor burden for both MTLn3 and NuTu-19 models was evaluated in cisplatin plus vehicle- and cisplatin plus SPI-3005/6-treated groups to assess the potential for reducing or enhancing the anti-neoplastic activity of cisplatin (Table 1). Evaluation of MTLn3 groups indicated that the majority of cisplatin plus vehicle-treated controls (80%) and cisplatin plus SPI-3005/6-treated animals (88%) demonstrated a partial or complete tumor re-sponse. A small number of MTLn3 animals (20% controls and 12% SPI-3005/6 treated) showed no tumor response to cisplatin. Tumor response results for controls with NuTu-19 tumor indicated that 100% of the animals showed a partial (43%) or complete (57%) response of the

tumor to cisplatin. However, tumor response rates for NuTu-19 animals treated with cisplatin plus SPI-3005/6 showed marked improvements: partial (8%) and complete (92%) response.

Weight loss was monitored throughout the study with the final (at sacrifice) measure expressed as a percent of the initial body weight. Mean weight loss (\pm SEM) was 30.5% (\pm 5.4) for the MTLn3 vehicle-treated controls and 26.0% (\pm 3.2) for the MTLn3 SPI-3005/6-treated group (Fig. 6A). Mean weight loss was 25.0% (\pm 3.8) for the NuTu-19 controls and 20.2% (\pm 3.1) for the NuTu-19 SPI-3005/6-treated animals (Fig. 6B). There were no significant differences in mean weight loss between vehicle-treated controls and SPI-3005/6-treated animals within either MTLn3 or NuTu-19 tumor groups, or between MTLn3 and NuTu-19 groups.

Fig. 5



Histologic evaluation of kidney sections. Representative kidney sections from cisplatin plus vehicle-treated rats with MTLn3 breast tumor (A) and NuTu-19 ovarian tumor (B) exhibit pyknotic nuclei and severe degeneration of the proximal tubules similar to that seen in non-tumor-bearing control rats treated identically with cisplatin and vehicle (C). Kidney sections from rats treated with cisplatin and SPI-3005/6 exhibit less severe necrosis and fewer pyknotic nuclei in the MTLn3 breast tumor model (D) and the NuTu-19 (E) ovarian tumor model. Kidney sections from untreated rats had normal cellular architecture and lack of pyknotic nuclei (F). Scale bar = 100 mm.

Table 1 Analyses of MTLn3 and NuTu-19 tumor burden in cisplatin plus vehicle-and cisplatin plus SPI-3005/6-treated groups

Tumor response (%)	MTLn3		NuTu-19	
	Cisplatin	Cisplatin + SPI-3005/6	Cisplatin	Cisplatin + SPI-3005/6
Complete	40	44	57	92
Partial	40	44	43	8
No	20	12	0	0

Response categories include the following: complete (no sign of tumor burden), partial (some evidence for tumor burden) and no (tumor was not responsive to cisplatin treatment) response. Increased response to cisplatin was notable in the NuTu-19 tumor group treated with SPI-3005/6.

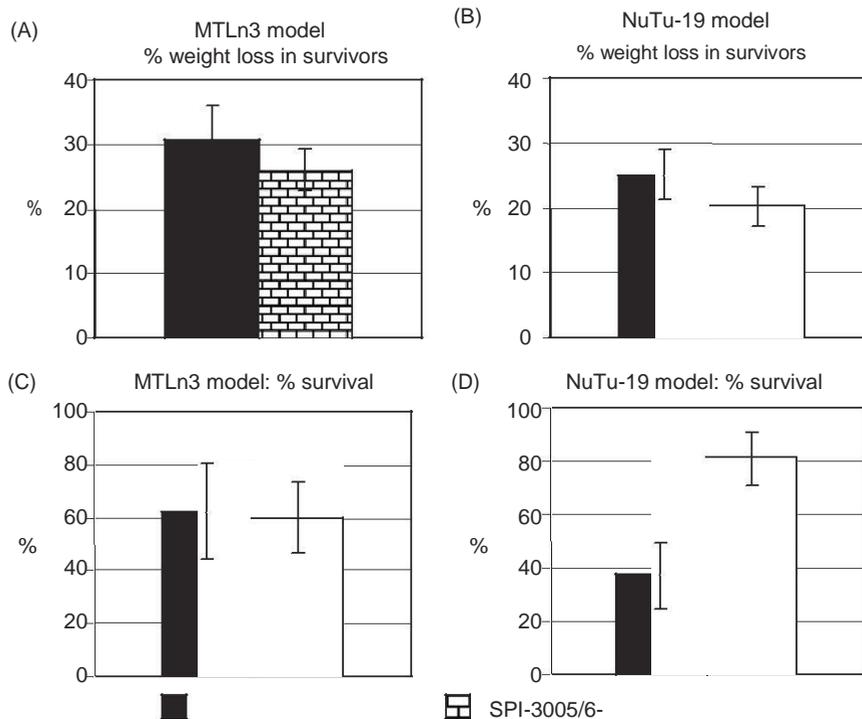
A total of 33 out of 55 animals survived the study, 11 out of 24 control animals and 22 out of 31 SPI-3005/6-treated animals. Of the control animals, five out of eight (62%) survived from the MTLn3 tumor group (Fig. 6C) and six

out of 16 (38%) survived from the NuTu-19 tumor group (Fig. 6D). SPI-3005/6-treated animals from the MTLn3 tumor group had nine out of 15 (60%) survivors, while treated animals from the NuTu-19 tumor group had 13 out of 16 survivors (81%). The highest survivability was seen in the cisplatin plus SPI-3005/6-treated NuTu-19 tumor group.

Discussion

The proven anti-tumor activity of cisplatin against solid tumors such as breast and ovarian cancer has been well established. Unfortunately, injury to the cochlea [5,28] and kidneys [23,24,29] due to cisplatin chemotherapy is also well documented. While several cytoprotective agents have shown the ability to ameliorate side-effects

Fig. 6



Weight loss in survivors. (A) MTLn3 tumor model: vehicle-treated control group $n = 5$, SPI-3005/6-treated group $n = 9$. (B) NuTu-19 tumor model: vehicle-treated control group $n = 6$, SPI-3005/6-treated group $n = 13$. Animal survivability. (C) MTLn3 tumor model: vehicle-treated control group $n = 5/8$, SPI-3005/6-treated group $n = 9/15$; (D) NuTu-19 tumor model: vehicle-treated control group $n = 6/16$, SPI-3005/6-treated group $n = 13/16$.

of cisplatin-induced oto- and nephrotoxicities in pre-clinical studies, issues such as route of administration, interference with cisplatin anti-tumor activity and dose level have limited the clinical potential of these strategies. Additionally, the simultaneous evaluation of oto-/nephrotoxicity and anti-neoplastic activity in the same tumor model has not been reported in cisplatin chemoprotection studies.

A combined oral formulation of ebselen and allopurinol (SPI-3005/6) was previously shown to be an effective chemoprotective strategy in an acute cisplatin toxicity model [10]. In an effort to extend these findings, we evaluated the combined formulation of SPI-3005/6 using a repeated cisplatin dosing schedule in F-344 rats with breast or ovarian cancer. The audiologic data presented indicate that SPI-3005/6 was most effective in preventing hearing loss in rats with breast cancer. Although, in the ovarian cancer model reduced threshold shifts were identified, they did not reach statistical significance. This difference is confusing since the level of OHC loss and the ABR latency shift in both chemoprotected cancer models appeared similar. Cisplatin is known to induce

changes in the vascular bed of the cochlea called the stria vascularis. Alteration in stria thickness has been correlated with loss of auditory function [30]. However, our previous experiments analyzed this structure and found no significant difference in stria thickness between SPI-3005/6 chemoprotected and vehicle-treated control groups [10]. One clear difference between our ovarian and breast cancer models is that the NuTu-19 model exhibits a greater morbidity, mortality and tumor response than the MTLn3 model. Here, we sought to determine if the protection occurred at the expense of cisplatin's anti-tumor activity. We chose two distinct, but clinically relevant, tumor models, breast cancer using MTLn3 cells, and ovarian cancer using NuTu-19 cells. It should be noted that both of these tumor models cause aggressive and highly metastatic tumors that are generally responsive to cisplatin treatment. Additionally, the MTLn3 and the NuTu-19 tumor lines, both syngenic to the F-344 rat, can be used without the confounding potential of immuno-suppressing drugs, some of which cause hearing loss.

In addition to interstrand DNA cross-linking leading to impairment of DNA replication, cisplatin also induces

reactive oxygen species (ROS) formation. The increase in ROS can lead to increased lipid peroxidation and subsequently cause the initiation of apoptosis in cells whose anti-oxidant defenses become overwhelmed or exhausted. The ROS-mediated toxicity of cisplatin is thought to be the major cause for cisplatin toxicity in non-dividing cells such as auditory hair cells in the cochlea and anuclear cells such as red blood cells. Ebselen, a GPx mimic, has been shown to be an excellent scavenger of peroxynitrite with the ability to protect against lipid peroxidation in the presence of glutathione or other thiols [31]. Ebselen has previously been shown to be effective in preventing noise [32,33] and cisplatin-induced ototoxicity [4,21] and nephrotoxicity [22] in animals while not impairing the anti-tumor activity of cisplatin [24]. Allopurinol is a XO inhibitor with the potential to reduce ROS generation and is used clinically in chemotherapy patients with acute hyperuricemia due to tumor lysis syndrome. Several studies have shown that platinum-based chemotherapeutics can increase XO activity in the cochlea [9] and kidney [8], which may exacerbate the ROS-associated toxicity, leading to the hypothesis that XO inhibition would be of benefit to patients receiving platinum chemotherapy. However, high-dose allopurinol has been shown to increase nephrotoxicity in rats treated with cisplatin [34], indicating that allopurinol displays pro-oxidative capacities at higher concentrations.

Our results indicate that the combined formulation of ebselen and allopurinol produced no inhibitory effect on cisplatin's anti-tumor activity in the MTLn3 breast cancer model, and in fact enhanced its efficacy in the NuTu-19 ovarian cancer tumor model. To our knowledge, this is the first description of a GPx mimic and XO inhibitor demonstrating enhanced tumoricidal activity in combination with chemotherapy in live animals. Current studies are aimed at addressing this additional benefit of ebselen and allopurinol co-treatment. It is unclear how these diverse mechanisms of ROS attenuation could augment the tumoricidal activity of cisplatin.

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