An Src-protein tyrosine kinase inhibitor to reduce cisplatin ototoxicity while preserving its antitumor effect

Eric C. Bielefeld[a], Chiemi Tanaka[b], Guang-di Chen[c], Donald Coling[c], Manna Li[c], Donald Henderson[c] and Anna R. Fetoni[d]

Ototoxicity remains a major dose-limiting side effect of cisplatin. The current studies were carried out to evaluate the effectiveness of a novel Src-protein tyrosine kinase inhibitor in protecting the ear from cisplatin ototoxicity without compromising cisplatin's antitumor effects. The Src inhibitor has been shown to be effective in protecting the ear from noise-induced hearing loss. Three studies were carried out to determine whether this compound has otoprotective activity in rats treated with cisplatin. The first two studies used the Src inhibitor as a cotreatment with single doses of cisplatin in Fischer 344/NHsd rats and nude rats, respectively. Cochlear damage was assessed by auditory brainstem response threshold shifts and outer hair cell loss. The third study was carried out in nude rats with implanted HT-29 tumors, and the Src inhibitor was administered as a cotreatment with a lower dose of cisplatin. Cochlear damage and changes in tumor volume were assessed in the third study. In the first two studies, cotreatment with the Src inhibitor reduced cisplatin-induced hearing loss significantly. In the third study, little hearing loss was induced because of the use of a lower dose of cisplatin. However, cotreatment with the Src inhibitor did not exert a negative effect on cisplatin's slowing of tumor growth in the treated rats. The findings suggest that the Src inhibitor may provide an effective cotreatment with cisplatin to reduce cisplatin's ototoxicity, without compromising its antitumor capability. ***Anti-Cancer Drugs*** **24:43–51 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2013, **24**:43–51

Keywords: apoptosis, cisplatin, cochlea, outer hair cell, Src

[a]Department of Speech and Hearing Science, The Ohio State University, Columbus, Ohio, [b]Oregon Hearing Research Center, Department of Otolaryngology, Oregon Health and Science University, Portland, Oregon, [c]Center for Hearing and Deafness, Department of Communicative Disorders and Sciences, State University of New York at Buffalo, Buffalo, New York, USA and [d]Institute of Otolaryngology, Catholic University School of Medicine, Rome, Italy

Correspondence to Eric C. Bielefeld, PhD, Department of Speech and Hearing Science, The Ohio State University, 110 Pressey Hall, 1070 Carmack Road, Columbus, OH 43220, USA
Tel: +1 614 292 9436; fax: +1 614 292 7504; e-mail: bielefeld.6@osu.edu

Received 8 February 2012 **Revised form accepted** 21 June 2012

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II) (CDDP)] is one of the most commonly used drugs for the treatment of testicular, ovarian, basal cell, and head and neck cancers. The side effects of CDDP include nausea and vomiting, neurotoxicity, nephrotoxicity, and ototoxicity. Of all the drugs used in oncology, CDDP has the highest potential for ototoxicity, and is the most common cause of drug-induced sensorineural hearing loss (see the review by Rybak *et al.* [1]). CDDP causes an initial high-frequency (8 kHz and above in human patients) hearing loss. The loss then spreads to the middle audiometric frequencies [2,3] as the dose increases or the number of treatments increases. The incidence of CDDP-related hearing loss varies between reports, with rates of significant hearing loss reported to be as high as 80% [2,4,5]. The damage induced by CDDP in the cochlea affects most of the key cell populations involved in the transduction of sound. The outer hair cells (OHCs) at the base of the cochlea are the most vulnerable cellular targets of CDDP-induced cochlear damage [6]. Loss of OHCs leads to loss of sensitivity to low-intensity sounds and loss of fine frequency discrimination abilities. With higher or multiple doses of CDDP, OHC losses spread into the middle turn of the cochlea, and pathology in the stria vascularis [7,8], supporting cells [9], inner hair cells, and spiral ganglion cells can be seen [10]. Damage to the inner hair cells and spiral ganglion cells results in a loss of the cochlea's ability to accurately transmit complex sound signals to the brain, manifesting commonly in a loss of speech discrimination ability in affected patients.

CDDP induces hearing loss through a multifaceted attack on cochlear tissue. Oxidative stress is a key factor, as CDDP induces a large burst of reactive oxygen species (ROS) at the level of the hair cells [11,12]. One of the key mechanisms for the increase in cochlear ROS is the generation of superoxide through the NADPH oxidase pathway. NADPH oxidase is an enzyme that catalyzes a reaction of molecular oxygen into superoxide [13]. NADPH oxidase has been shown to be active in the cochlea [14] and is upregulated by exposure to noise [15] and CDDP [16]. Blockade of NADPH oxidase in the cochlea reduces CDDP ototoxicity [17], implicating a role of NADPH oxidase in the oxidative stress induced by CDDP. While triggering an increase in ROS formation, CDDP also suppresses antioxidant defenses, including the suppression of glutathione [18]. The combined increase in ROS and decrease in antioxidants creates high levels of oxidative stress in cochlear tissues.

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32835739fd

The apoptotic cell death pathway has also been characterized as a key mechanism for CDDP ototoxicity. Increased oxidative stress triggers caspase-mediated apoptotic cell death, initially in the OHCs [19,20]. Alternatively, CDDP forms DNA adducts that signal arrest of progression of the cell cycle. Some cellular proteins that bind to these adducts are also known to signal induction of apoptosis [21]. CDDP has been shown to trigger the intrinsic apoptotic pathway in the cochlea, involving p53 signaling [22,23] caspase-3, caspase-9, and Bax [24,25].

Numerous drugs have been tested as cotreatments with CDDP to prevent the ototoxic side effect. Iron chelators have been tested extensively as otoprotectants against CDDP ototoxicity [26–28]. Although iron chelation was found to be an effective means of reducing CDDP's toxic side effects, chelators are believed to bind directly to CDDP, presumably reducing CDDP's tumorcidal ability.

In addition to chelator studies, other studies have produced evidence to indicate that the hearing loss from CDDP can be prevented with the use of antioxidants and proantioxidant drugs, including Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), D-methionine, *N*-acetyl-L-cysteine, and ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one). Each of these drugs provides protection against CDDP ototoxicity by strengthening cochlear antioxidant defenses [12,29–35].

KX1-004 is a non-ATP competitive Src-protein tyrosine kinase (PTK) inhibitor. Src, one of a family of nine oncogenes, was discovered ~25 years ago as viral Src (v-Src), the transforming gene in the rous sarcoma virus [36]. Since then, nine nonreceptor PTKs of the Src family [37] have been identified. Src (first known as cellular Src or c-Src), Fyn, and Yes are found in all mammalian cells, with the highest levels in the platelets, osteoblasts, and brain tissue. Src activity is elevated in many cancers [38], and is associated with the disassembly of adherens junctions, E-cadherin-mediated cell–cell adhesions associated with cortical actin filament networks [39], and with dysregulation of integrin signaling at junctions with the extracellular matrix [40].

Noise-induced hearing loss and cochlear damage can be reduced significantly with KX1-004 and its family of Src inhibitors [41–44]. With the numerous similarities between cochlear damage from noise and CDDP (including oxidative stress and apoptosis as mechanisms of cell death), the effectiveness of KX1-004 against noise led to the current studies examining its potential as a cotreatment with CDDP. The current report details a series of three experiments that tested whether the KX1-004 can protect against CDDP ototoxicity while maintaining or enhancing CDDP's antitumor effects. The first experiment was designed to test the effectiveness of KX1-004 against CDDP ototoxicity from a high dose of CDDP in a standard animal model of hearing loss. The second experiment was designed to test KX1-004 against a lower dose of CDDP in the nude rat model. The third experiment utilized implanted tumor cells to test the effect of KX1-004 on CDDP's ototoxicity as well as its antitumor effect.

Methods

Subjects

The current report encompasses results from three experiments. In the first study, eight adult Fischer 344/NHsd rats (mix of males and females) were used for the primary physiologic and anatomic testing. In the second and third studies, a total of 22 nude rats (athymic nude mutant rats, homozygous Hsd:RH-*Foxn1*^{rnu}/*Foxn1*^{rnu}) were used. Fischer 344 rats were obtained from Harlan Laboratories (South Easton, Massachusetts, USA) at 2–3 months of age. Nude rats (ages 3–5 months) were a gift from Dr Robert Plunkett of Roswell Park Cancer Institute (Buffalo, New York, USA). The animals were housed in a quiet colony (< 45 dBA). All procedures involving the use and care of the animals were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

For experiment 3, the nude rats were injected subcutaneously with HT-29 cells derived from a human colon adenocarcinoma [45]. Each rat was injected with a 20-G needle at two subcutaneous sites, one on each side, with 10^6 cells in 0.1 ml culture medium. The injection sites were in the dorsal torso near the rear leg. Care was taken to avoid intradermal and intramuscular placement of cells and to obtain a compact bolus of cells under the skin with minimal spreading. The tumor cells were allowed to grow *in situ* for 1 week before exposure to the Src-PTK inhibitor and/or CDDP.

Auditory brainstem response testing

To assess hearing thresholds, all rats used in the three studies were tested using free-field auditory brainstem response (ABR). The animals were anesthetized with inhalant isoflurane (4% for induction, 1.5% for maintenance, 1 l/min O_2 flow rate). Subcutaneous needle recording electrodes were placed at the vertex (noninverting), below the left pinna (inverting), and behind the shoulder blade (ground). During ABR recording, the rats were placed on a homeothermic blanket to maintain body temperature. Test stimuli consisted of alternating phase tone bursts at frequencies of 5, 10, 20, and 40 kHz. Signals were generated using Tucker Davis Technologies (TDT, Gainesville, Florida, USA) SigGen software. Each tone burst (1 ms duration) was gated through a Blackmann window, and had a 0.5 ms rise/fall time with no plateau. Stimuli were presented at a rate of 21/s. Signals were routed to a Leaf tweeter (model AS-TH400A, Hallam, Vic 3803, Australia) positioned at 0° azimuth, 17 cm from the vertex of each rat's head. Acoustic stimuli were calibrated before each testing

session by recording the output of the speaker with a microphone placed at the animals' head level. The rats' evoked responses were amplified with a gain of 50 000, using a TDT Headstage-4 bioamplifier, and bandpass filtered from 100 to 3000 Hz. A total of 250 sweeps were averaged at each stimulus level using TDT BioSig software. The level of the signal was decreased in 5 dB steps from 90 dB pSPL to a level 15 dB below that of the lowest level that induced a detectable and repeatable response. Threshold was recorded as the lowest level at which a detectable response was elicited and could be repeated.

Src inhibitor preparation and administration

KX1-004, the Src-PTK inhibitor, was obtained through collaboration with Kinex Pharmaceuticals (Buffalo, New York, USA; *http://www.kinexpharma.com*). The drug was dissolved in dimethyl sulfoxide and diluted to 2 mg/ml in sterile physiologic saline. In all experiments, the solution was delivered at 5 mg/kg by an intraperitoneal injection while the rats were awake and not under anesthesia. The dose was determined on the basis of a previous experiment that used KX1-004 to protect against noise-induced hearing loss [14]. Control animals were administered sterile physiologic saline by an intraperitoneal injection. For the first experiment in the Fischer 344/NHsd rats, the KX1-004 was delivered 30 min before CDDP infusion, and again 24 h after the CDDP infusion had ended. For experiment 2, the nude rats also received the KX1-004 injections at 30 min before and 24 h after the CDDP. In experiment 3, the KX1-004 was administered three times: 30 min before, 4 h after, and 24 h after the CDDP. The additional injection of KX1-004 at 4 h was administered in an attempt to optimize the protective effect of the KX1-004, and because no harmful effect of KX1-004 had been found in previous studies.

CDDP exposure

For each experiment, CDDP was obtained from Sigma (St Louis, Missouri, USA), and then dissolved in sterile physiologic saline. To facilitate the dissolution of CDDP, the solution was heated and stirred for a period of 20 min. During that time, the solution was covered to prevent any exposure to light. The rats were then anesthetized with isoflurane anesthesia (4% for induction, 1.5% for maintenance at a 1 l/min O_2 flow rate). The CDDP solution was then infused at a rate of 8 ml/h, delivered through a butterfly needle inserted into the abdomen for an intraperitoneal injection. For experiment 1, the CDDP was delivered at a dose of 16 mg/kg; therefore, the typical infusion was 35–45 min in duration. Although no mortality occurred in experiment 1, the rats showed significant weight loss, evidence of reduced food and water intake, and general lethargy from the CDDP treatments. To attempt to reduce those side effects, for experiment 2, the dose was reduced to 12 mg/kg; therefore, the typical infusion was 25–35 min in duration. For experiment 3, to further limit the nonauditory side effects of CDDP, the dose was reduced again to 10 mg/kg; therefore, the typical infusion was 20–27 min in duration.

Assessment of threshold shift

To assess the CDDP-induced threshold shift in the rats, pre-exposure thresholds were subtracted from postexposure threshold measurements to calculate the threshold shift at each time point. For experiment 1, the rats were tested three times, at 1, 3, and 5 days after CDDP. The nude rats in experiment 2 were tested once, at 7 days after CDDP. The test interval was extended from 5 to 7 days from experiment 1 to experiment 2 in an attempt to maximize the resultant hearing loss, and presumably maximize the demonstrated potential protective effect of KX1-004. Because the treated rats still showed weight loss, reduced appetite, and lethargy, the interval was reduced back to 5 days for experiment 3; therefore, the HT-29-injected nude rats in experiment 3 were tested once, at 5 days after CDDP.

Outer hair cell analyses

After the final physiological measurements were obtained, the animals were rapidly killed by inhalation of CO_2 and decapitation. For experiments 1 and 3, OHC cochleograms were obtained. Both auditory bullae were removed from each animal. The stapes was removed from each cochlea, and the cochleae were then perfused with 10% buffered formalin. The organs of Corti were then dissected out from the cochleae, and each was stained with propidium iodide to stain the nuclei of the hair cells. Specimens were placed in the propidium iodide solution (5 μg/ml in PBS) for 10 min. The stained specimens were mounted on slides and examined under a fluorescence microscope. Missing OHCs were counted along the entire length of the cochlea to allow assembly of a complete cochleogram for each ear. Any locations where OHC nuclei were expected but missing were labeled as 'missing' OHCs. The percentage of surviving OHCs was then calculated for four sections of the basilar membrane, the basal 25%, the first portion of the middle turn (26–50% from the base), the second portion of the middle turn (51–75% from the base), and the apex (76–100% from the base).

Tumor analyses

For the nude rats from experiment 3 with the HT-29 cells injected, tumor volumes were estimated before and after CDDP treatment. The tumors were identified under the skin and then measured for length, width, and height using measurement calipers. Gross tumor volume was then calculated from these three measurements. The criteria for early termination from the study were any tumors that were estimated to exceed 15% of the animal's body weight or any signs of the tumor that caused significant physical distress to the animal. None of the animals were excluded from the experiment on the basis of these criteria. Following postmortem cochlear removal,

the HT-29 tumors were removed and measured again with the calipers to calculate the post-treatment gross tumor volume. Two tumors were collected from each rat. Following measurement with the calipers, tumor volumes were confirmed by fluid displacement measurements.

Statistical analysis

For the first experiment, a three-factor analysis of variance (ANOVA) (drug group × frequency × test time) was used to analyze the differences between the means of the two experimental groups across the four different test frequencies at the three different time points after CDDP exposure. Group and frequency were analyzed as between-subjects variables and test time (days after CDDP exposure) was analyzed as a repeated measure. For experiments 2 and 3 with the nude rats, only one post-CDDP test time was used; therefore, analyses were carried out with two-factor ANOVAs (group × frequency). If a significant main effect occurred for frequency, post-hoc testing with Tukey's A tests was performed to delineate the nature of the differences. If a significant main effect of day occurred, the different days were compared using paired-subjects *t*-tests. For tumor volumes, each tumor (two tumors came from each rat) was treated as an individual data point. An independent-samples *t*-test was used to compare the CDDP-only and the CDDP + KX1-004 treatment groups.

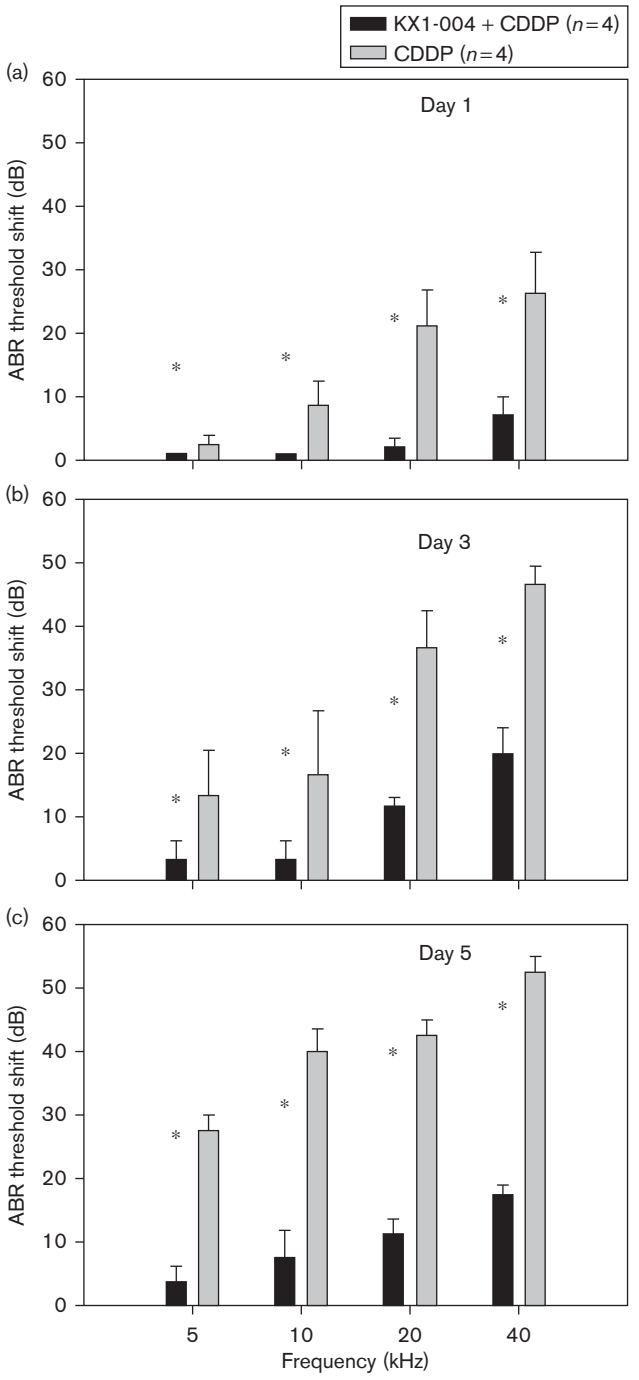
Results

No mortality occurred with the doses of CDDP used in the current experiments, although the studies only extended for 5–7 days after CDDP exposure. Weight losses and signs of digestive distress were evident in many rats within 3–5 days following the administration of CDDP, in a dose-dependent manner. As described in the Methods section, the doses were reduced in each experiment in the sequence to minimize the side effects, but they were still evident in experiments 2 and 3, despite the reduced doses.

Experiment 1 with Fischer 344/NHsd rats

For experiment 1, CDDP-induced ABR threshold shifts for the two groups (co-treated with KX1-004 and co-treated with saline) at the four frequencies tested are shown for day 1 (Fig. 1a), day 3 (Fig. 1b), and day 5 (Fig. 1c) after the administration of CDDP. At day 1, substantial threshold shifts occurred at 20 and 40 kHz in the saline co-treated control group. By day 3, the control group's mean threshold shifts at 20 and 40 kHz increased to 30–50 dB, whereas the lower frequencies of 5 and 10 kHz showed threshold shifts of less than 10 dB. The KX1-004 co-treated group showed no mean threshold shifts exceeding 20 dB at any frequency. At day 5, the threshold shifts had increased markedly for both the KX1-004 and the saline groups, but with a considerably lower threshold shift in the KX1-004 group. The three-way ANOVA (treatment group × test frequency × day after CDDP) showed a significant two-way interaction of group × day ($P < 0.001$) and a main effect of frequency ($P < 0.001$). The group × day interaction was broken

Fig. 1

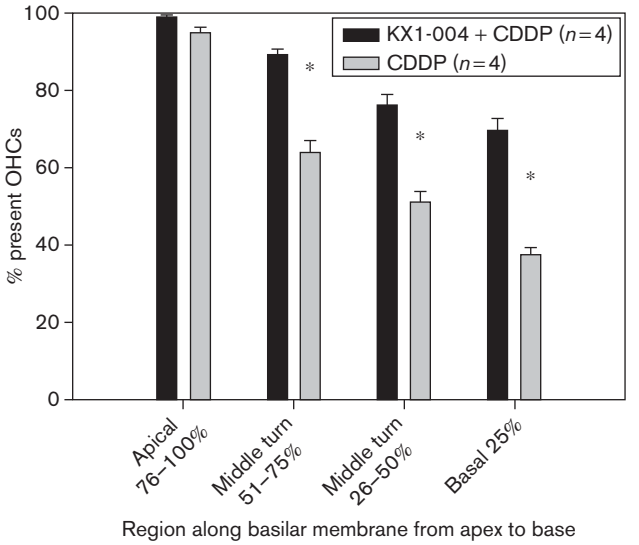


Auditory brainstem response (ABR) threshold shifts induced by the 16 mg/kg dose of CDDP at day 1 (a), day 3 (b), and day 5 (c) after exposure for the two experimental groups of Fischer 344/NHsd rats from experiment 1, plotted as an ABR threshold shift in dB against the frequency tested. Error bars are ±1 SEM. *Points at which the KX1-004-treated group had a significantly lower threshold shift than the control group. CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

down with a series of two-way (group × frequency) ANOVAs at each test day. The two-way ANOVAs on days 1, 3, and 5 each showed a significant main effect of group (Ps < 0.001). Thus, for each test day, the CDDP-induced threshold shifts were significantly lower in the KX1-004 co-treated group than in the saline co-treated group.

ABR threshold shift data were further confirmed with OHC cochleograms. The results are shown in Fig. 2. A two-factor ANOVA (treatment group × region along the basilar membrane) indicated a significant two-way interaction of group × location. Independent-samples t-tests were used to compare the KX1-004 co-treated and the saline co-treated groups at each location along the basilar membrane. The KX1-004 group had significantly less OHC loss than the saline group in the basal ($P < 0.001$), middle turn 1 ($P = 0.001$), and middle turn 2 ($P < 0.001$) regions.

Fig. 2

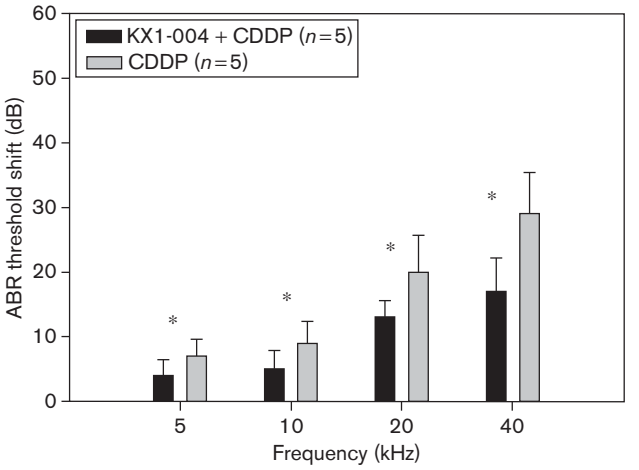


Outer hair cell (OHC) cochleograms from the Fischer 344/NHsd rats exposed to 16 mg/kg CDDP in experiment 1. Results are plotted as % present OHCs in each of four quadrants of the basilar membrane from apical to basal. *Points at which the KX1-004-treated group showed a significantly lower threshold shift than the control group. CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

Experiment 2 with nude rats without implanted tumors

For experiment 2, CDDP-induced threshold shifts for the KX1-004 and the saline co-treated groups at the four frequencies tested are shown for day 7 (Fig. 3). As in experiment 1, there was a greater threshold shift in the high frequencies of 20 and 40 kHz than in the lower frequencies. The lower dose of CDDP induced maximum threshold shifts in the 20–30 dB range, as opposed to the 16 mg/kg dose, which induced 40–60 dB of maximum threshold shift in the Fischer 344/NHsd rats by day 5. The two-way ANOVA (treatment group × frequency) showed significant main effects of group ($P < 0.05$) and frequency ($P < 0.001$). The main effect of frequency was expected because of the preferential effect of CDDP on the high frequencies (resulting from greater damage to the cells in the basal portion of the cochlea). The main effect of group confirms that the nude rats co-treated with KX1-004 showed less CDDP-induced ABR threshold shift than the saline co-treated rats.

Fig. 3



Auditory brainstem response (ABR) threshold shifts induced by the 12 mg/kg dose of CDDP at day 7 after exposure for the two experimental groups of nude rats from experiment 2, plotted as a threshold shift in dB against the frequency tested. Error bars are ±1 SEM. *Points at which the KX1-004-treated group showed a significantly lower threshold shift than the control group. CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

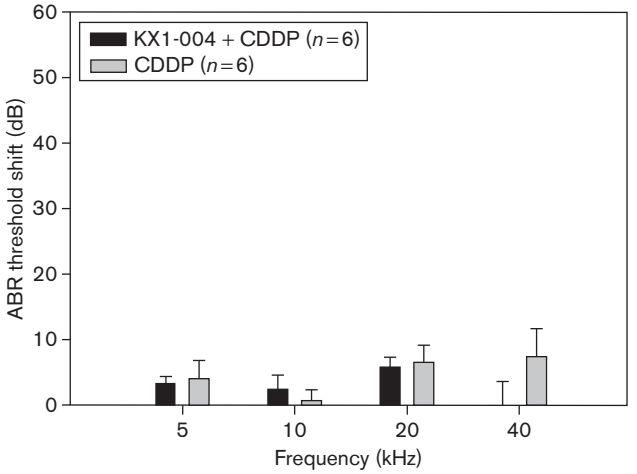
Experiment 3 with nude rats with implanted cancer cells

The lower dose of CDDP induced very low levels of threshold shift at the day 7 test time point. The mean threshold shifts are presented in Fig. 4. No mean threshold shift exceeded 10 dB for either group. The two-way ANOVA (treatment group × frequency) showed no significant differences between groups or test frequency. The lack of effect of frequency emphasizes the overall low amount of the threshold shift induced by the CDDP in these rats.

OHC cochleograms (Fig. 5) were consistent with the ABR threshold shift, in that relatively little OHC loss was detected. In the apical 50% of the cochlea, there was almost no OHC loss. In the basal 50% of the cochlea, the mean OHC losses were slightly greater, but still consistently below 20%. The two-way ANOVA (treatment group × region along the basilar membrane) showed no significant differences between the groups.

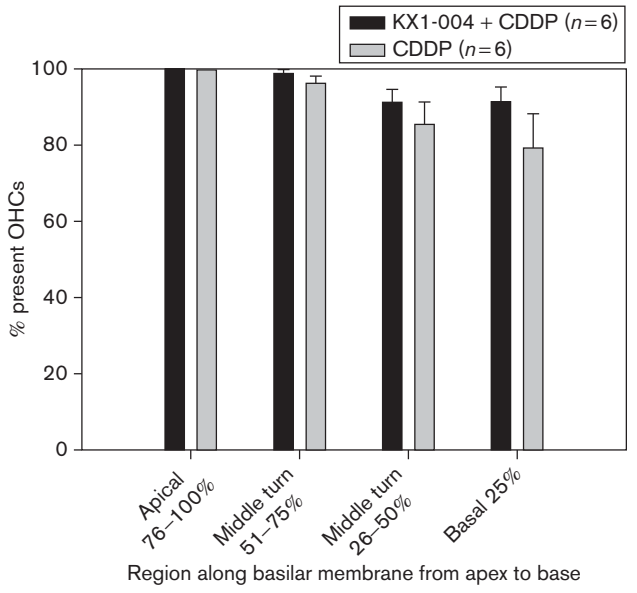
The final analysis that was carried out in experiment 3 was assessment of the tumor volumes to determine

Fig. 4



Threshold shifts induced by the 10 mg/kg dose of CDDP at day 5 after exposure for the two experimental groups of nude rats from experiment 3, plotted as a threshold shift in dB against the frequency tested. Error bars are ±1 SEM. Analysis showed no significant main effect of group ($P=0.283$) or group × frequency interaction ($P=0.391$). CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

Fig. 5



Outer hair cells (OHC) cochleograms from the nude rats exposed to 10 mg/kg CDDP in experiment 3. Results are plotted as % present OHCs in each of the four quadrants of the basilar membrane from apical to basal. Analysis showed no significant main effect of group ($P=0.402$) or interaction of group × distance along the basilar membrane ($P=0.305$). CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

whether the KX1-004 inhibited CDDP's antitumor effects. The mean tumor growth for the KX1-004 co-treated and saline co-treated rats is shown in Fig. 6. Tumors grew in the 5-day period between the initial measurement in both the treatment groups and the mean tumor volume after treatment was higher in the CDDP + saline treatment group. Statistical evaluation of the tumor sizes indicated no significant differences between the groups before or after CDDP treatment. The lack of difference shows that KX1-004 did not act to inhibit the antitumor effects of CDDP.

Discussion

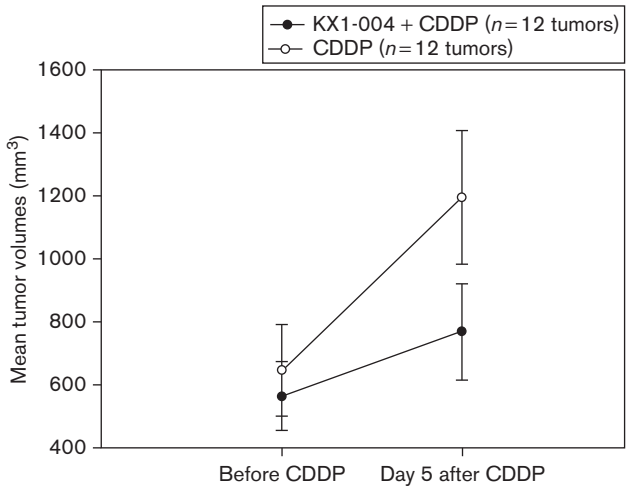
Protective effect of KX1-004

Experiments 1 and 2 showed that KX1-004 was able to protect the cochlea from a CDDP-induced threshold shift compared with the vehicle control solution when administered before CDDP exposure. Experiment 3 did not show this effect, but that can be attributed to a floor effect in which sufficient threshold shift or OHC loss was not induced by the CDDP to allow a protective effect from KX1-004 cotreatment to be shown. Experiment 3 did show that the KX1-004 did not work to inhibit the antitumor capacity of CDDP. Although the tumors grew in both the CDDP + saline treatment and the CDDP + KX1-004 treatment, the differences between the treatment groups were not statistically different before or after treatment. Although the different experimental parameters for experiments 1–3 make comparison across experiments in the current study difficult, overall, the current results indicate that KX1-004 does not inhibit the antitumor effects of CDDP. One of the primary concerns with any CDDP cotreatment aimed at reducing ototoxicity is the potential for that cotreatment to interfere or inhibit CDDP's antitumor effects. This is a significant concern with iron chelator cotreatments, and has limited their clinical utility for protection against CDDP ototoxicity. Although the current studies had small sample sizes, the effectiveness of KX1-004 as an otoprotectant in experiments 1 and 2 combined with the finding in experiment 3 that KX1-004 does not reduce CDDP's antitumor effects are interesting as they suggest a potential application for KX1-004 in minimizing CDDP ototoxicity. The current studies did not include a group that directly tested the potential ototoxicity of KX1-004. This was a decision on the basis of results from multiple experiments that used KX1-004 to protect against noise-induced damage to the cochlea [41–43]. In none of those studies was there any evidence of cochlear toxicity from KX1-004. Therefore, the direct effect of KX1-004 without CDDP was not tested in the current experiments.

Magnitude of the protective effect

As expected, the CDDP induced its greatest threshold shift and cochlear damage at the basal portion of the cochlea, corresponding to the high frequencies (20 and 40 kHz). Also expected was the dose dependence of the threshold shift. The Fischer 344/NHsd rats in experiment 1 received a single dose of 16 mg/kg, and showed the greatest mean threshold shifts by day 5 of the three

Fig. 6



Mean HT-29 cell tumor volumes before and following treatment with 10 mg/kg CDDP + saline or 10 mg/kg CDDP + KX1-004. Error bars are ±1 SEM. Sizes were not statistically different either before ($P=0.573$) or after ($P=0.145$) treatment. CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin).

studies. The nude rats in experiment 2 received a dose of 12 mg/kg and showed less threshold shift. Finally, the nude rats in experiment 3 received a 10 mg/kg dose and showed very little threshold shift at even the highest frequencies. The dose of KX1-004 was not varied between the experiments (5 mg/kg), although the first two experiments used two injections of KX1-004, whereas the third experiment used three injections. The CDDP-only group in experiment 1 developed 27–53 dB of mean threshold from 5 to 40 kHz. The CDDP-only group in experiment 2 developed 7–29 dB of mean threshold from 5 to 40 kHz, owing to the lower dose. In both experiments 1 and 2, the group co-treated with CDDP and KX1-004 developed 3–18 dB of the mean threshold shift. This is an interesting result, in that the protective effect was much greater in experiment 1 because of the higher thresholds in the CDDP-only group, rather than any differences between the threshold shifts of the CDDP + KX1-004 group. It is unclear whether rat strain differences in CDDP and KX1-004 metabolism and/or basal cochlear defenses may account for the differences in the protective effect or whether there is an initial amount of damage induced by CDDP in the cochlea against which the KX1-004 (at 5 mg/kg) is unable to protect. As for a dose-dependent effect of KX1-004, only one dose level was assessed in the current studies; therefore, no determination can be made. Future studies using KX1-004 to protect against cochlear damage from noise and CDDP aim to include dose–response testing to identify the optimal dose and schedule for protection against CDDP across a range of CDDP doses and treatment schedules. It is impossible to assess the potential therapeutic value of the third injection of KX1-004 used in experiment 3 because there was so little threshold shift induced by the CDDP that there was no room for any demonstrable protective effect of the KX1-004.

Mechanisms of otoprotection

The mechanisms by which KX1-004 exerts its protective effect on cochlear tissue are the subject of an ongoing investigation. The two key areas in which KX1-004 is believed to act are against oxidative stress and intracellular signaling in the apoptosis pathway. In previous studies using the Src inhibitor against noise [41–43], much of the focus was on the involvement of mechanical trauma-induced anoikis that can occur in hair cells stressed by high-level noise. Mechanical trauma is not a significant consideration with CDDP. Src inhibition has been linked to an inhibition of NADPH oxidase [46,47]. NADPH oxidase has been implicated as a mechanism in the CDDP-induced cochlear damage pathway [16,17]. Inhibition of NADPH oxidase in the CDDP-exposed cochlea would reduce superoxide levels, thus reducing the potential for oxidative stress.

Combined audiometric and xenograft model

Of particular interest in experiment 3 is the preservation of CDDP's antitumor effects when co-treated with KX1-004 in a model that utilized audiometric analyses of hearing loss from CDDP combined with assessments of antitumor effects using implanted xenografts of HT-29 cells. This combined audiometric and xenograft model has been used previously in the assessment of the effects of putative otoprotective compounds [34,48,49] and can be a direct and valuable means of assessing a putative otoprotective drug used in combination with an anticancer drug. A variant Src inhibitor compound related to KX1-004, KX2-391 was found to inhibit the proliferation of HT-29, BT-20 (human breast carcinoma), and CCRF-CEM (leukemia) cells *in vitro* [50], and KX2-391 was found in an unpublished preliminary work to interact synergistically with oxaliplatin to enhance its antitumor activity. These findings indicated that KX1-004 was a potential candidate to work synergistically with CDDP or inhibit *in vivo* tumor growth directly. This, combined with the effectiveness of KX1-004 in protecting OHCs from noise damage [39,40], led to the current studies. The results indicated that although KX1-004 was able to protect the cochlea from CDDP toxicity, it was also able to do so without compromising CDDP's ability to slow tumor growth. For this study, the human colorectal adenocarcinoma cell line HT-29 was tested because it is a cell line in which Src is known to be activated [51,52]. Although this cell line is sensitive to cisplatin, with an IC_{50} of 40–50 μmol/l [53,54], it is less sensitive to cisplatin than other cancer cell lines such as the ovarian carcinoma cell line A2780 [55]. Thus, a large impact of CDDP on tumor growth was not observed in the current study. Future studies designed to further test the combination of KX1-004 and CDDP on hearing on tumor

growth will use alternative cell lines that are known to have greater sensitivity to CDDP. In addition, more studies that use different CDDP doses and longer times after CDDP treatment are necessary to fully determine the effect that KX1-004 may exert on CDDP's antitumor action. The current study used a relatively low dose of CDDP and only tracked tumor growth up to 5 days after treatment. With a higher dose of CDDP or a longer treatment time window, it is possible that KX1-004 may exert more significant effects on the antitumor action of CDDP than were found in the current study. For any CDDP otoprotectant to be potentially clinically relevant, there must be no negative effect on the antitumor action. Therefore, future experiments are planned to test this relationship.

The current findings again suggest a complex role of Src in the stressed cochlea and in cancer cell proliferation. The exact nature of the KX1-004's effects on tumors is also the subject of future investigation. A systematic study of KX1-004 alone against tumor growth *in vivo* has not yet been carried out, but is the target of planned experiments.

Conclusion

The series of studies carried out in the current report showed that cotreatment of CDDP with KX1-004 may reduce CDDP's ototoxicity as indicated by ABR threshold shifts and OHC loss. The third experiment also showed that KX1-004 does not inhibit CDDP's antitumor capability as indicated by gross changes in tumor volume. Together, these experiments suggest that KX1-004 could be developed as a potentially effective cotreatment with CDDP to allow CDDP to be administered at higher doses without increasing the incidence of CDDP ototoxicity considerably.

Acknowledgements

The authors thank Dr David Hangauer and the Kinex Corporation for their contribution of the supply of KX1-004. They also thank Dr Irwin Gelman of the Roswell Park Cancer Institute (RPCI) for his generous gift of HT-29 cells and many thoughtful conversations, as well as Dr Robert Plunckett of RPCI for his donation of nude rats

Research was supported in part by the NIOSH Grant 1R01OH008113–01A1 and by a grant from the Kinex Corporation. Funding was used for supplies and animal purchasing. No salaries, portions of salaries, or honoraria were paid to the authors by the Kinex Corporation.

Conflicts of interest

There are no conflicts of interest.

References

1 Rybak LP, Whitworth CA, Mukherjea D, Ramkumar V. Mechanisms of cisplatin-induced ototoxicity and prevention. *Hear Res* 2007; **226 (1–2)**:157–167.
2 Van Zeijl LG, Conijn EA, Rodenburg M, Tange RA, Brocaar MP. Analysis of hearing loss due to *cis*-diamminedichloroplatinum-II. *Arch Otorhinolaryngol* 1984; **239**:255–262.
3 Madasu R, Ruckenstein MJ, Leake F, Steere E, Robbins KT. Ototoxic effects of supradose cisplatin with sodium thiosulfate neutralization in patients with head and neck cancer. *Arch Otolaryngol Head Neck Surg* 1997; **123**:978–981.
4 Hayes DM, Cvitkovic E, Golbey RB, Scheiner E, Helson L, Krakoff IH. High dose cis-platinum diammine dichloride: amelioration of renal toxicity by mannitol diuresis. *Cancer* 1977; **39**:1372–1381.
5 Reddel RR, Kefford RF, Grant JM, Coates AS, Fox RM, Tattersall MH. Ototoxicity in patients receiving cisplatin: importance of dose and method of drug administration. *Cancer Treat Rep* 1982; **66**:19–23.
6 Sergi B, Ferraresi A, Troiani D, Paludetti G, Fetoni AR. Cisplatin ototoxicity in the guinea pig: vestibular and cochlear damage. *Hear Res* 2003; **182**: 56–64.
7 Komune S, Asakuma S, Snow JB Jr. Pathophysiology of the ototoxicity of *cis*-diamminedichloroplatinum. *Otolaryngol Head Neck Surg* 1981; **89**:275–282.
8 Tange RA, Vuzevski VD. Changes in the stria vascularis of the guinea pig due to *cis*-platinum. *Arch Otorhinolaryngol* 1984; **239**:41–47.
9 Cardinaal RM, de Groot JC, Huizing EH, Veldman JE, Smoorenburg GF. Dose-dependent effect of 8-day cisplatin administration upon the morphology of the albino guinea pig cochlea. *Hear Res* 2000; **144**: 135–146.
10 Boheim K, Bichler E. Cisplatin-induced ototoxicity: audiometric findings and experimental cochlear pathology. *Arch Otorhinolaryngol* 1985; **242**:1–6.
11 Clerici WJ, Hensley K, DiMartino DL, Butterfield DA. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hear Res* 1996; **98**:116–124.
12 Kopke RD, Liu W, Gabaizadeh R, Jacono A, Feghali J, Spray D, *et al*. Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. *Am J Otol* 1997; **18**:559–571.
13 Heyworth PG, Peveri P, Curnutte JT. Cystolic components of NADPH oxidase: identity, function, and role in regulation of oxidase activity. In: Cochrane CG, Gimbrone J, editors. *Biological oxidants: generation and injurious consequences*. San Diego: Academic Press Inc. 1992. pp. 43–61.
14 Bielefeld EC, Hu BH, Harris KC, Henderson D. Damage and threshold shift resulting from cochlear exposure to paraquat-generated superoxide. *Hear Res* 2005; **207**:35–42.
15 Ramkumar V, Whitworth CA, Pingle SC, Hughes LF, Rybak LP. Noise induces A1 adenosine receptor expression in the chinchilla cochlea. *Hear Res* 2004; **188**:47–56.
16 Mukherjea D, Whitworth CA, Nandish S, Dunaway GA, Rybak LP, Ramkumar V. Expression of the kidney injury molecule 1 in the rat cochlea and induction by cisplatin. *Neuroscience* 2006; **139**:733–740.
17 Mukherjea D, Jajoo S, Kaur T, Sheehan KE, Ramkumar V, Rybak LP. Transtympanic administration of short interfering (si)RNA for the NOX3 isoform of NADPH oxidase protects against cisplatin-induced hearing loss in the rat. *Antioxid Redox Signal* 2010; **13**:589–598.
18 Ravi R, Somani SM, Rybak LP. Mechanism of cisplatin ototoxicity: antioxidant system. *Pharmacol Toxicol* 1995; **76**:386–394.
19 Liu W, Staecker H, Stupak H, Malgrange B, Lefebvre P, Van De Water TR. Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells. *Neuroreport* 1998; **9**:2609–2614.
20 Devarajan P, Savoca M, Castaneda MP, Park MS, Esteban-Cruciani N, Kalinec G, *et al*. Cisplatin-induced apoptosis in auditory cells: role of death receptor and mitochondrial pathways. *Hear Res* 2002; **174**:45–54.
21 Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003; **22**:7265–7279.
22 Zhang M, Liu W, Ding D, Salvi R. Pifithrin-alpha suppresses p53 and protects cochlear and vestibular hair cells from cisplatin-induced apoptosis. *Neuroscience* 2003; **120**:191–205.
23 Jamesdaniel S, Ding D, Kermany MH, Davidson BA, Knight PR III, Salvi R, *et al*. Proteomic analysis of the balance between survival and cell death responses in cisplatin-mediated ototoxicity. *J Proteome Res* 2008; **7**: 3516–3524.
24 Garcia-Berrocal JR, Nevado J, Ramirez-Camacho R, Sanz R, Gonzalez-Garcia JA, Sanchez-Rodriguez C, *et al*. The anticancer drug cisplatin induces an intrinsic apoptotic pathway inside the inner ear. *Br J Pharmacol* 2007; **152**:1012–1020.
25 Rybak LP, Mukherjea D, Jajoo S, Ramkumar V. Cisplatin ototoxicity and protection: clinical and experimental studies. *Tohoku J Exp Med* 2009; **219**:177–186.

26 Kameyama Y, Gemba M. The iron chelator deferoxamine prevents cisplatin-induced lipid peroxidation in rat kidney cortical slices. *Jpn J Pharmacol* 1991; **57**:259–262.

27 Liu H, Baliga R. Effect of iron chelator, hydroxyl radical scavenger and cytochrome P450 inhibitors on the cytotoxicity of cisplatin to tumor cells. *Anticancer Res* 2000; **20 (6B)**:4547–4550.

28 Dehne N, Lautermann J, Petrat F, Rauen U, de Groot H. Cisplatin ototoxicity: involvement of iron and enhanced formation of superoxide anion radicals. *Toxicol Appl Pharmacol* 2001; **174**:27–34.

29 Jamesdaniel S, Coling D, Hinduja S, Ding D, Li J, Cassidy L, *et al*. Cisplatin-induced ototoxicity is mediated by nitroxidative modification of cochlear proteins characterized by nitration of Lmo4. *J Biol Chem* 2012; **287**:18674–18686.

30 Campbell KC, Rybak LP, Meech RP, Hughes L. D-Methionine provides excellent protection from cisplatin ototoxicity in the rat. *Hear Res* 1996; **102**:90–98.

31 Campbell KC, Meech RP, Rybak LP, Hughes LF. D-Methionine protects against cisplatin damage to the stria vascularis. *Hear Res* 1999; **138**: 13–28.

32 Feghali JG, Liu W, Van De Water TR. L-*N*-Acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. *Laryngoscope* 2001; **111**:1147–1155.

33 Fetoni AR, Sergi B, Ferraresi A, Paludetti G, Troiani D. Protective effects of alpha-tocopherol and tiopronin against cisplatin-induced ototoxicity. *Acta Otolaryngol* 2004; **124**:421–426.

34 Lynch ED, Gu R, Pierce C, Kil J. Combined oral delivery of ebselen and allopurinol reduces multiple cisplatin toxicities in rat breast and ovarian cancer models while enhancing anti-tumor activity. *Anticancer Drugs* 2005; **16**:569–579.

35 Campbell KC, Meech RP, Klemens JJ, Gerberi MT, Dyrstad SS, Larsen DL, *et al*. Prevention of noise- and drug-induced hearing loss with D-methionine. *Hear Res* 2007; **226**:92–103.

36 Brugge JS, Erikson RL. Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* 1977; **269**:346–348.

37 Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 1997; **13**:513–609.

38 Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene* 2000; **19**:5636–5642.

39 Behrens J, Weidner KM, Frixen UH, Schipper JH, Sachs M, Arakaki N, *et al*. The role of E-cadherin and scatter factor in tumor invasion and cell motility. *EXS* 1991; **59**:109–126.

40 Playford MP, Schaller MD. The interplay between Src and integrins in normal and tumor biology. *Oncogene* 2004; **23**:7928–7946.

41 Harris KC, Hu B, Hangauer D, Henderson D. Prevention of noise-induced hearing loss with Src-PTK inhibitors. *Hear Res* 2005; **208**:14–25.

42 Bielefeld EC, Hynes S, Pryznosch D, Liu J, Coleman JK, Henderson D. A comparison of the protective effects of systemic administration of a pro-glutathione drug and a Src-PTK inhibitor against noise-induced hearing loss. *Noise Health* 2005; **7**:24–30.

43 Bielefeld EC, Wantuck R, Henderson D. Postexposure treatment with a Src-PTK inhibitor in combination with *N*-l-acetyl cysteine to reduce noise-induced hearing loss. *Noise Health* 2011; **13**:292–298.

44 Bielefeld EC, Hangauer D, Henderson D. Protection from impulse noise-induced hearing loss with novel Src-protein tyrosine kinase inhibitors. *Neurosci Res* 2011; **71**:348–354.

45 Von Kleist S, Chany E, Burtin P, King M, Fogh J. Immunohistology of the antigenic pattern of a continuous cell line from a human colon tumor. *J Natl Cancer Inst* 1975; **55**:555–560.

46 Yang S, Hardaway M, Sun G, Ries WL, Key LL Jr. Superoxide generation and tyrosine kinase. *Biochem Cell Biol* 2000; **78**:11–17.

47 O'Donnell RW, Johnson DK, Ziegler LM, DiMattina AJ, Stone RI, Holland JA. Endothelial NADPH oxidase: mechanism of activation by low-density lipoprotein. *Endothelium* 2003; **10**:291–297.

48 Fallah-Tafti A, Foroumadi A, Tiwari R, Shirazi AN, Hangauer DG, Bu Y, *et al*. Thiazolyl *N*-benzyl-substituted acetamide derivatives: synthesis, Src kinase inhibitory and anticancer activities. *Eur J Med Chem* 2011; **46**:4853–4858.

49 Wildrick DM, Alfaro SR, Gope R, Boman BM. A study of chromosome 6 allele loss in human colorectal carcinomas. *Anticancer Res* 1992; **12**: 1717–1719.

50 Park J, Cartwright CA. Src activity increases and yes activity decreases during mitosis of human colon carcinoma cells. *Mol Cell Biol* 1995; **15**:2374–2382.

51 Suzuki H, Tomida A, Tsuruo T. A novel mutant from apoptosis-resistant colon cancer HT-29 cells showing hyper-apoptotic response to hypoxia, low glucose and cisplatin. *Jpn J Cancer Res* 1998; **89**:1169–1178.

52 Bernhardt G, Biersack B, Bollwein S, Schobert R, Zoldakova M. Terpene conjugates of diaminedichloridoplatinum(II) complexes: antiproliferative effects in HL-60 leukemia, 518A2 melanoma, and HT-29 colon cancer cells. *Chem Biodivers* 2008; **5**:1645–1659.

53 Hector S, Bolanowska-Higdon W, Zdanowicz J, Hitt S, Pendyala L. In vitro studies on the mechanisms of oxaliplatin resistance. *Cancer Chemother Pharmacol* 2001; **48**:398–406.

54 Dimanche-Boitrel MT, Pelletier H, Genne P, Petit GM, Le Grimellec C, Canal P, *et al*. Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *Int J Cancer* 1992; **50**:677–682.

55 Branch P, Masson M, Aquilina G, Bignami M, Karran P. Spontaneous development of tumor resistance: mismatch repair and p53 defects in resistance to cisplatin in human tumor cells. *Oncogene* 2000; **19**: 3138–3145.