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Protection from impulse noise-induced hearing loss with novel Src-protein tyrosine kinase inhibitors

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Abstract

Apoptosis is a significant mechanism of cochlear hair cell loss from noise. Molecules that inhibit apoptotic intracellular signaling reduce cochlear damage and hearing loss from noise. The current study is an extension of a previous study of the protective value of Src-protein tyrosine kinase inhibitors against noise (Harris et al., 2005). The current study tested three Src-inhibitors: the indole-based KX1-141, the biaryl-based KX2-329, and the ATP-competitive KX2-328. Each of the three drugs was delivered into the chinchillas' cochleae by allowing the solutions to diffuse across the round window membrane thirty minutes prior to exposure to impulse noise. Hearing thresholds were measured using auditory evoked responses from electrodes in the inferior colliculi. Ears treated with KX2-329 showed significantly lower threshold shifts and outer hair cell losses than the control group. The cochleae treated with KX1-141 and KX2-328 did not show statistically significant protection from the impulse noise. The finding of protection with KX2-329 demonstrates that a biaryl-based Src inhibitor has protective capacity against noise-induced hearing loss that is as good as that demonstrated by KX1-004, a Src inhibitor drug that has been studied extensively as an otoprotectant against noise, and suggests that KX2-329 could be useful for protection against noise.

Keywords

Noise; Apoptosis; Src; Cochlea; Tubulin; Outer hair cell

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Conflict of Interest Statement

Dr. Hangauer is Senior Vice President of Research and Development at Kinex Pharmaceuticals (LLC). Drs. Bielefeld and Henderson have no financial interests in Kinex or the drugs tested.

1. Introduction

Noise-induced hearing loss (NIHL) continues to be a significant source of acquired hearing loss in the population of the world. One of the key pathologies underlying NIHL is loss of outer hair cells (OHCs) in the cochlea (Henderson et al., 2006). OHCs represent one of the key populations of sensory cells in the auditory system, and are responsible for the human ear's ability to hear low intensity sounds, as well as the ear's exquisite ability to discriminate sounds of different frequency. Loss of OHCs from noise exposure or other forms of insult leads to a loss of hearing sensitivity, frequency selectivity, and functional hearing in background noise. At the cellular level, apoptosis is a key mechanism in noise-induced death of the OHCs (Pirvola et al., 2000; Hu et al., 2000; Hu et al., 2002; Nicotera et al., 2003). In contrast with necrotic cell death, which is a passive process, apoptosis is an active, regulated cell death process that consumes energy (Majno and Joris, 1995). Through the activation of a family of specific cysteine proteases called caspases, the cell systematically disassembles (Kerr et al., 1972). Throughout the process of apoptosis, the cell membrane remains intact, and the cell condenses and pulls away from neighboring cells resulting in minimal damage to surrounding tissue. Apoptosis can be initiated by a number of triggers including mechanical stress (Frisch and Francis, 1994; Frisch and Sreaton, 2001) and reactive oxygen species (ROS) (McGowan et al., 1996), both of which occur in the cochlea as a result of noise exposure.

The discovery of the involvement of apoptosis in noise-induced OHC loss has led to a variety of intervention strategies designed to strengthen the ear and minimize the amount of OHC loss induced by high-level noise exposures. ROS have been detected in the cochlea after noise exposure (Yamane et al., 1995; Ohlemiller et al., 1999; Ohinata et al., 2000; Yamashita et al., 2004), and act as a putative trigger for apoptosis. Pretreatment of the cochlea with drugs to enhance antioxidant levels can attenuate noise damage and hearing loss (Seidman and Shivapuja, 1993; Quirk et al., 1994; Hu et al., 1997; Yamasoba et al., 1999; Kopke et al., 2000; Kopke et al., 2002; Hight et al., 2003; Kopke et al., 2005; Bielefeld et al., 2007; Hamernik et al., 2008). Other approaches have targeted signaling pathways within the cells that can culminate in apoptosis. The c-Jun NH₂-terminal kinase (JNK), a protein kinase signaling pathway, has been tested in multiple studies, using the JNK inhibitors CEP-1347 (Pirvola et al., 2000) and D-JNK-1 (Wang et al., 2003). Inhibition of JNK in those studies was found to reduce NIHL and limit OHC loss, indicating that interrupting the apoptosis signaling pathway can protect the cochlea from damage from noise.

Over the last several years, a series of studies have examined the protective effect of a group of Src-protein tyrosine kinase (PTK) inhibitors against noise-exposed cochlear damage. Src was targeted due to its possible role in signaling both mechanical stresses (impulse noise-related injuries) as well as metabolic changes (increases in ROS) that can trigger apoptosis. Mechanical stress is known to occur in the cochlea, and can result in disassociation of the OHCs from their supporting cells (Henderson et al., 2006), disconnections between the OHCs and the tectorial membrane (Nordmann et al., 2000), tears in the reticular lamina (Ahmad et al., 2003), and cleavage of F-actin in the cuticular plate (Hu and Henderson, 1997). Harris et al. (2005) examined the protective effects in chinchillas of several Src inhibitors on NIHL when administered through intra-cochlear infusion across the round window membrane. Following cochlear pre-treatment with the Src inhibitors, the chinchillas were exposed to either a four-hour continuous noise exposure or an impulse noise exposure with peak levels of 155 dB SPL. The ears pretreated with one of the Src inhibitors had substantially less hearing loss and OHC loss than the control ears in both the continuous and impulse noise conditions.

The most effective of the Src inhibitors tested by Harris et al. (2005) was KX1-004. KX1-004 is an indole-based non-ATP competitive Src-PTK inhibitor that was originally synthesized as an anti-cancer chemotherapeutic drug. The purpose of the current study was to extend the work of Harris et al. (2005) by examining the potential protective effects of three additional Src inhibitors delivered locally to the cochlea via the round window. KX1-141 is also an indole-based non-ATP competitive Src protein kinase inhibitor. KX2-329 is a biaryl-based non-ATP competitive Src protein kinase inhibitor that inhibits Src-driven cell growth with a GI_{50} of 35 nM. KX2-329 is also a tubulin polymerization inhibitor (D. Hangauer unpublished results) which contributes to the drug's potent anti-cancer effect (Li and Sham, 2002; Checchi et al., 2003). KX2-328 is an ATP-competitive Src protein kinase inhibitor (Ple et al., 2004) and inhibits Src-driven cell growth with a GI_{50} of 14 nM (D. Hangauer unpublished results). See Figure 1 for the structures of the three compounds tested, and the structure of KX1-004. All three compounds were provided by Kinex Pharmaceuticals LLC, Buffalo, NY. The purpose of this study was to determine if the more structurally complex indole-based Src inhibitor KX1-141, or the biaryl-based Src inhibitor KX2-329, was effective in protecting against NIHL, and how these compounds compare to the ATP-competitive Src inhibitor KX2-328.

2. Methods

A total of 28 ears from 17 chinchillas (mix of male and female) weighing between 400 and 700 g were divided into four different experimental groups (n=6 ears per treated group and n=10 ears in the control group for a total of 28; the other six ears were used for data collection from a different study). Prior to surgical procedures and between test times, the animals were housed in a quiet colony. All procedures involving 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.

2.1 Inferior Colliculus (IC) Electrode Implantation

To test hearing sensitivity, all animals were implanted with IC electrodes. The animals were intramuscularly injected with ketamine (60 mg/kg) and acepromazine (0.5 mg/kg) to achieve a deep plane of anesthesia. The animals were then placed in a stereotaxic apparatus. The electrodes were inserted bilaterally into the region of the IC. A ground electrode was placed in the rostral cranium (Henderson et al., 1973). The animals were allowed to recover for two weeks prior to evoked potential testing.

2.2 Evoked Potential Testing

During evoked potential threshold testing, the chinchillas were awake and secured in a restraint tube (Snyder and Salvi, 1994). Test stimuli consisted of alternating phase tone bursts at frequencies of 0.5, 1, 2, 4, and 8 kHz. Signals were generated using Tucker Davis Technologies (TDT) SigGen software. Each tone burst had a 2 ms rise/fall time, a 1 ms plateau with a cosine ramp, and were presented at a 21/sec rate. Signals were generated digitally with a TDT D/A converter at a 50 kHz sampling rate, and were 20 kHz low-pass filtered. Signals were routed to an earphone (Etymotic ER-1) that was inserted into the canal of the test ear. Acoustic stimuli were calibrated prior to each testing session, by inserting the earphone into a 2 cc coupler and recording output with a probe tube microphone (Etymotic ER-7C). The evoked responses were amplified by 20,000 times by a TDT Headstage-4 bioamplifier, and bandpass filtered at a range of 100–3000 Hz. One hundred samples were taken at each stimulus level. Averaged responses were displayed on a PC monitor using TDT BioSig software. The level of the signal was decreased in 5-dB steps from 90 to a point at which no response was detectable. Threshold was determined as the midpoint between the level at which a clear response was elicited and the next lowest level.

2.3 Src Inhibitor Solution Preparation and Application Surgery

KX1-141, KX2-329, and KX2-328 were obtained through collaboration with, Dr. David Hangauer, Senior Vice President for Research and Development Kinex Pharmaceuticals, LLC (www.kinexpharma.com). Prior to administration onto the round window, the compounds were first dissolved in dimethyl sulfoxide (DMSO) (10 mM), and then further diluted in phosphate buffered saline (PBS) to generate the desired concentration of 50 μ M. The concentration of DMSO never exceeded 0.5% of the total volume of solution. The drugs were applied at room temperature via surgical delivery onto the round window. The animals were again intramuscularly injected with ketamine (60 mg/kg) and acepromazine (0.5 mg/kg) to achieve deep anesthesia. An incision was made to expose the auditory bulla on the right side. A hole was then formed in the bone using a scalpel. A portion of the basal turn of the cochlea, and a portion of the round window were identified under an operating microscope. Using a 10- μ L microsyringe (World Precision Instruments Inc) 10 μ L of one of the three Src inhibitors was applied to the round window, or the control vehicle solution of 50 μ M DMSO in PBS. Ears were randomly assigned one of the four solutions, but no animal received the same solution on both ears. The process was repeated for the left ear of the animal, timed so that the drug was applied to each round window within two minutes of each other. All of the 10 μ L drop was placed on the round window niche to permit as much of the solution to diffuse across the round window membrane as possible. Ten minutes after application of the Src inhibitor onto the round window niche, excess solution that remained on the round window niche was wicked off using bits of paper. The holes in the auditory bullae were covered with dental cement, which was given ample time to harden prior to suture of the incisions (silk 3/0 suture). For each ear, the bulla was open for twenty-three minutes. Upon cochlear removal at the conclusion of the study, all middle ear spaces were examined for any signs of infection.

2.4 Impulse Noise Exposure

Thirty minutes after the Src inhibitor application surgery, the animals were exposed to impulse noise. The impulse noise exposure signal was generated utilizing a Spectrum TMS 320C25 signal processing board in a personal computer. The noise was routed through an amplifier (NAD 2200 PE) and transduced using a high frequency acoustic driver (JBL L455J) with a pass-band of 20–20,000 Hz. The width of the impulses was 100–18,000 Hz. The animals, still under anesthesia from the Src inhibitor application surgery, were placed directly in front of the speaker with their ears exactly 3 inches away, so that the speaker was located at zero degrees azimuth relative to the animal. Each animal was exposed to a total of 150 impulses, delivered in 75 pairs, with a 1 second silent interval between the onset of each pair. The peak sound pressure level of the impulses was 155 dB SPL in order to remain consistent with the noise exposure procedures of the previous Src-PTK inhibitor experiment (Harris et al, 2005).

2.5 Assessment of Threshold Shift

To assess noise-induced threshold shift in the animals, IC evoked potential testing was performed at three time points following noise exposure: 24 hours post application (Day1), seven days post (Day7), and twenty-eight days post (Day 28). Pre-exposure thresholds were subtracted from the post-exposure threshold measurements to calculate threshold shift at each time point. The Day 28 measurement provided the data for calculation of permanent threshold shift (PTS).

2.6 OHC Histology

After the final physiological measurements were obtained, the animals were rapidly decapitated following inhalation of CO₂. Both auditory bullae were removed from each

animal. The stapes was removed from each cochlea, and the cochlea was perfused with an incubation solution containing 0.05% tetranitro blue tetrazolium, 0.05 M sodium succinate, and 0.05 M phosphate buffer. The cochlea was then incubated in the solution for 1 hour at 37° C. The cochlea was then rinsed and fixed in 10% buffered formalin until dissection. For OHC analysis, the organs of Corti were then dissected from the cochleae and mounted as surface preparations. Missing OHCs were counted along the length of the organ of Corti. Missing OHCs were expressed as cochleograms displaying percent missing OHCs from the apex to the base.

2.7 Statistical Analysis

A three-factor ANOVA (Treatment Group $\times$ Frequency $\times$ Day post noise) was used to analyze differences between the means of the four experimental groups (KX1-141, KX2-329, KX2-328, and Controls) across the five different test frequencies at the three different time points post noise. Group and Frequency were analyzed as between-subjects variables, and Day post noise was analyzed as a repeated measure. If a significant main effect occurred for Group or Frequency, post hoc testing with Tukey A tests was performed to delineate the nature of the differences. If a significant main effect of Day occurred, the different days were compared with paired subjects t-tests.

For comparing differences between Groups with respect to OHC loss, the length of the basilar membrane was divided into seven segments with roughly 14% of the length of the basilar membrane encompassed in each segment. OHC losses were averaged within each subject across each of those segments, and those loss data were used for statistical analysis. The groups were compared using a two-factor ANOVA (Group $\times$ Distance along the basilar membrane), with post-hoc Tukey A tests to delineate any main effect of Group. Statistical analyses were performed in SPSS version 17.0.

3. Results

3.1 Pre-exposure thresholds

Group sizes for the four experimental groups were: of $n=6$ ears for the KX1-141 group, the KX2-329 group, and the KX2-328 group, and $n=10$ for the vehicle control group. The control was expanded to help control for the high level of variability inherent in threshold shift and OHC loss from the impulse noise condition based on past experiments (Harris et al., 2005; Kopke et al., 2005). Mean pre-exposure thresholds are displayed in Figure 2. Statistical analysis using a two-factor ANOVA (Group $\times$ Frequency) revealed a significant main effect of Frequency, but no significant main effect of Group or Group $\times$ Frequency interaction. The main effect of Frequency was anticipated due to the inherent differences in chinchilla hearing sensitivity across the frequency range that was tested. The absence of differences between groups prior to the treatments and noise exposures indicate that the differences observed in noise-induced threshold shift between groups were a result of the treatment solutions that each group received.

3.2 Threshold shifts induced by the impulse noise

Threshold shifts for the four groups at the five frequencies tested are shown for Day 1 (Fig. 3), Day 7 (Fig. 4), and Day 28 (Fig 5). At Day 1, substantial threshold shifts occurred across the frequency range, consistent with past experiments that used the same impulse noise exposure protocol (Harris et al., 2005; Kopke et al., 2005). All groups exhibited recovery during the period of time from Day 0 to Day 28. The three-factor ANOVA (Group $\times$ Frequency $\times$ Day) showed significant main effects of Day ($p<0.001$) and Group ($p<0.01$), without any significant interactions. The effect of Day was anticipated in light of the amount of compound threshold shift known to occur with the impulse noise (Harris et al. 2005;

Kopke et al., 2005) and the subsequent recovery between Day 1 and Day 28. Post hoc Tukey A testing revealed that the group pre-treated with the KX2-329 exhibited significantly lower threshold shifts than the vehicle control group ($p < 0.01$). No other groups were significantly different from one another, though there was a trend toward the group treated with KX1-141 being lower than the control group ($p = 0.101$). In order to determine if ear distribution for the three drug treatment groups had any influence, the left and right ears from the three treatment groups were collapsed and compared to one another using a three-way ANOVA (Ear $\times$ Frequency $\times$ Day). The KX1-141 group consisted of two left ears and four right ears. The KX2-329 group consisted of two left and four right ears. The KX2-328 group consisted of three left and three right ears, for totals of seven left ears and eleven right ears. The statistical analysis revealed no differences between the left and right ears, suggesting that the ear distribution did not significantly influence the threshold shift results.

3.3 OHC loss

Figure 6 displays the percent missing OHCs for the three experimental groups plotted against the missing OHCs for the control group as a function of the distance along the basilar membrane from the apex of the cochlea. Panel A shows KX1-141 compared to the controls; Panel B shows KX2-329 compared to the controls; Panel C shows KX2-328 compared to the controls. For all groups, the lesions of missing OHCs were interspersed throughout the length of the cochlea, as compared to a focused lesion associated with a narrow band noise exposure. The two-factor ANOVA revealed a significant main effect of Group ($p < 0.01$) with no significant interaction. Post hoc Tukey A testing revealed that the group treated with KX2-329 had significantly less OHC loss than the group treated with the vehicle solution and the group treated with the KX1-141.

4. Discussion

The purpose of the current study was to examine the protective effects of three Src-PTK inhibitors against high-level impulse noise. KX1-141 is an indole-based compound that builds upon the chemical structure of KX1-004, which has been shown to demonstrate significant protective effects against noise (Harris et al., 2005; Bielefeld et al., 2005). KX2-329 is a biaryl compound, and represents a chemically different class of compounds from the indole compounds. Both KX1-141 and KX2-329, like KX1-004, are non-ATP-competitive Src inhibitors. KX2-328 is an ATP-competitive compound, and was used in the current study to supply some insight into the potential benefits of using non-ATP-competitive Src inhibitors. The hearing losses following the noise exposure revealed that KX2-329, the biaryl compound, was the most effective of the three in protecting against NIHL at 4 and 8 kHz. It produced threshold shift reductions and reductions in OHC loss that were equivalent to those demonstrated by KX1-004 in a previous study (Harris et al., 2005).

KX1-141-treated ears showed the lowest mean PTS at 0.5–2 kHz, and the compound showed a statistical trend toward providing protection from threshold shift. The OHC loss in the ears treated with KX1-141 was not statistically different from the control ears, and the mean OHC losses were actually greater in the treated ears than the controls at sections of the middle turn of the cochlea. This finding is surprising in the chinchilla, since impulse NIHL in the chinchilla has been closely correlated with OHC loss (Hamernik et al., 1989). The level of OHC loss in the control ears is within the expected range based on the amount of PTS (Hamernik et al., 1989). Thus, the disconnect between apparent protection from PTS and lack of protection from OHC loss can likely be attributed to one of two explanations, either the statistical trend for protection from PTS is spurious, or the KX1-141 exerts a protective effect on a cell population other than the OHCs. While the sample size of six ears per treatment group was made based on a power analysis utilizing variance data from previous studies using the impulse noise (Harris et al., 2005; Kopke et al., 2005), a larger

sample size for the KX1-141 group may have compensated for the large variance found in the KX1-141 group and given the group the statistical power to reach significance (or conversely, to have revealed a true lack of difference in threshold shift between the groups). The hearing loss protection accorded by KX1-141 would also be further clarified by the use of a more direct assessment of the auditory periphery (near-field electrodes for compound action potential measurement or measurement of the P1 wave of the far-field auditory brainstem response). These assessments would possibly have a closer correlation with the hair cell damage than the IC evoked potentials do. Such assessments would also potentially provide the ability to reliably test higher frequencies (12 and 16 kHz) which would give more insight into the base of the cochlea, where drugs delivered on the round window are reaching at their highest concentrations (Salt and Ma, 2001; Laurell et al., 2002).

If the protective effect is legitimate for a population of cells other than the OHCs, other possible targets include stria vascularis, the inner hair cells (IHCs), or cells of the spiral ganglion. Though the previous Src inhibitor studies (Harris et al., 2005; Bielefeld et al., 2005; Bielefeld et al., 2011) have not focused on these structures, they cannot be ruled out as possible targets of protection afforded by the Src inhibitors, specifically KX1-141 in this case. IHC damage from impulse noise has been well-documented at 30 days post-exposure, with considerable cell death and morphological disruption in surviving cells (Hamernik et al., 1984), and a past study with the same impulse noise parameters (Kopke et al., 2005) showed a lesion of missing IHCs (5–20%) in the 0.5 to 2 kHz range of the chinchilla cochlea. With the disconnect between OHC loss and hearing loss differences in the KX1-141-treated ears, IHC damage may explain the differences seen in hearing loss. Additionally, no measurements were taken in the current study of threshold status following application of the solutions onto the round window prior to the noise exposure. The possibility that the drug solutions exerted a direct effect on threshold, and could have altered noise-induced threshold shifts in the treated and control groups, cannot be ruled out. Such an effect could potentially explain the lack of consistency between the threshold shift and OHC loss results from the KX1-141 group.

KX2-328, the ATP-competitive Src inhibitor, demonstrated no protective effect on PTS or OHC loss. ATP-competitive kinase inhibitors compete with ATP for binding sites within the cells, and therefore can induce significant unwanted physiological side effects (Kiselyov et al., 2006) or result in a reduced inhibitory potential of the drug (Garuti et al., 2010). Though no side effects were demonstrated in the current study, likely due to the local administration of the drug into the cochlea, the lack of a protective effect is consistent with a lack of Src inhibition, or an opposing inhibition of another tyrosine kinase. Therefore, the ATP-competitive drug may indeed be failing to effectively or selectively inhibit Src in the cochlea, resulting in a lack of a demonstrated protective effect in comparison to KX2-329, or the three non-ATP-competitive Src inhibitors tested by Harris et al., (2005). The primary advantage of the non-ATP-competitive PTK inhibitors is their kinase inhibitory selectivity. KX1-004, a non-ATP-competitive indole-based Src inhibitor, has been tested a variety of systemic studies against noise (Bielefeld et al., 2005; Bielefeld et al., 2011) without notable side effects or weight changes.

The biaryl Src inhibitor, KX2-329, has a dual mechanism of action in cells, with tubulin polymerization inhibition as the second mechanism. Both the Src inhibition and tubulin polymerization inhibition effects promote cell death or arrest of cell mitosis in cancer cells. Yet KX2-329 appears to generate the paradoxical effect of promoting cell survival in OHCs stressed by noise. The inhibition of Src and the inhibition of tubulin polymerization may both be playing roles in generating the protective effect of KX2-329 against NIHL. Currently, it cannot be stated which of the two mechanisms is the more potent in protecting against NIHL. Src activation is hypothesized to act as a trigger for apoptosis in the OHCs

when the OHCs are mechanically stressed by noise (Harris et al., 2005; Bielefeld et al., 2005). OHCs have high levels of tyrosinated tubulin that is found in highly dynamic cell populations undergoing frequent cell division (Slepecky et al., 1995). The tubulin is frequently being polymerized and depolymerized in a cyclical fashion. In the case of the OHCs, the tyrosinated tubulin may be involved in the cells' shape changes that occur during electromotility (Slepecky and Ulfendahl, 1992). What is currently unknown is the effect that high-level noise exposure has on the OHCs' tubulin and microtubules. Noise is known to alter the spatial distribution of both actin and tubulin in the OHCs (Liberman, 1987; Raphael et al., 1993; Hu and Henderson, 1997; Spongr et al., 1998), but the effect that those distribution changes have on cell death versus cell survival and retention of functionality is unknown. The possibility exists that noise exposure alters the pattern of tubulin polymerization, which could lead to loss of OHC electromotility or cell death. Future studies will focus on the mechanism(s) of action of KX2-329 in the noise-exposed cochlea, in addition to assessment of the effectiveness of the drug in a variety of systemic administrations, culminating in oral dosing. These studies will permit the evaluation of any side effects from the drug as well as its capability of crossing the blood-labyrinth barrier. In addition, future studies will also test the drug's effectiveness against a variety of noise exposures with different acoustic parameters. The introduction of multiple possible mechanisms of action for the Src inhibitor drugs also creates the potential for benefit from combining the drugs in order to address the anti-apoptotic effect, the anti-ROS effect, and the tubulin polymerization inhibition effect of the drugs. Using combinations of the Src inhibitors to optimize benefit will also be explored in future studies.

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Abbreviations

DMSO	dimethyl sulfoxide
IC	inferior colliculus
IHC	inner hair cell
JNK	c-jun terminal kinase
NIHL	noise-induced hearing loss
OHC	outer hair cell
PBS	phosphate buffered saline
PTK	protein tyrosine kinase
PTS	permanent threshold shift
ROS	reactive oxygen species
TDT	Tucker Davis Technologies

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Highlights

Src inhibitors used to protect against acoustic trauma to the inner ear

Biaryl-based Src inhibitor, KX2-329, protected hearing and sensory cells

Indole-based Src inhibitor did not provide statistically significant protection

KX2-329 inhibits Src and inhibits tubulin polymerization

Inhibiting tubulin polymerization is a novel mechanism to protect the ear from noise

Figure 1.
Molecular structures of KX1-004, KX1-141, KX2-329, and KX2-329.

Figure 2.
Mean pre-exposure thresholds for the four experimental groups. Error bars are +1 SEM.

Figure 3. Mean threshold shifts 24 hours after the impulse noise exposure. Error bars are ± 1 SEM.

Figure 4. Mean threshold shifts one week after the impulse noise exposure. Error bars are ± 1 SEM.

Figure 5.
Mean threshold shifts 28 days after the impulse noise exposure. Error bars are ± 1 SEM.

Figure 6. Mean OHC loss cochleograms for the four experimental groups, plotted as % OHC lost against % distance from the apex of the cochlea.