Postexposure treatment with a Src-PTK inhibitor in combination with N-l-acetyl cysteine to reduce noise-induced hearing loss

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Abstract

Both the antioxidant, N-l-acetyl cysteine (NAC), and the Src inhibitor, KX1-004, have been used to protect the cochlea from hazardous noise. In order to extend our previous work on KX1-004 with noise exposure, the current studies were undertaken with two goals: (1) to test the effectiveness of NAC and KX1-004 in combination with one another when given in a protection paradigm, and (2) to test the NAC+KX1-004 combination in a postexposure rescue paradigm. The noise exposure for the first experiment consisted of a 4-kHz octave band of noise at 107 dB SPL for 2 hours. The combination of NAC and KX1-004 were administered either prior to the noise exposure or post exposure (rescue). The second experiment was undertaken to extend the findings of the first experiment's rescue paradigm. The 4 kHz octave band noise was delivered at 112 dB SPL for 1 hour, with the experimental drugs delivered only in a rescue paradigm. In Experiment 1, animals treated before the 2-hour noise exposure with the combination of NAC and KX1-004 had from 12 to 17 dB less permanent threshold shift when compared to control saline treated animals. Treatment in the rescue paradigm did not produce any reductions in threshold shift from the 2-hour exposure. In the second experiment, with the 1-hour noise, rescue with KX1-004 or KX1-004 plus NAC yielded small, but significant, reductions in threshold shift. There was no additional benefit from the combination of NAC and KX1-004 over KX1-004 by itself.

Keywords: Apoptosis, cochlea, glutathione, noise, reactive oxygen species, Src

Introduction

Several past investigations into noise-induced hearing loss (NIHL) have revealed two important mechanisms involved in noise-induced hair cell death, leading to discoveries about key methods for pharmacological interventions to prevent NIHL. The first of the mechanisms is that noise causes an increase in reactive oxygen species (ROS) leading to hair cell loss[1,2] from ROS activity.[3,4] Pretreatment of the cochlea with antioxidants (which scavenge ROS or convert them to less harmful molecules) or pro-antioxidant drugs can attenuate noise damage and hearing loss.[5-12] The second key mechanism is the involvement of apoptosis in the growth of the outer hair cell (OHC) lesion following a traumatic noise exposure.[13,14] Pre-treating the cochlea with anti-apoptotic agents has also been demonstrated to be an effective strategy to protect against NIHL.[12,15,16]

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The current report details two experiments that are extensions of previous studies that used interventions with N-acetyl, l-cysteine (NAC)[11,12,17,18] or the Src inhibitor, KX1-004[12,16] to protect against noise damage. Both drugs have shown substantial protection when given systemically. NAC, a thiol-containing amino acid derivative, is a supplier of cysteine for endogenous antioxidant glutathione synthesis. NAC is used in the United States as a nutritional supplement as well as prescription drug to treat acute acetaminophen overdose, which causes over production of a highly reactive free radical, *N*-acetyl-*p*-benzoquinoneimine in the liver.[18,19] It has been shown in animal models to be protective against various types of noise exposure, such as continuous, impulse, high-kurtosis noise (combination of continuous noise and impact noise), and nonGaussian noise[9,11,12,17,20-23] In the noise-exposed cochlea, NAC is thought to scavenge ROS.[24] replenish GSH,[25,26] protect mitochondria,[27] reduce glutamate excitotoxicity,[28,29] block apoptosis through inducing specific gene expression,[30] and reduce activation

of caspase and MAPK/JNK.[31,32] KX1-004 is believed to inhibit Src, one of a family of nine oncogenes that is found in all mammalian cells, with greatest levels in the platelets, osteoblasts, and brain tissue. Src activity is associated with the disassembly of adherens junctions, E-cadherin-mediated cell-cell adhesions associated with cortical actin filament networks,[33] and with disregulation of integrin signaling at junctions with the extracellular matrix,[34] culminating in anoikis, a form of apoptosis that results from mechanical trauma to a cell or stress to the cell's extra-cellular matrix or connections with other cells.[35] The triggering mechanism for Src activation is not well understood, but involves intramolecular interaction, autophosphorylation of tyrosine 416 and dephosphorylation of tyr 527.[36] One of the characteristics of NIHL is that traumatic noise can cause the complex cellular interconnections to become stressed and/or broken,[13,37,38] leading to anoikis.

The current study encompasses two individual experiments. The goal of the studies was to begin to develop an effective noise protection and rescue protocol using KX1-004 in combination with NAC. "Rescue" is the term used for drug treatments that are only given after the noise exposure in the hopes of minimizing the resulting hearing loss. It is a relevant treatment paradigm for noise exposures that are either completely unanticipated or whose severity is greater than anticipated. In those cases, drug treatments given prior to the noise are impossible or impractical, and the best option available to those noise-exposed individuals is a post-exposure rescue treatment to minimize the resulting hearing loss. The purpose of Experiment 1 in the current study was to test the effectiveness of NAC and KX1-004 in combination with one another when given before (protection) or after (rescue) a noise exposure. In our previous study,[12] the two drugs were given separately to compare their effectiveness. As discussed above, both drugs have proven to provide significant protection from noise when given separately. The purpose of Experiment 2 was to test NAC/KX1-004 in a postexposure rescue paradigm with a different noise exposure that was designed to induce a combination of mechanical trauma and metabolic ROS damage to the OHCs.

Methods

The current report encompasses results from two experiments. In the first experiment, seventeen adult chinchillas weighing between 400 and 700 g were divided into three different experimental groups. In the second experiment, another seventeen chinchillas were divided into three experimental groups. Prior to noise experimental procedures and in 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 (Protocol HER 14045Y).

Auditory brainstem response test

For both experiments, free-field auditory brainstem response (ABR) thresholds were obtained in a sound booth before the noise exposure, and 15 minutes, 7 days, and 21 days after the noise. The duration of the testing was 30-40 minutes. The animals were anesthetized with inhalant isoflurane (5% for induction with 3 L/min O_2 flow rate, 2% for maintenance with 1 L/min O_2 flow rate) and placed on a homeothermic blanket to maintain body temperature during ABR recording. Platinum subdermal needle electrodes (Grass Technologies, West Warwick, RI) were placed at the scalp vertex (non-inverting), below the left pinna (inverting), and below the shoulder (ground) to record ABRs. The test stimuli were alternating phase tone bursts (1-ms duration with 0.5-ms rise/fall time) at frequencies of 2, 4, 6, and 8 kHz (in that order for each test) presented at a rate of 21/s. TDT hardware and software (Sig-Gen) were used to generate the stimuli. Acoustic stimuli were calibrated prior to each testing session (each session typically consisting of 4–6 animals being tested) by recording the output of the speaker with a microphone placed at the animals' ear level. Stimuli were attenuated with a programmable attenuator (TDT PA-4) and presented through a speaker placed at zero degrees azimuth, 10 cm from the vertex of each chinchilla's head. Responses were bandpass filtered (100–3000 Hz), amplified (50,000X), and averaged for 250 stimulus presentations using TDT hardware and software. Replications were obtained at stimulus levels near and at threshold. The lowest stimulus level that elicited a repea waveform was considered as threshold.

Noise exposure

For Experiment 1, the noise exposure was a continuous octave-band noise centered at 4 kHz at 107 dB SPL for two hours. For Experiment 2, the 4 kHz octave band noise was raised to a level of 112 dB SPL, and the duration was reduced to one hour. The rationale for the change in noise exposures was that the rescue paradigm may be optimally applied to shorter-duration, higher-sound pressure level noise exposures since those exposures will create more of their cochlear damage after cessation of the noise. The cochlear damage that develops post-noise is that which can be prevented using the rescue paradigm. In addition, the higher-level, shorter-duration noise was designed to induce a combination of mechanical trauma to the OHCs, as well as metabolic damage mediated by ROS. Noise that combines mechanical and metabolic OHC damage is common in conditions of impulse or impact noise, as well as some of the peak noise levels that can be delivered by speaker systems in automobiles, night clubs, and concert venues. Therefore, in an attempt to assess the therapeutic potential of the NAC/KX1-004 combination for a commonly-occurring type of noise that blends mechanical and metabolic damage, the 112 dB SPL, one-hour noise was used for Experiment 2. The noises were generated by a D/A converter on a signal processing board in a personal computer. The noises were routed through an

attenuator (HP 350 D), a filter (Krohn-Hite 3550R) and an amplifier (NAD 2200 PE). The acoustic horn (JBL 2360) was suspended directly above the chinchillas' cages. Prior to the noise exposures, the noise levels were calibrated with a Larson Davis 800B sound level meter.

Drug preparation and administration

NAC (Sigma Chemicals A7250) was dissolved in physiological saline at a concentration of 81.25 mg/ml. The solution was then brought up to physiological ph with the addition of sodium hydroxide. Animals were given the NAC solution at a level of 325 mg/kg (equal to 4.0 ml of the solution per kg) by intraperitoneal injection. KX1-004, the Src-PTK inhibitor, was obtained through collaboration with Kinex Pharmaceuticals (www.kinexpharma.com). The drug was dissolved in dimethyl sulfoxide (DMSO) and diluted to 2 mg/ml in sterile physiologic saline. The solution was delivered at 10 mg/kg by intraperitoneal injection. Control animals were given sterile physiological saline by intraperitoneal injection.

For Experiment 1, the two drugs were given in combination in two paradigms. In the protection paradigm, the NAC plus KX1-004 injections were given at 48, 24, 20, and 1 hour before the noise exposure. In the rescue paradigm, the NAC plus KX1-004 injections were given at 1, 20, 28, and 48 hours after the noise exposure. For Experiment 2, all drugs were given in rescue paradigms after the noise exposure in a different schedule compared with Experiment 1. In the first experimental group, KX1-004 alone was given at 1 and 24 hours after the noise exposure. In the second experimental group, the combination of NAC plus KX1-004 was given at 1 and 24 hours after the noise. In comparison with Experiment 1, the rescue protocol in changed in Experiment 2 from four total injections in 48 hours to two injections in 24 hours. The rationale for the change in injection schedules was based on unpublished pilot observations with KX1-004 that the injections occurring after 24 hours did not contribute to improved effectiveness of rescue. Therefore, if the rescue paradigm with KX1-004 was effective, it would only require injections to take place within 24 hours of the noise exposure. Experimental conditions are summarized in Table 1.

Assessment of threshold shift

To assess noise-induced threshold shift in the animals, pre-exposure thresholds were subtracted from the three post-exposure threshold measurements (15 minutes, 7 days, 21 days) to calculate threshold shift at each time point. The 15-minute and 7-day measurements provided indices of temporary threshold shift (TTS). The 21-day measurement provided the data for calculation of permanent threshold shift (PTS).

Statistical analysis

For each of the two experiments, 3-factor ANOVAs (Drug Group x Frequency x Test time) were used to analyze differences between the means of the three experimental groups across the four different test frequencies at the three different time points post noise exposure. Group and Frequency were analyzed as between-subjects variables, and Test time (Days post noise) was analyzed as a repeated measure. If a significant main effect occurred for Group or 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 with paired subjects *t*-tests.

Results

Pre-exposure thresholds

Pre-exposure thresholds for all groups in each of the two experiments were confirmed as being the same. For Experiment 1, the control group and the NAC/KX1-004 protection group had six animals each, and the NAC/KX1-004 rescue group had five animals. Statistical analysis (Two-way ANOVA) revealed a significant main effect of frequency, but no main effect of Group or Group x Frequency interaction. For Experiment 2, the control group and the KX1-004 rescue group had six animals each, and the NAC/KX1-004 rescue group had five animals. Statistical analysis (Two-way ANOVA) revealed a significant main effect of frequency, but no main effect of Group or Group x Frequency interaction. The absence of differences between groups prior to noise exposure gave confidence that the differences observed in threshold shift between groups were a result of the protective drug administrations the groups received before each day of the noise exposure.

Threshold shifts induced by noise

For Experiment 1, threshold shifts for the three groups at the four frequencies tested are shown for Day 0 [Figure 1a], Day 7 [Figure 1b], and Day 21 [Figure 1c]. At Day 0, substantial threshold shifts occurred at 2-8 kHz, consistent with the magnitude and duration of the noise exposure. All groups exhibited recovery during the period of time from Day 0 to 21. The three-way ANOVA showed a significant two-way interaction of Group x Day. The Group x Day interaction was broken down with a series of two-way

Table 1: Summary of the experimental conditions in Experiments 1 and 2

Drugs	Experiment 1	
	Paradigm	Times given
KX1-004 and NAC	Protection	48, 24, 20, and 1 hrs before noise
KX1-004 and NAC	Rescue	1, 20, 24, and 48 hrs after noise
Control saline	Protection or rescue	
	Experiment 2	
	Paradigm	**Times given**
KX1-004 alone	Rescue	1 and 24 hrs after noise
KX1-004 and NAC	Rescue	1 and 24 hrs after noise
Control saline	Rescue	1 and 24 hrs after noise

(Group x Frequency) ANOVAs at each test day. The two-way ANOVA on Day 0 found no main effects or interactions. The two-way ANOVA on Day 7 revealed a significant main effect of Group (P=0.001). Tukey A post hoc testing revealed that the group pre-treated with the NAC/KX1-004 combination had significantly lower threshold shift that the control group or the group treated with NAC/KX1-004 in the rescue paradigm. The two-way ANOVA on Day 21 revealed a significant main effect of Group (P=0.050). Tukey A post hoc testing revealed that the group pre-treated with the NAC/KX1-004 combination had significantly lower threshold shift that the control group or the group treated with NAC/KX1-004 in the rescue paradigm. The NAC/KX1-004 group treated in the rescue paradigm was not significantly different from the control group at any time point.

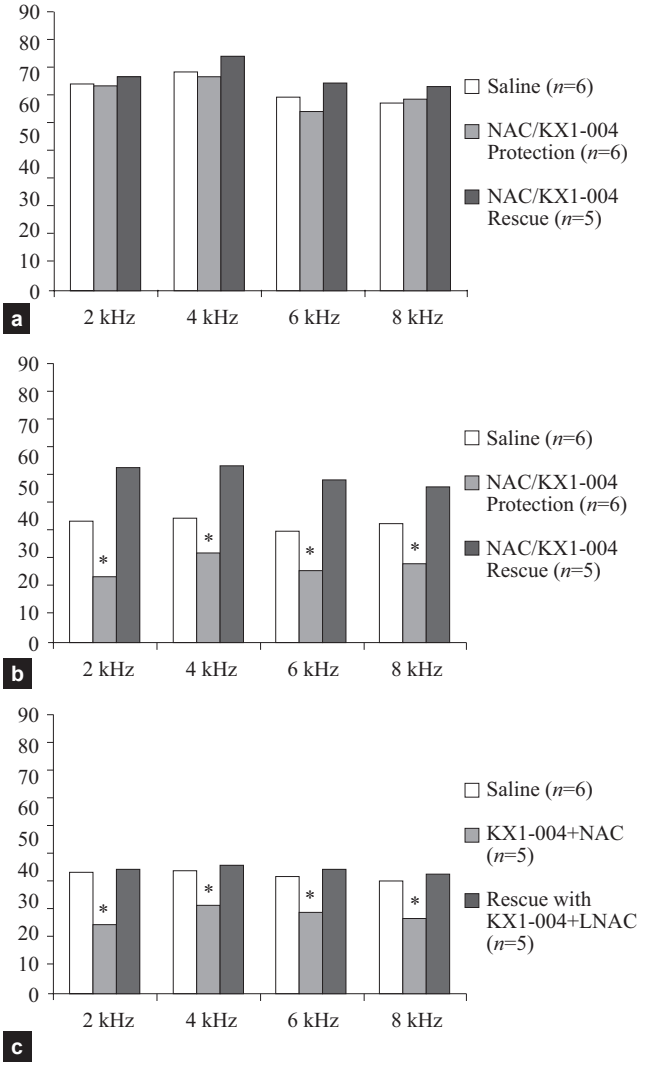

Figure 1: Threshold shifts at a) Day 0, b) Day 7, and c) Day 21 for the three experimental groups from Experiment 1, plotted as Threshold shift in dB against the frequency tested. Error bars are +1 SEM. On Day 7 and 21, the group pre-treated with KX1-004 and NAC had significantly lower threshold shifts at all frequencies compared to the control group and the group treated with Kx1-004 and NAC in the rescue paradigm (marked with *)

For Experiment 2, threshold shifts for the three groups at the four frequencies tested are shown for Day 0 [Figure 2a], Day 7 [Figure 2b], and Day 21 [Figure 2c]. As in Experiment 1, there was substantial TTS at Day 0, followed by all groups exhibiting recovery during the period of time from Day 0 to 21. The three-way ANOVA showed a significant two-way interaction of Group x Day. The Group x Day interaction was broken down with a series of two-way (Group x Frequency) ANOVAs at each test day. The two-way ANOVA on Day 0 found no main effects or interactions. The two-way ANOVA on Day 7 revealed a significant main effect of Group (P<0.001). Tukey A post hoc testing revealed that the group treated with KX1-004 in the post-noise rescue paradigm had significantly lower threshold shift that the control group, and the group treated with the NAC/KX1-004 in the post-noise rescue paradigm had significantly less threshold than both the control group and the KX1-004 group. The two-

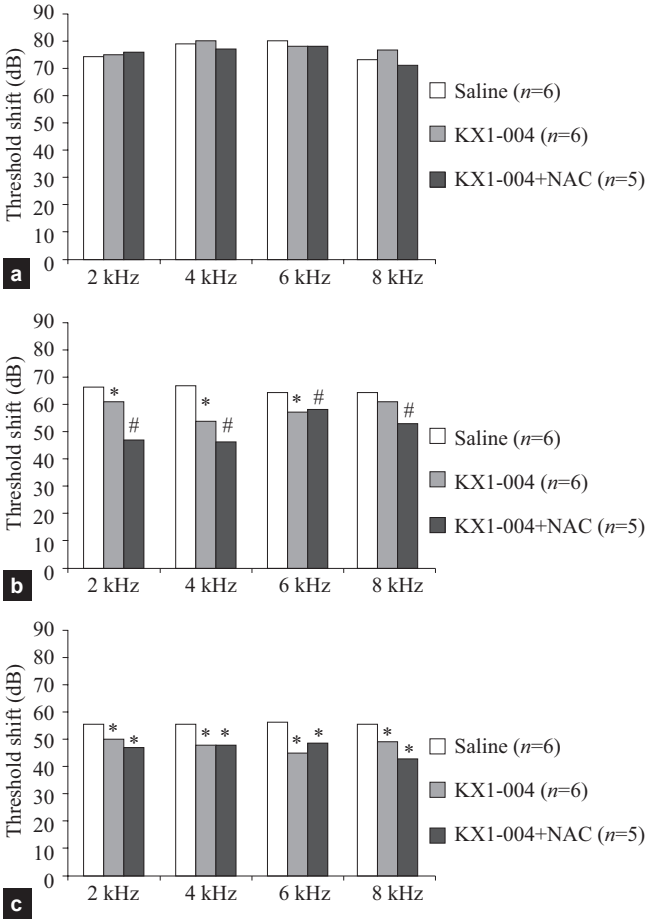

Figure 2: Threshold shifts at a) Day 0, b) Day 7, and c) Day 21 for the three experimental groups from Experiment 2, plotted as Threshold shift in dB against the frequency tested. Error bars are +1 SEM. Groups significantly different from the control are marked with*. Groups significantly different from both the control and the KX1-004 treated group are marked with #

way ANOVA on Day 21 revealed a significant main effect of Group ($P<0.01$). Tukey A post hoc testing revealed that the group treated with KX1-004 in the post-noise rescue paradigm and the group treated with the NAC/KX1-004 in the post-noise rescue paradigm both had significantly lower threshold shift that the control group, but the two treated groups were not different from one another.

Discussion

Consistent with expectations, the combination of NAC and KX1-004 was able to reduce TTS and PTS compared to the vehicle controls when given prior to noise exposure. TTS at Day 0 was not affected by the protection paradigm. This may have been due to the high level of TTS that was achieved (60 dB or more) or that the mechanisms believed to create TTS (stereocilia damage, damage to junctions of inner hair cells and afferent auditory nerve fibers[39]) may have been unaffected by the drug treatments. Contrary to expectations, the combination of NAC and KX1-004 did not provide more protection than the two drugs individually have provided in previous studies.[11,12,17] The lack of a synergistic effect of combining the two drugs can be explained in several ways. It is possible that one of the drugs did not adequately penetrate the cochlea. It is also possible that there is more redundancy in the drugs' mechanisms than expected. KX1-004, through inhibition of Src, is believed to inhibit apoptosis. Much of the noise-induced apoptosis may be triggered by ROS, and scavenging ROS with increased glutathione (from NAC) can act to minimize apoptosis, thus rendering the KX1-004 unnecessary. The Src pathway may also be involved in the generation of superoxide and other downstream ROS through a complex pathway involving the activation of NADPH oxidase, an enzyme shown to be active in the cochlea.[40-42] Inhibition of the Src pathway may serve to restrict noise-induced ROS generation in the cochlea, thus rendering the antioxidant treatment unnecessary. The potential redundancy of the two drug treatments is a relevant topic for future investigation.

The other key finding from Experiment 1 was that the combination of NAC and KX1-004 was ineffective when given as a rescue. Any study of pharmacological protection from noise that yields negative results is somewhat difficult to interpret, in that the dosing, scheduling, noise parameters, route of administration, etc can be a part of the reason for the negative results. Because of the failure of the NAC/KX1-004 in the rescue paradigm in Experiment 1, a second experiment was added to further test the NAC/KX1-004 combination, focusing only on the rescue paradigm. In the second experiment, we chose to pursue a study with a shorter noise duration and higher sound pressure level. The shorter noise duration creates the potential for more post-noise cochlear damage, damage that can be prevented with a rescue drug treatment. Experiment 2 utilized a one-hour, 112 dB SPL 4 kHz noise to create more direct mechanical trauma and significant post-noise OHC lesion growth. The experiment found a positive rescue effect of both KX1-004 and the NAC/KX1-004 combination as rescue treatments. The effect was fairly modest (~10 dB) for PTS, but the positive effect establishes that the Src inhibitor can be effective as a rescue treatment. There was also an additional protective effective from the NAC/KX1-004 combination for Day 7 TTS, but not for the PTS measurement. The lack of synergistic effect of NAC and KX1-004 in the rescue paradigm can be attributable to the same set of possible factors as discussed above for the drugs in Experiment 1. The improved effectiveness of the NAC/KX1-004 as a rescue treatment in Experiment 2 compared with Experiment 1 provokes multiple possible explanations. First, the reason that a shorter-duration (one hour), higher-level (112 dB SPL) noise was utilized in Experiment 2 was to create a noise exposure in which more of the cochlear damage was taking place after cessation of the noise. The goal of the experiment was to demonstrate possible rescue with NAC/KX1-004, and a noise with more post-noise cochlear damage is an optimal noise exposure for rescue intervention. With the two-hour noise in Experiment 1, much of the cochlear lesion may have already been formed by the end of the noise exposure. Rescue injections after that sort of noise may have come too late to be effective. If the duration and level of the noise exposure were indeed the cause for the differences in rescue effectiveness, this implies that rescue may best applied to clinical patients that sustained short-duration, high-level exposures, such as impulse or impact trauma. A second possible explanation lies with the mechanisms of action of KX1-004. It is an apoptosis inhibitor that may preferentially target anoikis. Anoikis is a subset of apoptosis that results from mechanical trauma to the cell that disrupts the cell's connections to its support cells and extra-cellular matrix.[35] The 112 dB SPL noise in Experiment 2 likely induced more mechanical stress to the OHCs than did the 107 dB SPL noise from Experiment 1. Therefore, KX1-004 may have been a more effective rescue intervention for the noise in Experiment 2 compared with the noise in Experiment 1 because of the greater potential for OHC death through anoikis in the 112 dB SPL noise.

While the current experiments demonstrated a proof of concept that KX1-004 can be successfully used in a rescue paradigm, the significance of the results is limited by the relatively small magnitude of the rescue effect. What remains unknown from the current studies is the ideal injection schedule and dosing regimen for KX1-004 or the NAC/KX1-004 combination in the rescue paradigm. It is possible that higher doses, or more frequent injections within the 24-hour window, after the noise exposure would optimize the effectiveness of the approach. Future studies will focus on developing optimal dosing regimens and assessment of rescue against a variety of noise conditions, and will utilize OHC cochleograms to further support the threshold shift findings and elaborate on the mechanisms of the rescue effect demonstrated.

Side effects from the drugs were minimal. None of the groups' mean weights in either experiment changed significantly (data not shown). Overall, the findings of the current study extend the findings of our previous study[12] that the Src inhibitor, KX1-004, can be effective as an otoprotective drug against noise exposure when delivered systemically. The effective use of the drug in a rescue paradigm is a step forward toward fully realizing the drug's protective value against NIHL.

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