

Post-Exposure Oral Afatinib Mitigates Noise-Induced Hearing Loss at Clinically Translatable Doses

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ABSTRACT

Noise-induced hearing loss (NIHL) remains a major occupational hazard in modern society, with limited effective options for prevention or treatment. We previously demonstrated that oral administration of Afatinib, a potent and selective epidermal growth factor receptor inhibitor approved by the U.S. FDA for cancer treatment, protects against NIHL in both zebrafish and mouse models. In the present study, we found that plasma exposure to orally administered Afatinib exhibits linear dose–exposure relationships across humans, rats, and mice. Based on this cross-species analysis, we extrapolated that a dose of 6 mg/kg in the mouse would elicit a similar clinical exposure to that observed in humans with the FDA-approved 40 mg daily dose. To define the therapeutic time window, we evaluated oral Afatinib administration initiated at different time points relative to moderate noise exposure in mice. The greatest protective effect was observed when treatment was initiated 1-hour post-exposure. Notably, protection was achieved across a broad dose range (0.6–90 mg/kg), corresponding to a therapeutic dose window exceeding 150-fold. The optimal effective dose was 0.6 mg/kg—equivalent to one-tenth of the human daily dose administered to lung cancer patients. Given its established human safety profile, low effective doses, wide therapeutic window, and post-exposure efficacy, Afatinib represents a promising and clinically translatable candidate for trials targeting NIHL.

KEY WORDS

Afatinib, noise, hearing loss, cancer, epidermal growth factor receptor, kinase inhibitors, pharmacokinetics, therapeutic window, preclinical, clinical trial, clinical translation

INTRODUCTION

Noise-induced hearing loss (NIHL) is a major occupational hazard and a leading cause of permanent sensorineural hearing loss worldwide. According to the American Academy of Otolaryngology–Head and Neck Surgery, approximately 23 million Americans aged 20 and older suffer from this condition¹. Those at greatest risk include individuals in high-noise occupational and recreational settings, such as military personnel, construction and manufacturing workers, and musicians²⁻⁶. The loss of hearing significantly impairs quality of life, contributing to communication difficulties and reduced workplace productivity. Furthermore, NIHL carries consequences that extend far beyond physical hearing, significantly degrading an individual's social and emotional quality of life⁷. Despite its substantial burden, effective preventive and therapeutic options remain limited.

NIHL arises from irreversible damage to the cochlear sensorineural pathways—the sensory and neural structures responsible for converting sound vibrations into electrical signals transmitted to the brain. Within this pathway, mechanosensory hair cells, including inner and outer hair cells (IHCs and OHCs), convert acoustic vibrations into electrical signals, a process known as mechanotransduction. These signals are then relayed to the auditory nerve via synaptic ribbons—specialized presynaptic structures at the IHC base that enable the rapid, high-fidelity neurotransmission to the brain^{8,9}. Several overlapping mechanisms have been proposed to account for the damage to cochlear sensorineural pathways following prolonged or moderate to intense noise exposure. These pathological mechanisms—including mechanical trauma, metabolic exhaustion, oxidative stress, glutamate excitotoxicity, cochlear synaptopathy, inflammation, and apoptosis—frequently converge on cochlear hair cells and synaptic ribbons, resulting in their irreversible loss and permanent hearing impairment^{9,10}. Effective pharmacological intervention must therefore target these pathways within a critical time window to halt ongoing damage and promote recovery.

In our previous study¹¹, we compared published cochlear gene expression profiles from noise-exposed mice with the LINCS L1000 database (>1 million drug-induced transcriptomic profiles) to identify candidate therapeutics without direct compound screening in noise exposure animal models. Among the top candidates, Afatinib, a potent and selective epidermal growth factor receptor (EGFR) inhibitor and an FDA-approved drug for lung cancer, demonstrated robust protective effects in both a zebrafish excitotoxicity model and a mouse NIHL model with a pre-exposure treatment regimen. Consistent with these findings, conditional knockout mice lacking EGFR in the cochlea exhibited significantly reduced hearing loss following noise exposure, implicating EGFR signaling as a key mediator of NIHL and supporting Afatinib as a promising preventive therapeutic candidate.

In the present study, we evaluated the otoprotective efficacy of orally administered Afatinib in a clinically relevant mouse model of NIHL. We first assessed dose translation by comparing plasma pharmacokinetic profiles across mice, rats, and humans. We then investigated the therapeutic time window and found that treatment initiated 1 hour after noise exposure provided maximal protection against hearing loss. Notably, this protection was achieved at doses as low as one-tenth of the human-equivalent daily dose. Overall, Afatinib conferred protection across a broad dose range, corresponding to a therapeutic dose window exceeding 150-fold. These findings demonstrate robust post-exposure efficacy at clinically relevant doses, highlighting the translational potential of Afatinib and providing mechanistic insight into the temporal dynamics of acoustic injury and repair.

MATERIAL AND METHODS

Animal experiments

Noise exposure

Noise-induced ototoxicity mouse model was employed in CBA/CaJ mice (Strain #:000654, Jackson Laboratory [JAX], Bar Harbor, ME) with minor modifications from previously published methods^{11,12}. CBA mice (9 to 10 weeks old) placed in small open-walled cylindrical containers inside a reverberant chamber were exposed to octave band noise (8-to 16-kHz at 103-dBSPL) for 2 hours delivered via an exponential horn fitted onto a titanium horn driver (JBL 2426H) and driven by a power amplifier (Crown XTi1000) using RpvdsEx circuit and a RZ6 Multi-I/O processor (Tucker Davis Technologies, Alachua, FL). Before each session, the overall noise level was measured and calibrated at the center and four quadrants of the reverberant chamber using a calibrated 6.35-mm microphone (PCB 378C01). Noise exposure level was based on our previously published work¹¹⁻¹⁷ showing 8-to16-kHz octave band noise at 100 dB for 2 hours causes 20-dB ABR threshold shift and IHC synaptic ribbon loss in FVB mice and adjusted to 103 dB due to lower sensitivity of CBA mice to the noise.

A total of 127 mice, aged 8–10 weeks at the start of the 14-day experimental protocol, were used. The animals were given 1 week to acclimate to the Animal Resource Facilities (ARF) at the Scintillon Institute (San Diego, CA, USA). All animals were housed in standard cages with *ad libitum* access to food and water in accordance with the NIH NIDCD/NINDS guidelines and the Institutional Animal Care and Use Committee-approved protocol (2024-JZ-001). At the beginning of the study, the average body weight ranged from 17.9 to 22.5 (mean $\pm$ SD: 20.7 $\pm$ 1.2, n=22) for females and ranged from 21.8 to 33 (mean $\pm$ SD: 27.3 $\pm$ 2, n=105) for males. Animals were randomly assigned to all experimental groups, with approximately equal numbers of males and females for key experimental groups.

For noise-induced ototoxicity, mice received 103 dB noise 8-16 kHz for 2 hrs. Each treatment scheme consisted of 2 hrs of noise exposure followed by a 14-day recovery period (**Fig. 2A and 3A**). Control animals received the same manipulation except the noise exposure. Mouse overall condition and body weight were monitored throughout studies to assess their general health status.

For otoprotectant treatment, Afatinib (# 21567) was obtained from Cayman Chemical (Ann Arbor, MI, USA), dissolved in a vehicle composed of 0.5 w/v% Methyl Cellulose 400 solution (#133-17815, Fujifilm Wako Pure Chemical, Japan), and administered to mice via oral gavage (OG). Various doses (0.04, 0.6., 2.3, 18, and 90 mg/kg) were given to mice with one- or two-day intervals between administrations for a total treatment of 5 times for each dose. The treatment timing was based on plasma pharmacokinetic data of Afatinib in mice, prior FDA approval documents, as well as our *in vivo* efficacy data on Afatinib¹¹.

Auditory testing

For all auditory assessments, animals were anesthetized via intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. To maintain proper thermoregulation during the entire recording session, mice were placed on heating pads that maintained their body temperature at 37 ± 0.2 °C. Ophthalmic ointment was applied to the eyes to prevent corneal desiccation during the procedure.

ABR and wave I amplitude measurements

ABRs were recorded from the left ear only, as previously described with minor modifications¹². Briefly, following anesthesia, ABR waveforms were acquired in a sound-attenuated chamber (Industrial Acoustics Company) using a closed-field configuration. Saline was applied to the electrode sites, and subdermal needle electrodes were inserted at the vertex (active), behind the left pinna (reference), and at the base of the tail (ground). The response signals were amplified using a low-impedance digital biological amplifier (Medusa RA4L system; Tucker-Davis Technologies [TDT]; Alachua, FL, USA) with a 20 dB gain. Three stimulus

frequencies (8, 16, and 32 kHz) were tested. For each frequency, the sound pressure level (SPL) was decreased in 5 dB steps from 90 to 0 dB to determine the auditory threshold, defined as the lowest SPL eliciting a response above background noise. Each ABR waveform was averaged over 500 tone bursts. Signals were bandpass filtered between 300 Hz and 3 kHz. Baseline ABR recordings were performed within 1 week prior to any treatment (when mice were 7–8 weeks old), and final recordings were conducted immediately after the 14-day experimental protocol concluded (mice aged 10–11 weeks). Thresholds were independently determined by 2 to 3 experimenters per mouse. ABR wave 1 amplitudes were measured as the difference between the positive peak and the following negative trough. Data analysis was performed using BioSigRZ software.

DPOAE measurements

Distortion product otoacoustic emissions (DPOAEs) were measured from the left ear only, as previously described with slight modifications¹⁸. Following anesthesia, DPOAEs were recorded in a sound attenuated chamber (Industrial Acoustics Company) using the RZ6 Multi-I/O processor (TDT). To ensure clear access to the tympanic membrane, a DPM1 microphone system (TDT) was inserted into the ear canal. Three stimulus frequencies (10, 14, and 29 kHz) were tested using an f_2/f_1 ratio of 1.2. At each frequency, the stimulus intensity was decreased in 5 dB steps from 75 to 0 dB SPL to determine the DPOAE threshold, defined as the lowest SPL producing an emission detectable above background noise. Changes in DPOAE thresholds were calculated by subtracting baseline values from those obtained at the end of the experimental protocol. Thresholds were independently determined by 2 to 3 experimenters per mouse. Data analysis was performed using BioSigRZ software.

Cochlea dissection and immunohistochemistry

Following auditory testing at the end of the 14-day protocol, mice were euthanized via CO₂ inhalation followed by decapitation as a secondary confirmatory method, in accordance with institutional guidelines. The temporal bones were immediately harvested and rinsed with PBS

until all visible blood was removed. Cochleae were then fixed in 4% paraformaldehyde (PFA) at 4 °C for six days. Fixed tissues were washed three times with PBS and decalcified in 150 mM EDTA (pH 8.0) for 72 to 96 hours at 4 °C. Once decalcified, each cochlea was micro-dissected and incubated in a blocking solution consisting of 0.1% Triton X-100 (Bio-Rad) in PBS (PBST) with 10% normal horse serum, with gentle rocking. Samples were then incubated overnight at 4 °C with the anti-myosin-VIIa primary antibody (rabbit anti-myosin VIIa; 1:300; Proteus Biosciences #26-6790) and anti-C-terminal binding protein 2 (CtBP2) antibody (mouse anti-CtBP2; 1:200; BD Biosciences #BDB612044) diluted in the same blocking solution. After four 10-minute washes in PBST at 4 °C with rocking, samples were incubated overnight at 4 °C with secondary antibody conjugated to Alexa Fluor™ 568 (anti-rabbit, 1:300, Invitrogen #A10042) and secondary antibody conjugated to Alexa Fluor™ Plus 488 (anti-mouse, 1:400, Invitrogen #PIA32766). Following four additional 10-minute washes in PBST at 4 °C with rocking, samples were incubated with 4',6-diamidino-2-phenylindole, dilactate (DAPI, dilactate, Invitrogen #D3571, 10 ug/ml) for 15-minutes at room temperature, and then washed thoroughly with PBST to remove excess DAPI. Each cochlea was then dissected into three segments (apical, middle, and basal turns) and mounted using ProLong™ Gold Antifade mounting media (Invitrogen #P36930).

Imaging and data acquisition

To count IHCs, OHCs, and CtBP2 puncta, images were acquired at room temperature using a Zeiss LSM-880 confocal microscope with the Airyscan mode at a Z stack of 40 slices with a total thickness of 2.4 µm. Images acquired using the Zeiss system were processed with Airyscan process and max projections of the 3D z-stack images. IHC and OHC counts were expressed as the number of cells per 135 µm segment of the organ of Corti. All counts were performed manually at regions corresponding to 16 and 32 kHz along the cochlear spiral. To map cochlear positions to their respective frequency regions, a cochlear frequency map was generated using the Measure Line plug-in in ImageJ software. Minor linear adjustments to brightness and contrast

were applied uniformly across images, and final figures were assembled using Adobe Photoshop and Illustrator software.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (version 10.3.1; GraphPad Software, San Diego, CA, USA). Differences among multiple groups were assessed using two-way analysis of variance (ANOVA), with noise exposure and drug treatment as the two independent factors. Post hoc multiple comparisons were performed using Tukey's test when comparing all group pairs, or Dunnett's test when comparing treatment groups against the noise exposure group. Statistical significance was defined as $p < 0.05$.

RESULTS

Plasma pharmacokinetics of single oral Afatinib and dose-dependent AUC_{0-24h} across mice, rats, and humans

To better understand the pharmacokinetics of orally administered Afatinib in mice, we compiled literature data available in mice, rats, and humans following a single oral administration. For purposes of comparison across species, doses were normalized to a human equivalent dose using the well-established cross-species body surface ratios and a 60 kg human body weight²⁶, and tabulated with their corresponding exposure values AUC_{0-24h} (**Table S1 and Fig. 1**). In mice, Afatinib reached an AUC_{0-24h} of 2188 ± 282 h·ng/mL (red triangles, **Fig. 1**) in plasma after oral administration at 30 mg/kg in mice (equivalent to ~150 mg in humans)^{19,20}. Published rat data were consistent with the mouse data, and showed a linear increase in AUC_{0-24h} with increasing dose across a wide dose range (1.5 to 18 mg/kg in rats, equivalent to 14.6 to 175.1 mg in humans) (**Table S1 and Fig. 1**, green squares)²¹. Similarly, available human Afatinib data from 4 clinical studies (**Table S2 and Fig. 1**, blue circles) showed AUC_{0-24h} values increased in a dose-dependent manner in both healthy individuals and cancer patients²²⁻²⁵. Importantly, when converted to human equivalent doses, the regression linearity curves for mice, rats, and humans ($R^2 > 0.9$) demonstrate an excellent correlation between average plasma AUC_{0-24h} values and administered doses of Afatinib, indicating that the dose-proportional pharmacokinetics scale allometrically within the tested range across the three species. These agreements indicate that AUC_{0-24h} is a suitable parameter to compare human and mouse exposure following oral Afatinib dosing and that an Afatinib dose in mice can be reasonably scaled to a human equivalent dose that will elicit similar exposure.

To benchmark a dose range for our hearing loss studies and to use as a reference for estimating a safety margin, we converted the clinical dose of Afatinib to a mouse equivalent dose. Studies in cancer patients identified dose limiting toxicities at ≥ 50 mg in a continuous, once daily dosing schedule. The FDA approved daily dose is 40 mg corresponding to a human equivalent

mouse dose of 8 mg/kg based on body surface area^{21,26}. Extrapolating from the data relating dose to AUC_{0-24h} (**Fig. 1**), we estimate that a dose of 6 mg/kg in the mouse would elicit a similar clinical exposure to that observed in humans with the 40 mg dose.

Oral Afatinib administration is effective in protecting against noise-induced hearing loss when given post-noise exposure

Prior to conducting dose response studies and refining the dosing scheme, we first sought to evaluate the otoprotective efficacy of Afatinib in a NIHL mouse model in which treatment was initiated at different times relative to noise exposure¹¹⁻¹⁷. CBA/CaJ (CBA) mice received 8-16 kHz octave noise exposure for 2 hrs at 101.6, 103, 105, and 107 dB SPL levels followed by 14 days of recovery (**Fig. S1**). Consistent with other studies^{11,14}, the 103-dB SPL noise exposure resulted in moderate permanent hearing loss as assessed by two auditory tests: auditory brainstem response (ABR) threshold shifts and distortion product otoacoustic emission (DPOAE) threshold shifts.

With this moderate level of noise exposure, we then tested three different Afatinib prevention or treatment schemes, including treatment starting at 1 day prior to the noise exposure (pre-treatment 1d), 1 hr (post-treatment 1hr) and 1 day (post-treatment 1d) after the noise exposure (**Fig. 2A**). In each scheme, oral doses of Afatinib were administered for a total of 5 times starting daily for the first two doses and then every other day for the remaining three doses. The Afatinib dose (18 mg/kg) selected for this treatment timing experiment, though ~3-fold higher than the equivalent clinical human dose, was previously found to be otoprotective in the NIHL mouse model¹¹.

Both ABR and DPOAE auditory tests were performed on all animals at the beginning and end of the 14-day protocol (**Fig. 2A**). Body weights were recorded for each mouse, and no significant differences were found among all experimental groups (**Fig. S2, Table S3**). The ABR baseline thresholds were comparable among all experimental groups at the beginning of the study (**Fig. 2B, Table S4**). The ABR threshold shifts at 8, 16, and 32 kHz in the NIHL group were 21-52

dB and those in the vehicle only treated group (Control) were close to zero (**Fig. 2C**, blue and black lines, respectively). These threshold shifts (both means and SEM) are consistent with other reports using similar mouse models¹¹⁻¹⁷, demonstrating the reproducibility and reliability of noise ototoxicity using this model as well as the accuracy of our measurements. Consistent with our previous report¹¹, the group with the pre-treatment significantly reduced the noise-induced ABR threshold shifts. Interestingly, the post-treatment 1 hr group significantly reduced the noise-induced ABR threshold shifts, while the treatment at 1-day post-noise exposure group did not (**Fig. 2C, Table S4**), suggesting a limited time window (<1 day) for effective post-exposure intervention.

To further examine functions of OHCs, we analyzed DPOAE thresholds. Similarly, the DPOAE baseline thresholds were comparable among all experimental groups at the beginning of the study (**Fig. S3A, Table S5**), whereas significant differences were detected between NIHL group and vehicle control group at all tested frequencies, consistent with previous studies¹¹. No significant protection was observed between any treatment groups and the NIHL only group, although there were trends of improvement by Afatinib (**Fig. S3B, Table S5**). Together, these studies demonstrate that oral Afatinib administration is an effective early treatment strategy against NIHL in mice.

We also tested oral Afatinib at as high as 90 mg/kg in the 24-hour pre-exposure scheme and found that Afatinib is still otoprotective even at such a high dose without any obvious overall adverse effects during the experiment (**Fig. S4**). These findings demonstrate a wide therapeutic dose range and favorable tolerability profile for oral Afatinib when administered as a short-term otoprotective treatment.

Oral Afatinib administration is effective in protecting against NIHL at one-tenth of the FDA-approved human equivalent daily dose

Next, we focused on the post treatment scheme at 1 hr after the noise exposure to study the therapeutic dose range of Afatinib (**Fig. 3A**). Since the FDA-approved human equivalent mouse dose is 6 mg/kg based on AUC_{0-24h} , we chose four doses (0.04, 0.6, 2.3 and 18 mg/kg) of oral Afatinib plus a vehicle negative control for otoprotection experiments in mice, with the highest dose (18 mg/kg) selected as a positive control to confirm Afatinib otoprotection in the NIHL mouse model. ABR and DPOAE were performed on all animals at the beginning and end of the 14-day protocol (**Fig. 3A**). Body weights were recorded for each mouse and no significant differences were found among all experimental groups (**Fig. S5, Table S6**).

The ABR baseline thresholds were comparable among all experimental groups at the beginning of the study (**Fig. 3B, Table S7**). Noise exposure at 103 dB for two hours resulted in dramatic ABR threshold shifts (ABR traces in **Fig. S6**). At 8, 16, and 32 kHz in the NIHL group, ABR threshold shifts were 25-41 dB and those in the vehicle only (Control) group were close to zero (**Fig. 3C** blue and black lines, respectively), again consistent with previous studies (**Fig. 2C**)¹¹⁻¹⁷. The groups with Afatinib alone and no noise exposure did not show any ABR threshold shifts, indicating that Afatinib at these doses are not ototoxic. Among the 4 doses tested in this post-exposure treatment efficacy experiment, oral Afatinib administration at 0.6, 2.3, and 18 mg/kg significantly reduced the noise-induced ABR threshold shifts, while the treatment at 0.04 mg/kg noise exposure group did not (**Fig. 2C and S6**). No statistically significant differences were observed among the 0.6, 2.3, and 18 mg/kg treatment groups, although the 0.6 mg/kg treatment group showed a trend toward greatest otoprotection.

Consistent with the significant ABR threshold shifts, analysis of the wave 1 amplitudes of ABR tests at 16 kHz and 32 kHz revealed a significant decrease of wave 1 amplitudes in the noise exposure group compared to the non-exposed controls (control and Afatinib only groups). Treatment with Afatinib at the dose of 0.6 mg/kg had significant protection against amplitude reductions following noise exposure at the sound levels of 50 and 55 dB at the frequencies of 16 and 32 kHz, respectively (**Fig. S7A and B**). Analysis of the latency to wave 1 revealed significant

delay of latency amplitudes in the noise exposure group compared to the non-exposed controls (control and Afatinib only groups). However, treatment with Afatinib had no significant protection against noise-induced Wave 1 delay over the range of sound levels (20-90 dB SPL), although there were trends of improvements by Afatinib particularly again at the dose of 0.6 mg/kg (**Fig. S7C and D**).

For DPOAE, baseline thresholds were comparable among all experimental groups at the beginning of the study (**Fig. S8A, Table S8**) and significant differences were detected between NIHL group and vehicle control group at all tested frequencies (**Fig. S8B and S9**). After Afatinib treatment, significant protection was observed between the noise exposure group and the Afatinib treatment group at the dose of 0.6 mg/kg at 10, 14 and 29 kHz and at the dose of 2.3 mg/kg only at 10 kHz (**Fig. S8B and S9** for DPOAE traces). In addition, we analyzed DPOAE amplitudes at 14 kHz and 29 kHz and found significant decreases of DPOAE amplitudes in the noise exposure group compared with the control group (**Fig. S10**). Treatment with Afatinib at the dose of 0.6 mg/kg had significant protection against noise-induced decrease of DPOAE amplitudes over the range of sound levels 40-70 dB except the 45 dB at 14 kHz. Similarly, treatment with Afatinib at the dose of 2.3 mg/kg had significant protection against noise-induced decrease of DPOAE amplitudes, but only at the stimulus sound level of 40 dB (**Fig. S10A**). At 29 kHz, significant protection against noise-induced decrease of DPOAE amplitudes by Afatinib was found only at 0.6 mg/kg and at the stimulus sound level of 50 dB (**Fig. S10B**). In this post-exposure treatment efficacy experiment, all key treatment groups included both male and female mice. Sex-stratified analysis of the efficacy experiment revealed no significant difference in Afatinib-mediated otoprotection against NIHL (**Fig. S11**).

To determine cochlear hair cell and presynaptic ribbon changes following NIHL and the Afatinib treatment, we sacrificed the mice at the end of the entire protocol. Cochlear morphological analysis included hair cell counts using myosin 7a immunostaining and IHC presynaptic puncta quantification using CtBP2 staining. Hair cell counts, including IHCs and OHCs, by using myosin

7a immunostaining, showed that no treatment caused any hair cell loss (**Fig. S12A-C**), consistent with prior studies on similar moderate levels of noise injury in mice^{11,12,27}. Next, we examined and quantified synaptic ribbons in the IHCs in two tonotopic regions of the cochlea, 16-to 22-kHz, and 32-to 40-kHz regions (**Fig. S12D-F**). After noise exposure, there were significantly lower CtBP2 presynaptic puncta in IHCs. Furthermore, there were more CtBP2 presynaptic puncta in the Afatinib-treated groups than the noise group treated with the vehicle in 16-and 32-kHz regions, suggesting that post-exposure Afatinib protects against noise-induced cochlear synaptopathy.

DISCUSSION

The present study demonstrates that orally administered Afatinib, an FDA-approved anticancer drug with an established safety profile in humans, confers robust otoprotection in a clinically relevant mouse model of NIHL. Pharmacokinetic profiling across mice, rats, and humans enabled cross-species dose translation, establishing a dose range for testing in the NIHL model which can be potentially translated to a dose for clinical testing. Treatment initiated one hour after noise exposure provided maximal hearing protection, defining a clinically actionable therapeutic window. Remarkably, this protection was achieved at a dose equivalent to one-tenth of the human daily dose administered to lung cancer patients, with efficacy maintained across a broad dose range corresponding to a therapeutic dose window exceeding 150-fold. Collectively, these findings support the translational potential of Afatinib and provide mechanistic insight into the temporal dynamics of noise-induced cochlear injury and repair.

The finding that Afatinib remains protective when administered 1 hour after noise exposure strongly supports its clinical relevance. Because noise exposure is often unpredictable, pre-treatment strategies—commonly employed in preclinical studies, including our prior work¹¹—are impractical in many real-world settings such as military operations and industrial environments. Consistent with this, prior studies have shown that MAPK pathway inhibitors (**Fig. 4**), including Dabrafenib (BRAF inhibitor), Trametanib (MEK inhibitor), Tizaterkib (ERK1/2 inhibitor), and Oseltamivir phosphate, can confer protection when administered within a post-exposure window of 1–48 hours^{13–18}. Together, these findings support a shift from prevention-only approaches to clinically feasible early intervention strategies. The observed post-exposure efficacy further indicates that the molecular cascade triggered by acoustic trauma persists and remains pharmacologically targetable after the initial insult. This is consistent with established NIHL pathophysiology, in which glutamate excitotoxicity, oxidative stress, inflammation, and aberrant kinase signaling—including EGFR/BRAF/MEK/ERK pathway activation—continue beyond the

period of noise exposure. Our prior observations of increased MEK and ERK phosphorylation in cochlear tissues and supporting cells 30–120 minutes following noise or cisplatin insult further support this temporal window^{11,13-18}. These findings suggest that Afatinib acts on downstream injury pathways rather than solely preventing the initial acoustic damage. While Afatinib did not demonstrate significant protection when administered 24 hours post-exposure, future studies are needed to define the upper temporal limit of efficacy and to evaluate combination strategies that may extend the post-exposure therapeutic window.

Another key translational finding is that robust otoprotection is achieved at doses substantially lower than those used in oncology. Afatinib demonstrated efficacy at 0.6 mg/kg—approximately one-tenth of the human-equivalent FDA-approved dose—and across a broad dose range (0.6–90 mg/kg), corresponding to a therapeutic dose window exceeding 150-fold. To establish human dose relevance, we compared plasma pharmacokinetic profiles across species and found that oral Afatinib exhibits a consistent linear dose–exposure relationship in mice, rats, and humans. Based on this cross-species analysis, we estimated that a mouse dose of approximately 6 mg/kg corresponds to the FDA-approved human dose (**Fig. 1**), consistent with conventional body surface area–based scaling²⁶. Accordingly, the efficacious dose range identified in mice (0.6–2.3 mg/kg) falls below the FDA-approved human-equivalent exposure range in cancer patients. However, treatment of these patients with Afatinib at 40 mg/day results in an array of serious adverse effects also associated with other EGFR inhibitors, including diarrhea, rash/acneiform dermatitis, stomatitis, paronychia, dry skin, decreased appetite, nausea, vomiting, and pruritus that occurred in $\geq 20\%$ patients in the trials. The more severe effects, though less frequent ($<2\%$), were bullous and exfoliating skin disorders, interstitial lung disease, hepatic toxicity, keratitis, and fetal toxicity in pregnant women. Lower effective doses as well as the shorter duration of treatment that would be needed for otoprotection are expected to reduce the incidence and severity of these adverse effects. This is particularly important for potential use

in otherwise healthy populations, such as military personnel and industrial workers. A wide therapeutic dose window is also highly desirable for preventive or early-intervention applications. Notably, other MAPK pathway inhibitors, including Tizaterkib and Dabrafenib, have demonstrated similar translational characteristics (**Fig. 4**), supporting the broader relevance of this signaling axis as a therapeutic target in NIHL^{13,14,16,18}.

The noise exposure paradigm used in this study (103 dB, 8–16 kHz octave band noise for 2 hours in CBA/CaJ mice) produced permanent hearing loss of 30–50 dB, modeling clinically relevant acoustic injury encountered in both military and civilian settings without protective equipment. It remains to be determined whether Afatinib and related pathway inhibitors provide comparable protection across a broader range of noise intensities. Lower-intensity exposures may result in transient threshold shifts and synaptopathy, whereas higher-intensity exposures can cause more extensive structural damage to the inner ear. It is also conceivable that mitigating noise-induced damage with Afatinib could reduce cumulative injury over time and thereby contribute to the prevention of age-related hearing loss.

Mechanistically, our prior studies using genetic knockout and knockdown models, in conjunction with pharmacological interventions, demonstrate that the otoprotective effects of Afatinib and related EGFR/BRAF/MEK/ERK inhibitors are mediated through on-target modulation of signaling pathways rather than off-target effects (**Fig. 4**)^{11,14}. The high efficacy of these agents at doses substantially lower than those used in oncology suggests that EGFR/MAPK signaling in the cochlea—comprising largely post-mitotic wildtype cells—may be particularly sensitive to inhibition under conditions of stress. This may reflect enhanced pathway activation, altered accessibility, or distinct downstream signaling dynamics in injured cochlear tissue. Alternatively, partial pathway inhibition may be sufficient for otoprotection, whereas more complete inhibition is required in proliferative cancer cells. In addition, immune responses, which are known to be

activated in the days following acoustic or ototoxic injury, may contribute to the protective effects observed and warrant further investigation^{16,17}.

Overall, these findings support the advancement of Afatinib into Phase I/II clinical trials designed around post-exposure, low-dose administration in individuals at risk of NIHL. In contrast to most otoprotective candidates that require pre-treatment at full therapeutic doses, Afatinib offers a clinically practical, mechanistically grounded, and readily translatable strategy for early intervention following acoustic injury.

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FIGURE LEGENDS

Figure 1. Observed plasma AUC of single oral Afatinib dosing in mice, rats and humans.

Average plasma AUC_{0-24h} values of Afatinib were obtained from 4 clinical studies (blue circles for humans), the FDA approved NDA file (green squares for rats), and two independent mouse PK studies (red triangles for mice). Both rat and mouse plasma AUC values were scaled to human oral doses using the reference of human body weight of 60 kg. A linear regression analysis was performed on the mean AUC versus the administered doses. The solid lines represent the linear regression fit, with a coefficient of determination (R^2) > 0.9. The dotted lines represent the FDA-approved human daily dose of 40 mg and its corresponding plasma AUC_{0-24h} of 450 h·ng/mL; based on this, the mouse equivalent dose of the human daily oral dose of 40 mg was extrapolated to be 6 mg/kg.

Figure 2. Oral administration of Afatinib starting at different time points protects against NIHL.

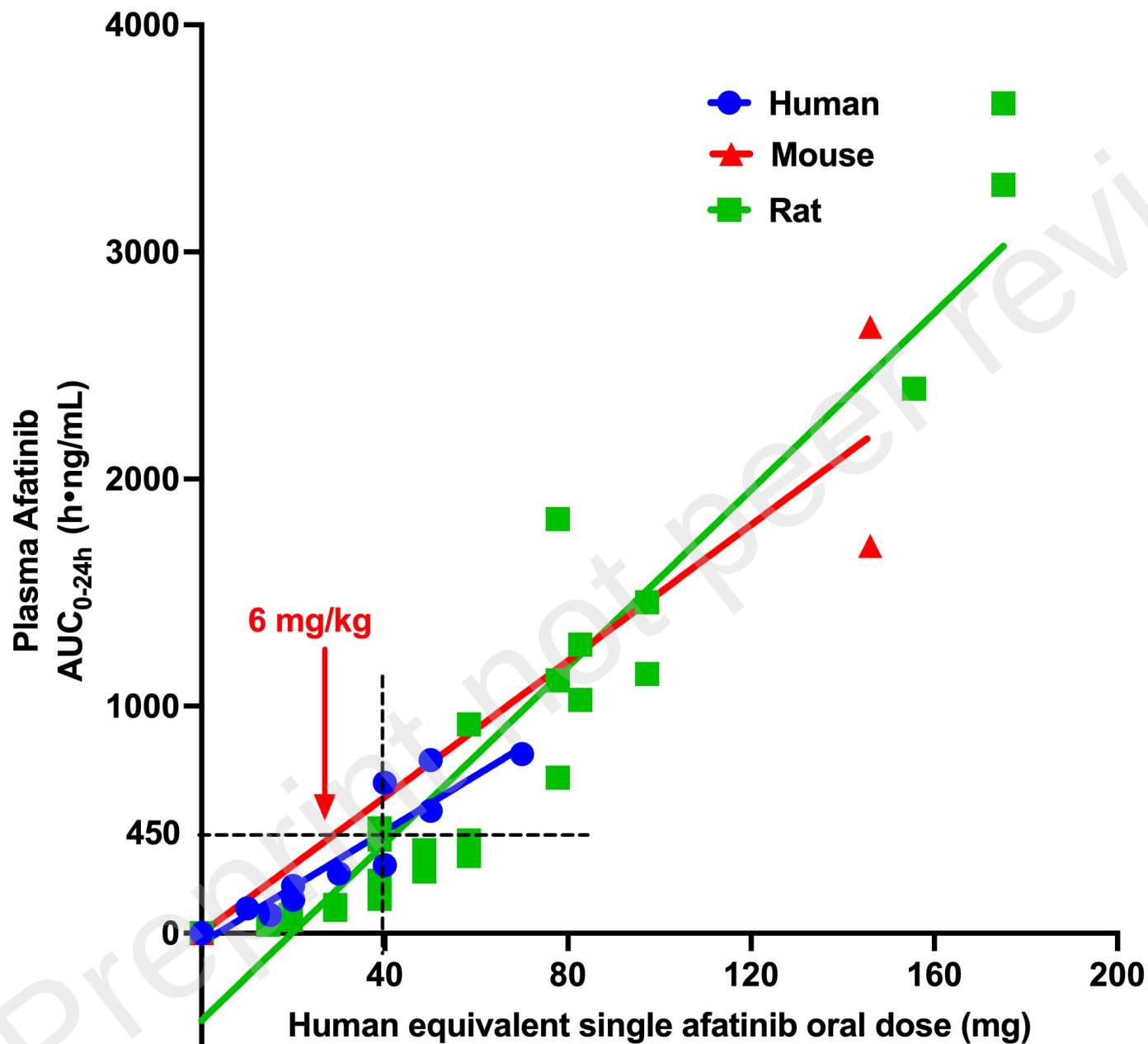
A) Experimental design of the NIHL in CBA mice and 3 different Afatinib administration schedules, each labeled with a different color. **B, C)** Hearing thresholds were assessed by ABR before (**B**, baseline) and 14-days after the treatment. Hearing loss is reported as threshold shifts (**C**, the difference between baseline and end point ABR thresholds). N: number of animals in each group. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparisons test. *** represents $p < 0.001$, compared to the NIHL group. Means $\pm$ SEM are plotted in all panels.

Figure 3. Lower doses and post-exposure oral Afatinib protect against NIHL.

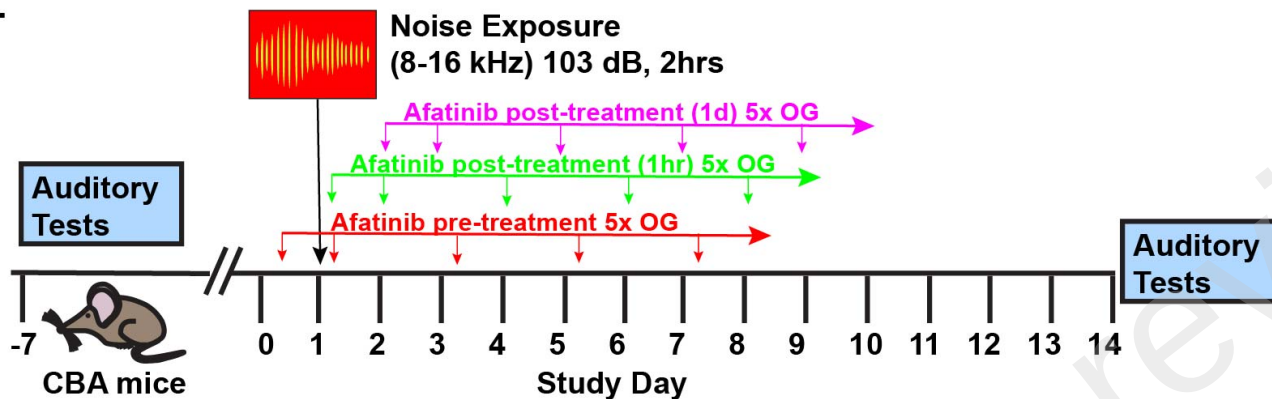
A) Experimental design of the NIHL and various doses of Afatinib administration in CBA mice. **B, C)** Hearing thresholds were assessed by ABR before (**B**, baseline) and after the treatment. Hearing loss is reported as threshold shifts between post-treatment end point and the baseline ABR thresholds (**C**). Statistical analysis was performed using two-way ANOVA with Dunnett's multiple

comparisons test. Different colors depict different animal groups. N: number of animals in each group. ns, not significant. **, ***, and **** represent $p < 0.01$, 0.001 , and 0.0001 , respectively, compared to the NIHL group. Means $\pm$ SEM are plotted in all panels.

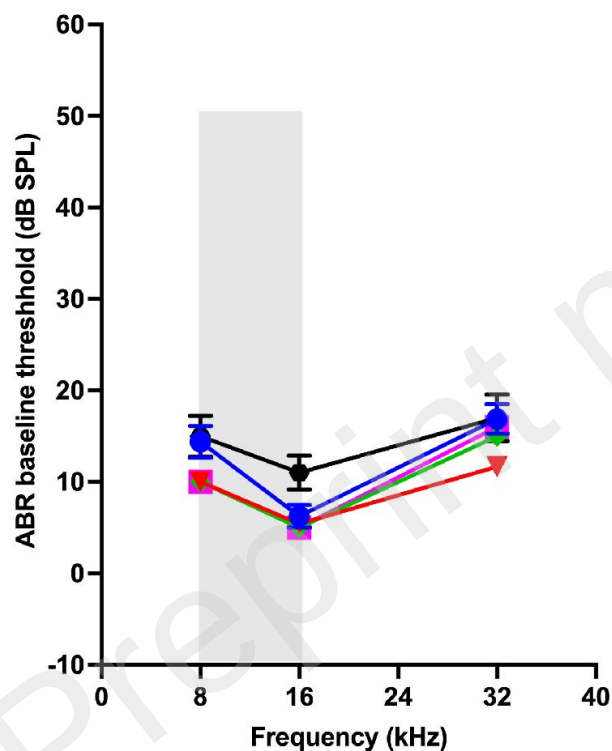
Figure 4. Inhibition of the EGFR/BRAF/MEK/ERK signaling cascade protects against noise- and cisplatin-induced hearing loss in mice. In the present study, we demonstrate that disruption of the EGFR signaling by Afatinib significantly reduces permanent hearing loss caused by noise exposure. Together with previous findings, blocking EGFR and its downstream kinase targets such as BRAF, MEK and ERK also prevents NIHL or cisplatin-induced hearing loss. The convergence of data validates that the specific EGFR signaling pathway is a key player involved in NIHL and cisplatin-induced hearing loss.



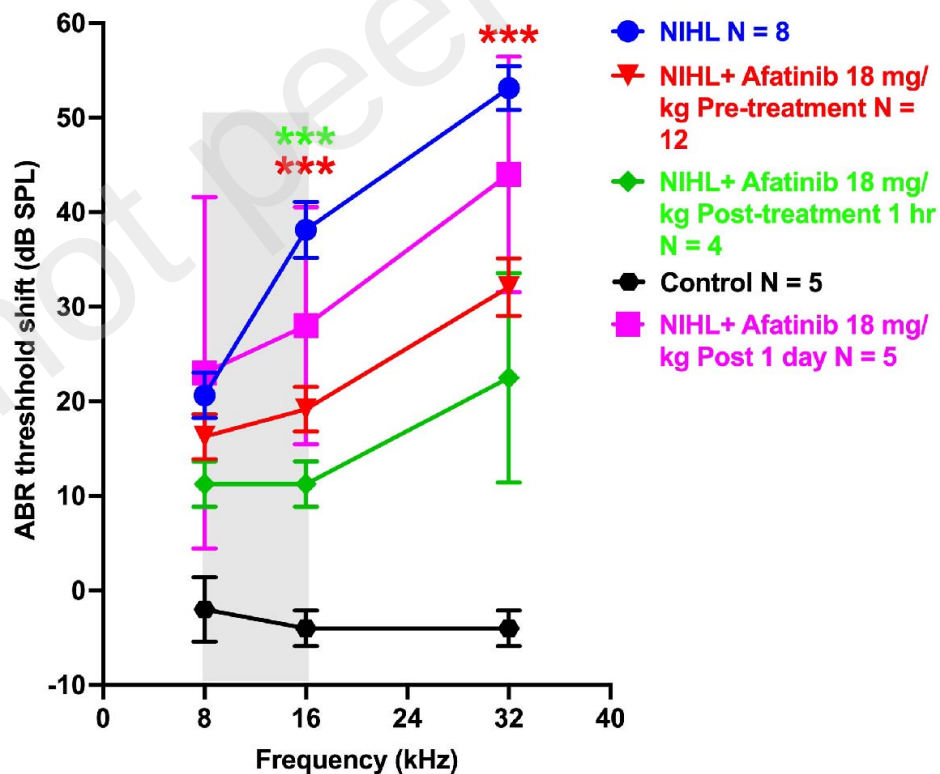
A.



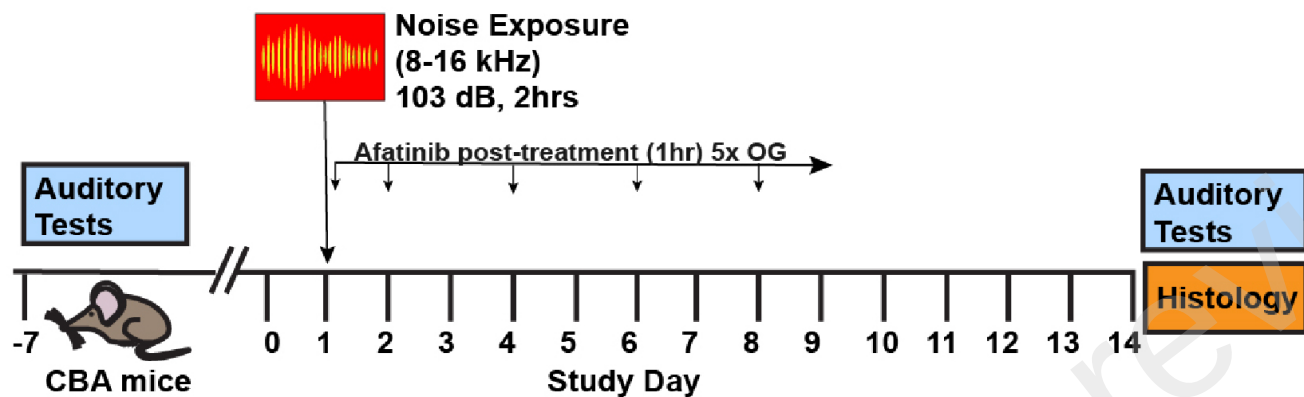
B.



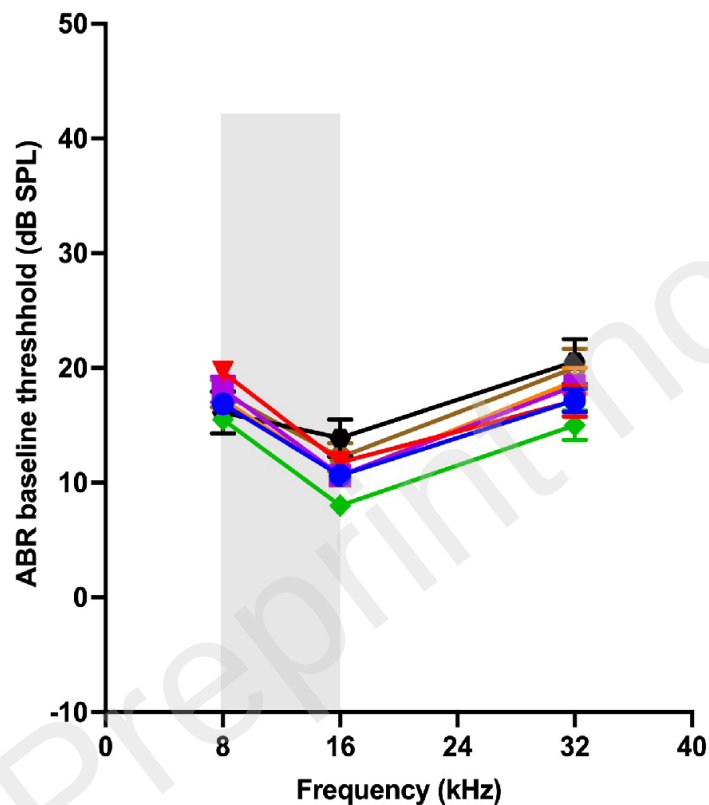
C.



A.



B.



C.

