Supplementary Information

A single dose of a novel pyridoindole restores hearing in a guinea pig model of noise-induced hearing loss to almost pre-noise levels.

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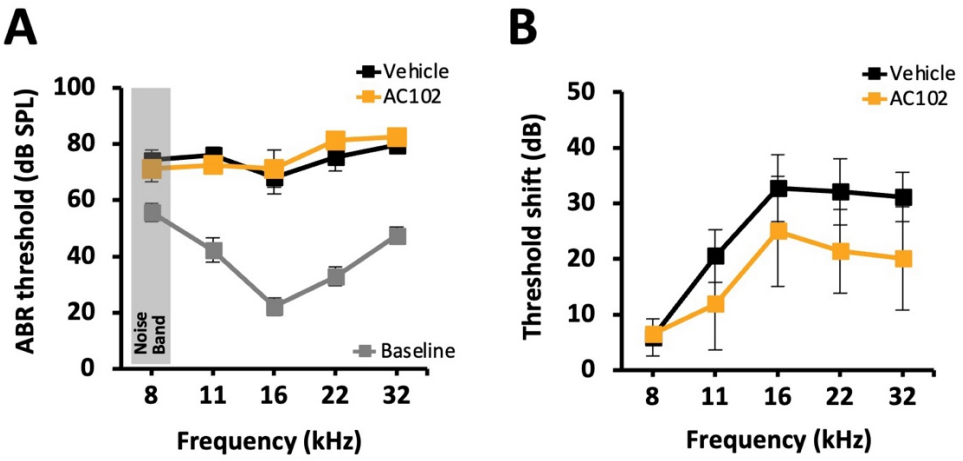
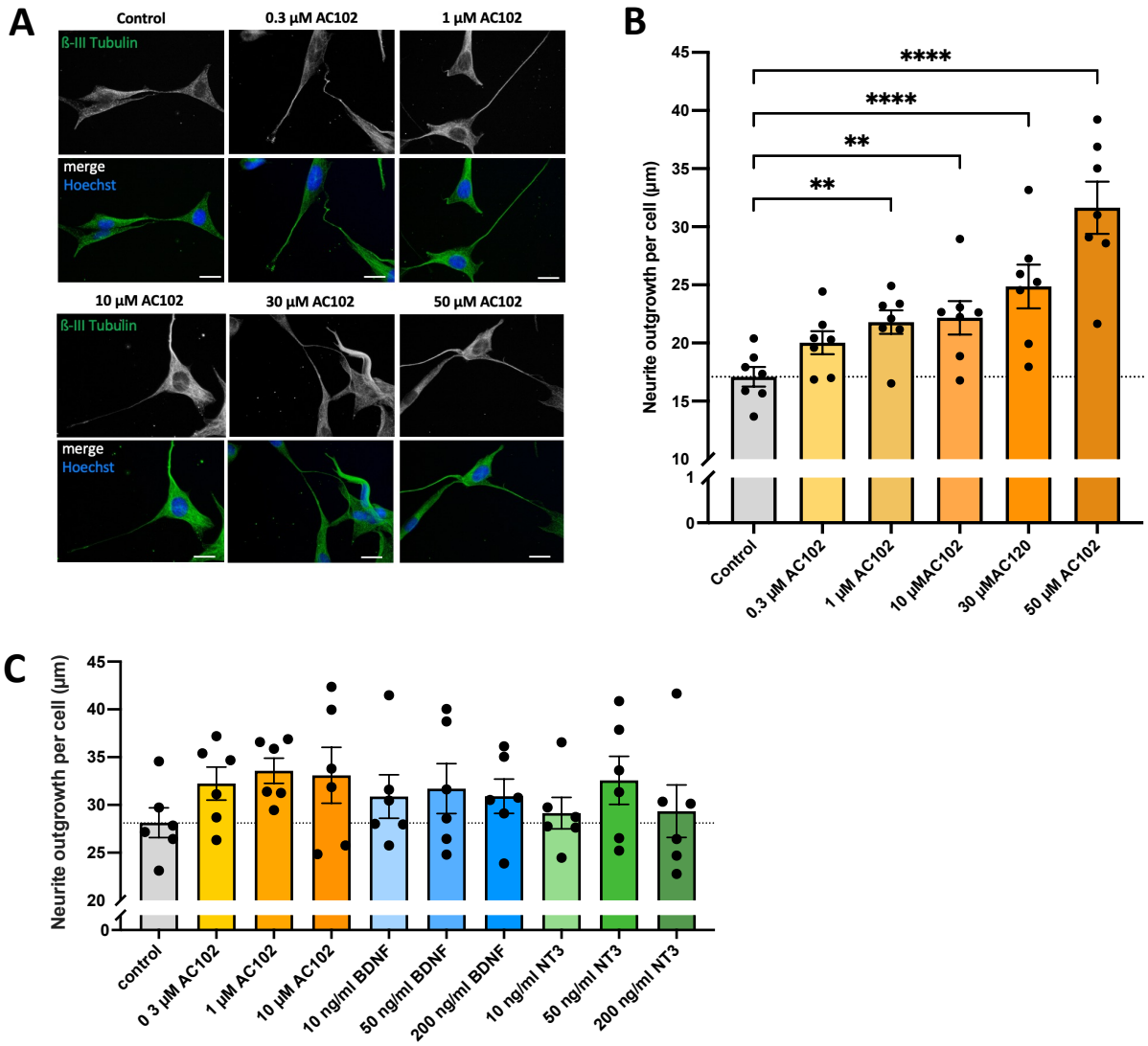
Suppl. Fig. 1.



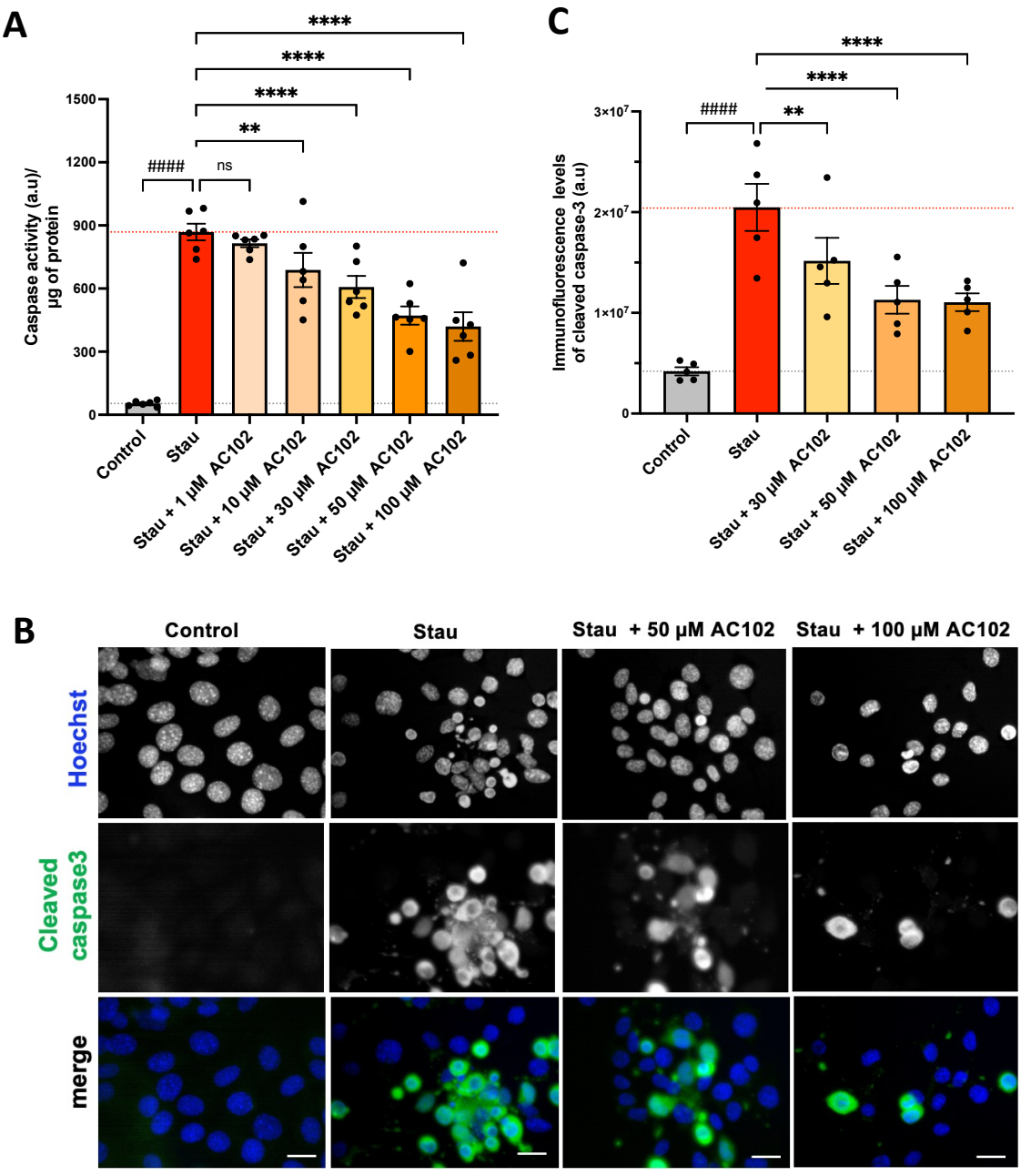
Suppl. Fig. 1. ABR data after double injection (72h + 6 days) of AC102. (**A**) Audiogram illustrates the hearing loss induced by noise exposure (AC102: orange, vehicle: black) compared to the averaged pre-noise hearing thresholds of both groups (baseline, gray). Noise exposure led to a mean hearing loss of 75 ± 4 dB SPL over the measured frequencies. (**B**) PTS 14 days after noise exposure show a higher hearing recovery in the AC102-treated group (mean PTS 17 ± 7 dB) compared to vehicle controls (mean PTS 24 ± 5 dB), even if treatment starts with a delay of 72h after noise exposure. N=7 (vehicle), n=4 (AC102).

Suppl. Fig. 2



Suppl. Fig. 2. Neuroregenerative properties of AC102. (**A**) Representative fluorescence images of HT22 cells immunostained with ß-III Tubulin. Nucleus are shown by Hoechst staining. Scale bar = 50 µm. (**B**) Quantification of the effects of varying concentrations of AC102 on neurite outgrowth in HT22 cells determined by ß-III Tubulin. Shown are mean ± SEM (n=7). (**C**) Effects of varying concentrations of AC102 and selected neurotrophins on neurite outgrowth in HT22 cells. Shown are mean ± SEM (n=6). Repeated measures one-way ANOVA and Fisher's LSD test for comparison of AC102-treated groups against control (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Suppl. Fig. 3



Suppl. Fig. 3. Anti-apoptotic properties of AC102. (**A**) Quantification of caspase 3/7 activity in presence of apoptosis inducer staurosporine alone or co-treatment with varying concentrations of AC102 (n=6). (**B**) Immunofluorescence levels of cleaved caspase-3 in presence of apoptosis inducer staurosporine alone or co-treatment with varying concentrations of AC102 (n=5). (**C**) Representative images showing increased levels of cleaved caspase. One-sided paired t-test ($^{\#\#\#\#}p \leq 0.0001$). Repeated measures one-way ANOVA and Fisher's LSD test for comparison of AC102-treated groups against control ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). Scale bar = 50 µm.

SI Materials and Methods

Anesthesia, analgesia and euthanasia of animals

Application of AC102 formulation as well as ABR recordings were performed under general anesthesia, using a combination of fentanyl (0.025 mg/kg), medetomidine (0.2 mg/kg) and midazolam (1 mg/ kg). The anesthesia was partially antagonized by atipamezole (1 mg/kg), flumazenil (0,1 mg/kg) and naloxone (0,03 mg/kg). Local anesthesia was accomplished by 2% lidocaine subcutaneous injection. For analgesia, 0.2 mg/kg meloxicam was applied. After the final hearing measurement and under deep general anesthesia, animals were euthanized by decapitation before temporal bones were harvested.

Animal model of NIHL

All animal experiments were approved by the Animal Use and Care Committee of the Senate of Berlin (reference no. G0199/15, A0187/17) and were conducted in accordance with the regulations based on the EU-directive 2010/63/EU. Fully anesthetized male Dunkin Hartley albino guinea pigs (GP, Envigo, 450-1400g) were placed inside a sound-proof chamber and exposed to a single continuous noise band of 1 octave centered at 4 kHz (125 dB SPL, 1h) or at 8 kHz (120 dB SPL, 30 min). The noise band was amplified (Yamaha A-S201) and presented by a loudspeaker (BMS 4538-8) 25 cm above the animal's left ear, while the contralateral ear was closed with a plug. In total 57 animals were used for the *in vivo* experiments (experiment 1: n=6 (vehicle), n=7 (AC102); experiment 2: n=8 (vehicle), n=7 (AC102); experiment 3: n=11 (vehicle), n=9 (AC102). Furthermore, three naïve animals served as untreated controls for each experiment.

Drug administration *in vivo*

AC102 was applied intratympanically as a sterile suspension in a poloxamer-based hydrogel (e.g. 1,2) at defined time points after the noise trauma (see **Fig. 1A** for an overview of experimental details). After a retroauricular skin incision, the bulla of the left ear was opened to visualize the round window (RW) niche. A volume of 10 µL (0.12 mg) of AC102 suspension or vehicle, respectively, were applied into the RW niche using a Hamilton syringe (Model 1705 RN, Hamilton, Bonaduz, Switzerland) assembled on a motorized micropump (UMP3, World Precision Instruments, Friedberg, Germany). The bulla was closed with dental cement (Durelon® Carboxylate Luting Cement, 3M, Saint Paul, MO, USA) and the skin incision was sutured. For double injections a second dose of 5 µL (0.06 mg AC102) was applied three days after the first dose (1h or 72h after noise overexposure) by the same administration route. Results from a previously performed *in vivo* dose-response study served as basis for the selected concentration of 0.12 mg AC102.

Hearing assessment

Auditory function was measured by recording auditory brainstem responses (ABRs). The TDT System 3 (Tucker-Davis-Technologies, Alachua, FL, USA) was used for presentation of stimuli and recording of ABRs. Hearing thresholds were measured three days before, on the day and 14 days after noise overexposure. Anesthetized animals were presented sinus tones with frequencies between 2 - 32 kHz for a duration of 10 ms in 5 dB steps from 0-90 dB SPL in a free field configuration. Recorded signals were pre-amplified (RA4PA 4 channel preamplifier), band pass filtered above 300 Hz and below 3 kHz and obtained as time locked averages of 128 measurements. ABR analysis was performed under fully blinded conditions using the TDT BioSigRZ software.

Immunohistochemistry

Cochleae from treated GPs were dissected out of the temporal bone at the end of each in-life phase (D14) and directly placed in a cold (4°C) 4% paraformaldehyde (PFA) solution for up to 2h. Following fixation, PFA was replaced with Phosphate Buffered Saline (PBS), and cochleae stored at 4°C for 24h. Next, samples were washed once with PBST (PBS/1% Triton X-100), the bony cochlear capsule removed, and the inner ear tissue carefully collected. The tissues were washed twice in PBST for 10 min on an orbital shaker, then placed into a 30% sucrose solution in PBST and transferred in a -80°C freezer for 30 min for permeabilization. Two washing steps with PBST for 15 min followed before tissues were placed into 5% Normal Horse Serum in PBST (blocking solution) for 2h at room temperature. After blocking, cochlear tissues were exposed to primary antibodies for 24h at 37°C. The following primary antibodies were used: mouse (IgG1) anti-CtBP2 at 1:200 (anti-CtBP2, #612044, BD, Franklin Lakes, NJ, USA) and mouse (IgG2a) anti-GluA2 at 1:2000 (anti-GluA2, #MAB397, Millipore, Burlington, MA, USA). Samples were then washed three times with PBST for 15 min and stained with secondary antibodies and DAPI at 1:200 (4',6-Diamidin-2-phenylindol, #D1306, Invitrogen, Waltham MA, USA) in blocking buffer for 2h at room temperature. Secondary antibodies used were goat anti-mouse (IgG1)-AF568 at 1:500 (#A21124, Invitrogen, Waltham MA, USA), goat anti-mouse (IgG2a)-AF488 at 1:500 (#A21131, Invitrogen, Waltham MA, USA) and phalloidin 405 at 1:200 (phalloidin 405, #A30104, Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, tissues were washed twice with PBST for 10 min, the sensory epithelium dissected out from the cochlear tissue, cut into sections and flat mounted in a tonotopic manner on microscope slides using VECTASHIELD® Hard Set (Cat. #H-1400, Vector laboratories, Newark, CA, USA) as a mounting agent.

Image acquisition and histological assessment

Leica SP5 confocal microscope (Wetzlar, Germany) was used for the imaging of the stained cochlear sections. Images were acquired using a 40X oil immersion lens and a 1.5X digital zoom, with the resolution of 1024x1024 pixels, and a bidirectional scanning mode with scanning speed of 600 kHz. Lower magnification images were acquired using 20X oil immersion lens for quantification of OHCs. For the histological analysis of OHCs and ribbon synapses, all images were processed using ImageJ (version 1.53t, NIH, USA). To quantify cell survival, OHCs were counted manually. OHC loss was considered to have occurred if both the stereocilia and the cuticular plate could not be identified. To obtain the average number of OHCs per µm, the total number of OHCs in all three rows was divided by the corresponding length of the sensory epithelium that was measured in each picture. The scoring of ribbon synapses was also performed manually. Each punctum with co-localized CtBP2 and GluA2 signal was scored as intact ribbons synapse (3,4). Synapses with CtBP2 but without GluA2 staining were defined as orphan, disconnected synapses. IHCs were counted using DAPI staining. The average number of intact or orphan synapses per IHC was obtained by dividing the number of synapses scored by the total number of IHC nuclei in each picture. A minimum of four z-stacks were collected per cochlea and synapses from at least ten IHCs per z-stacks quantified.

Analysis of cell damage and neurite connections

Mouse hippocampal neuron-derived immortalized neuronal cells (HT-22; SCC129, Sigma-Aldrich, St. Louis, MO, USA) were seeded and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, D6429, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). Cells were treated 24h after seeding with 10µM rotenone (R8875-1G, Merck, Darmstadt, Germany) alone or co-treated with 0.1, 0.3, 1, 10, 30, or 100 µM AC102 for 20min in serum-free medium. After the incubation imaging was performed using an Axiovert Inverted Microscope (Zeiss, Oberkochen, Germany) equipped with a 20X

objective lens and Cam SC500 5.1 MP (SWIFT, La Hulpe, Belgium) mounted on the eyepiece. Images were taken using SWIFT Imaging 3.0 software for Windows (SWIFT, La Hulpe, Belgium). Damaged cells (identified by round morphology) and healthy cells (rather oval shaped or looking like typical neuronal cells, often with neurites) were quantified using imageJ (version 1.53t, NIH, USA). For the neurite connection analysis time-lapse videos were acquired for 30min and videos were analyzed by ImageJ software. Number of neurite connections (neurites touching a cell or another neurite) per cell were quantified at time '0' and '30' min, and connections lost between the time points were reported as % of original connections.

Neurite outgrowth assay

HT22 cells were treated with medium containing appropriate concentration of either AC102, or one of the neurotrophins BDNF (Biochrom AG, Berlin, Germany), or NT-3 (Peprotech, Cranbury, NJ, USA) 24h after seeding. For the rotenone damage model cells were treated with 10 µM rotenone for 2h in serum-free DMEM medium. Afterwards cells were washed with PBS and the media with rotenone was exchanged with fresh DMEM media containing vehicle or varying concentrations of AC102. After 24h treatment, image acquisition was performed as described above. Neurites and cells were quantified using ImageJ (version 1.53t, NIH, USA) and represented as total neurite length per cell.

ROS Assay

HT22 cells they were first treated with 5µM Carboxy-H2-Dichlorodihydrofluorescein diacetate (DCFDA) reagent (C400, Thermo Fisher Scientific, Waltham, MA, USA) in HBSS medium (14175-095, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 30min. After washing with HBSS, cells were treated with 100 µM of ROS inducer antimycin A (J63522, Sigma-Aldrich, St. Louis, MO, USA) alone or with different concentrations of AC102 (1 – 100µM) or DMTU (D188700, Sigma-Aldrich, St. Louis, MO, USA) or Tiron (A0447328, Thermo Fisher Scientific, Waltham, MA, USA) in DMEM without phenol red and FCS for 20min. After the incubation, DCFDA fluorescence (Ex: 485nm; Em: 535nm) was measured at a ClarioStar cell analyzer (BMG Labtech, Ortenberg, Germany) and blanks from cell-free wells were subtracted. For cell-free ROS assay 1 µM H_2O_2 was incubated with AC102 or with DMTU for 2h and levels of H_2O_2 was determined by luminescence-based assay (ROS-Glo, G8820, Promega, Madison, Wisconsin, USA).

All the data are normalized against the protein levels determined by Bicinchoninic Acid (BCA) assay (23223, Pierce, Thermo Fisher Scientific, Waltham, MA, USA) under the same experimental condition.

Total ATP Assay

HT22 cells were treated with varying concentrations of AC102 for 4h. Total ATP production was measured with the Luminescence ATP Detection Assay kit (Cayman Chemical, 700410) according to the manufacturer's instructions. In brief, luciferase was provided as substrate, which ATP converts to oxyluciferin and luminescence that can be measured at a plate reader. The use of a standard curve with reagents provided by the manufacturer allows the calculation of exact amounts of ATP present in the samples. All the data are normalized against the protein levels determined by BCA assay under the same experimental condition.

Caspase activity

For caspase 3/7 activity assay HT22 cells were incubated with 1 µM staurosporine alone or co-treated with varying concentrations of AC102 for 5h. After the incubation the activity of cleaved caspase 3/7 were determined by luminescence-based assay (Caspase Glo, G8091, Promega, Madison, Wisconsin, USA) and normalized by protein concentration. For determining the immunofluorescence levels of cleaved caspase-3 cells were plated on coverslips and treated with

staurosporine alone or in combination with AC102 as mentioned above. Following the treatment cells were fixed with 4% PFA, permeabilized with 0.2% Tween-20 in PBS and blocked with 0.2% Tween-20 in PBS containing 5% normal goat serum. For immunostaining cleaved caspase antibody (CST9661, cell signaling technology, Danvers, MA, USA) was used at 1:200 dilution. Nuclei were stained with Hoechst dye. The images were acquired using Zeiss fluorescence microscope equipped with 40X objective lens. The integrate densities were quantified using image J. For each biological replicate 10-15 images were quantified per individual group and the representative graphs are derived from 7 biological replicates.

Statistics

Data for all experiments were analyzed with PRISM (Version 9.5, GraphPad Software, San Diego, CA, USA). For *in vivo* experiments, treated and vehicle groups were compared with one-sided unpaired t-tests (Fig. 1-2). For experiments with 3 groups, these were compared with one-way ANOVA and Tukey's posttest (Fig. 3). For *in vitro* experiments, repeated one-way ANOVA was used for all graphs unless indicated otherwise. Dunnett's multiple comparisons tests was used for comparison of independent groups, and uncorrected Fisher's LSD was displayed for comparison of varying concentrations versus vehicle. For comparison between two groups with expected outcome (downregulation for damage model, or upregulation for positive control) one-sided paired t-tests were applied.

References

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