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Evaluation of the Selective Glucocorticoid Receptor Agonist Compound A for Ototoxic Effects

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Abstract

Objective—To evaluate the selective glucocorticoid receptor agonist (SEGRA) compound A, a potential novel therapeutic for inner ear disorders, for ototoxic effects.

Study Design—Laboratory animal study

Methods—Experimental guinea pigs were grouped as follows: 1 & 2) systemic application of compound A (1.5 mg/kg and 4.5 mg/kg; n=6/group); 3 & 4) intratympanic application of compound A (1 mM and 10 mM; n=6/group). Contralateral ears in topically treated animals served as controls. Hearing thresholds were determined by ABR before and directly after the application of compound A, as well as on days three, seven, 14, 21 and 28. At the end of the experiments temporal bones were harvested for histological evaluation.

Results—Systemic administration of compound A (1.5 mg/kg & 4.5 mg/kg) did not cause hearing threshold shifts, whereas the intratympanic injection (1 mM & 10 mM) resulted in a hearing loss. Histological analysis of the middle and inner ears after topical compound A application showed alterations in the tympanic membranes, the auditory ossicles and the round window membranes, whilst spiral ganglion cells and hair cells were not affected.

Conclusion—SEGRAs like compound A could provide novel therapeutic options with reduced metabolic side-effects for the treatment of inner ear disorders. Whereas intratympanic application

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Level of Evidence: N/A

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of compound A resulted in hearing loss, the systemic application of compound A merits evaluation for otoprotective effects in trauma models.

Keywords

selective glucocorticoid agonists; SEGRAs; compound A; ototoxicity

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Introduction

Treatment of diseases affecting the inner ear like idiopathic sudden sensorineural hearing loss or Menière's disease is still largely dependent on glucocorticoids (GCs), which cause multiple side effects including diabetes and osteoporosis.1–3 Although these undesired ramifications tend to be less deleterious in short-term GC treatment, even single-shot applications carry a certain risk for adverse effects - severe hyperglycemia in diabetic patients being one of them. Recent attempts to increase the therapeutic index of anti-inflammatory drugs resulted in the development of selective glucocorticoid receptor agonists (SEGRAs). These compounds bind to the GC-receptor (GR) and selectively activate the transrepression of pro-inflammatory transcription factors like NF-κB, without causing the binding of GR-dimers to glucocorticoid responsive elements, which is the molecular mechanisms underlying most side-effects.4,5 The quest for such substances started after the finding that dexamethasone keeps its anti-inflammatory properties in a GR dimerization-defective mouse model, while causing fewer side effects.6,7 Since then, various SEGRAs, which bind to the GR receptor without causing receptor dimerization, have been evaluated for their anti-inflammatory potential *in-vitro* as well as *in-vivo*.8–14 In contrast to most of the other SEGRAs, which were developed by pharmaceutical companies, compound A (CpdA) is a chemical analog of a hydroxyphenyl aziridine which was discovered in the Namibian shrub *Salsola tuberculatiformis Botschantzev* when the cause of prolonged gestation in sheep was investigated.15 So far, the anti-inflammatory effects of CpdA have been demonstrated in zymosan-induced paw inflammation and collagen-induced rheumatoid arthritis as well as in different models for neuroinflammatory diseases.12,16–18 Up to date, SEGRAs have not been evaluated as potential therapeutic agents for conditions affecting the inner ear. In this first study to introduce a SEGRA into the field of inner ear research, we aimed to exclude potential ototoxic effects by evaluating hearing thresholds and histological alterations, after systemic as well as intratympanic (IT) administration of CpdA.

Materials and Methods

All animal experiments were approved by the local animal welfare committee and the Austrian Federal Ministry for Science and Research (BMWF-66.009/0293-II/3b/2012). In total, 24 pigmented guinea pigs (weight: 424 - 666 grams) were used. Each experimental group consisted of 6 animals (3 males and 3 females). In the first phase of the experiment, one group received a single shot of a 10 mM solution of CpdA IT. For this purpose, the bulla was completely filled with the Cpd A solution (approx. 200-250µl). The second set of guinea pigs received 1.5 mg/kg CpdA intraperitoneally every 24 hours on three consecutive days. After evaluation of the outcomes, doses were reduced to 1 mM CpdA for the IT injection

and raised to 4.5 mg/kg for the systemic application of CpdA in the second phase of the experiment.

Compound A Preparation

Compound A, or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, (Enzo Life Sciences AG, Lausen, Switzerland), was stored at −80° C. Because of the reported instability of CpdA in buffered solutions18, aliquots were prepared in water for injection, stored at −80° C and only thawed once directly prior to use.

Anesthesia

IT application of CpdA as well as auditory brainstem response (ABR) recordings were performed under general anesthesia, using medetomidine (0.3 mg/kg), midazolam (1 mg/kg), fentanyl (0.03 mg/kg) and ketamine (10 mg/kg). To aid recovery, anesthesia was partially antagonized by atipamezole (1 mg/kg). Heart rate and vascular pO_2 were measured using a pulse oximeter during IT injections. Body temperature was maintained at 38°C with a heating plate whenever the animals were anesthetized.

Application Procedures

For systemic application, CpdA was injected intraperitoneally on three consecutive days. To standardize the trauma during the IT application of CpdA, the injection was performed under a surgical microscope (Carl Zeiss, Jena, Germany) using a YOU-1 micromanipulator (Narishige, Tokyo, Japan), and Hamilton syringes (Hamilton, Bonaduz, Switzerland) with blunt 29G needles. The eardrum was incised in the upper posterior as well as upper anterior quadrant to allow for air to escape and the middle ear to fill completely. In each animal one tympanic bulla was filled with CpdA at the respective concentration, whereas control fluid (water for injection) was applied in the same way to the opposite ear. The side receiving the CpdA application, as well as the order of the applications, was randomized to exclude effects of possible fluid loss during the injection procedure on the contralateral side.

Auditory brainstem responses

ABRs were recorded in a soundproof chamber (mac-2, Industrial Acoustics Company, Winchester, UK). A DT-48 speaker (Beyerdynamic, Heilbronn, Germany) was placed 3 cm from the tested ear to generate the sound field, and a K2 microphone (Sennheiser, Wedemark-Wennebostel, Germany) was positioned at the level of the pinna for calibration. For acoustic isolation of the tested ear, the contralateral ear was plugged with Ohropax classic (Ohropax, Werheim, Germany). A custom-made setup, including a PC system equipped with a multifunction I/O card (National Instruments, Austin, TX, USA) and AudiologyLab software (Otoconsult, Frankfurt am Main, Germany), was used for the measurement of auditory potentials. The stimuli used in this study included clicks and tone bursts (3 ms duration; 1 ms rise/fall) presented in the frequency range of 1-32 kHz (one step per octave). For the determination of click thresholds, the sound pressure was changed in 2 dB steps, while 5 dB steps were used for the determination of frequency-specific thresholds. Signals were amplified (80 dB), band-pass filtered between 10 Hz and 10 kHz and averaged (512x for clicks, 256x for tone bursts). Baseline ABR thresholds were determined one week

before the IT application of CpdA. Further measurements were performed directly after the IT injection of CpdA (day 0) as well as on days three, seven, 14, 21 and 28. In the systemically treated animals, preoperative hearing was determined directly before the first injection (day -2), 6 hours after the last application (day 0) and on days three, seven, 14, 21 and 28.

Histology

After the last audiometry, the animals were perfused intracardially with 4% buffered paraformaldehyde (PFA, Sigma Aldrich, Seelze, Germany) and randomly assigned to the classic histological evaluation or to the preparation of organ of Corti whole-mounts. After isolation of the temporal bones and removal of soft tissue, the bullae were opened carefully to allow entry of the fixative and the samples were postfixed in 4 % buffered paraformaldehyde for at least 2 days.

For histological examination, the temporal bones were decalcified in EDTA (8%), trimmed and precisely positioned parallel to the modiolus of the cochlea prior to embedding in paraffin, which was chosen to allow for serial sectioning and to maintain options for immunohistochemical stainings. The preparation was cut at a thickness of 4 μm and every fifth section was stained with hematoxylin-eosin (H&E). Thirty sections were examined per specimen and representative sections were photographed under a light microscope (Polyvar, Reichert-Jung, Vienna, Austria). The following regions of interest were evaluated for alterations: Tympanic membrane, tympanic bulla, cochlea, spiral ganglia, round window membrane (RWM) and ossicles. Deviances of the physiological state were considered pathological and were evaluated according to the following criteria: increase in thickness, composition of tissue and appearance of cells, modified attachments as well as infiltration.

To prepare organ of Corti whole-mounts, the bony capsule was carefully removed from the fixed cochlea. The samples were stained with Phalloidin-Tetramethylrhodamine B isothiocyanate (0.3 μg/ml PBS; Phalloidin-TRITC; Sigma-Aldrich, Vienna, Austria) and Hoechst 33342 trihydrochloride trihydrate (0.05 mg/ml PBS; Molecular Probes®, Invitrogen Corp., Carlsbad, CA, USA) for 30 minutes at room temperature. Thereafter each turn of the cochlea was separately embedded in Fluorsave™ Reagent (Calbiochem, Darmstadt, Germany) and analyzed by means of confocal microscopy. As previously published, hair cells were counted in 3 randomly selected 200 μm sections of each turn.19 The numbers of viable hair cells were expressed as a percentage of their expected total numbers, which were determined for every 200 μm section by counting the viable hair cells plus the missing hair cells as if they were still in existence. All histopathological examinations were performed blinded to the assigned treatment.

Statistics

Data are presented as mean values. Error bars represent the SD. Data were analyzed using IBM SPSS (Version 21). One-way ANOVA (Tukey's HSD for post-hoc analysis) was used for the statistical evaluation of the datasets. Results were considered statistically significant when $p<0.05$.

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Results

Intratympanic compound A causes hearing threshold shifts

The IT injection of CpdA resulted in a statistically significant hearing loss (Fig. 1A). Click threshold shifts were 28 and 21 dB higher in the groups treated with 1 mM and 10 mM CpdA as compared to the control group. This effect did not resolve until day 28, when differences in threshold shifts of 19 and 12dB for 1 mM and 10 mM of CpdA respectively, were still persistent ($p<0.05$ for both groups at all points in time). The IT application of water for injection resulted in a temporary click threshold shift, which resolved by day 7. Similar results were obtained in the frequency specific measurements. The IT application of both concentrations of CpdA resulted in pure tone threshold shifts, which partially recovered until day 28 and were more prominent in the higher frequencies (Fig. 2). At 1 kHz thresholds differed significantly between both treatment groups and the control group on days 7, 21 and 28. Mean thresholds on day 28 were 33.3 dB, 56.7 dB and 44.2 dB SPL for the control-group, the 1 mM CpdA IT-group and the 10 mM CpdA IT-group, respectively. At 2 kHz the mean hearing-thresholds measured on day 28 were 33.8 dB, 55 dB and 40 dB for the controls, the 1 mM IT and the 10 mM IT CpdA group. At 4 and 8 kHz day 28 thresholds were 38.3 dB, 57.5 dB and 49.2 dB SPL, and 22.9 dB, 40.8 dB and 35 dB SPL, for the control group, the 1 mM group and the 10 mM group, respectively. At these 2 frequencies the measured threshold shifts reached statistical significance only in the 1 mM Cpd A group. At 16 and 32 kHz, hearing threshold shifts of both treatment groups differed statistically significantly from the control group at all measured time points. 16 kHz-thresholds on day 28 were 10.8 dB, 32.5 dB and 35 dB SPL, and 32 kHz-thresholds were 16.25 dB, 50 dB and 66.7 dB SPL, for the control- , the 1 mM CpdA- and the 10 mM CpdA-group, respectively (Fig. 2).

No ototoxicity after systemic compound A application

The systemic application of 1.5 and 4.5 mg/kg of CpdA did not result in clinically relevant or statistically significant changes in click hearing thresholds as compared to baseline values (Fig 1b). Except for the day 21 result of the 1.5 mg/kg CpdA group (threshold shift: 5dB, $p=0.048$), no statistically significant threshold shifts were found in the frequency-specific ABRs following the intraperitoneal application of CpdA as compared to the baseline measurements (Fig. 3).

No hair cell loss after compound A treatment

Treatment with CpdA did not result in a statistically significant loss of outer or inner hair cells in any of the treatment groups. In the samples evaluated, the percentage of viable outer hair cells ranged from 99.5% to 99.9% in the basal turn and from 98% to 99.5% in the second turn of the cochlea. In the third turn between 97.4% and 99% of the outer hair cells were still existent. In the very apical region of the cochlea 93.8% to 97.7% of the expected outer hair cells were found (Table I). Between 99.6% and 100% of the inner hair cells were preserved in all treatment groups and portions of Corti´s organ (Table II).

Histological evaluation of the middle and inner ears after compound A treatment

The topical administration of CpdA caused multiple changes in the middle ears of the guinea pigs (Figs. 4 and 5; table III). The H&E stained histological sections showed a thickening of the tympanic membrane in 3/3 animals in both the 1 mM and the 10 mM IT groups (Fig. 4 B, C). The thickening was due to increase of epithelial cell layers and hyperplasia of connective and bony tissue from the region of the tympanic annulus. The ossicular chain was also affected by hyperplasia of connective tissue as well as infiltration of inflammatory cells and local bleeding (Fig. 4 E, F). Even freshly mineralized bone tissue could be examined between the malleus and the cochlea and connected these structures. The bulla wall was increased in its thickness by the means of new bone growth and an associated hyperplasia of mucosa which could be found in 3/3 animals in both treated groups (Fig. 4 H, I). Additionally, the RWM, especially the connective tissue layer was thickened in 3/3 animals in the 1 mM CpdA group and in 3/3 animals in the 10 mM CpdA group (Fig. 5 B, C). The attachment of the RWM was strengthened by new bone formation that partially involved the RWM itself. No substantial differences in appearance of spiral ganglion cells could be examined in all IT and systemically treated animals (Fig. 5 D, E, F and Table III). Neither the IT injection of water (Fig. 4A, D, G, Fig. 5A, D; Table III), nor the systemic administration of CpdA (Table III) did cause histological changes in the middle ear.

Discussion

This study is the first to evaluate the suitability of a SEGRA as a potential alternative to the classic glucocorticoids used to treat patients with inner ear diseases. The data presented herein demonstrate that the IT application of the SEGRA CpdA causes hearing threshold shifts and histological alterations in the middle ears of possible pathological nature, while no such effects were found after systemic administration. Indeed, it is still unclear if these negative effects on hearing thresholds after topical application of CpdA are compound-specific, or if other SEGRAs would also cause such a hearing loss. Taking into account the previously described instability of CpdA in buffered solutions, which results in the generation of the alkylating metabolite N-methyl-2-(4-acetoxyphenyl)-aziridine18, these toxicities are most likely CpdA specific in our eyes. As this group of drugs holds the potential to significantly reduce the side effects of GC therapy, further SEGRAs should therefore be evaluated in the setting of inner ear disease.

We chose CpdA as a model SEGRA because - in contrast to the SEGRAs developed by pharmaceutical companies - it is commercially available and has therefore been successfully tested by different groups and in various models for inflammatory diseases.12,18,20 This advantage of CpdA is flawed by its instability18 and the resulting side effects. Most frequently a reduced weight gain was described.21,22 Such a weight loss was also observed in our animals after systemic application. Other side effects reported in the literature include diarrhea and even the need to euthanize the animals after high-dose treatments.18,20 Histologic studies in CpdA treated animals showed normal liver histology, but also reduced anti-apoptotic effects in the intestine and abdominal necrosis after the application of high doses.18,23,24 In our study, the histologic analysis of the peritoneum of selected animals did not show any inflammatory or necrotic alterations after the i.p. application of CpdA.

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The different results after IT and systemic application of CpdA could possibly be explained by its toxic metabolites and the stabilization of CpdA by plasma protein binding.21 Given the known variability of inner ear drug levels after IT application of GCs25,26 and the ototoxic effects of IT CpdA, other SEGRAs with a broader dosing range appear to be more appealing candidates for a translation into clinical studies. Nevertheless, CpdA - applied systemically – merits investigation with respect to otoprotective effects in trauma models, as this compound could pave the way for the application of other SEGRAS in the treatment of inner ear disorders. Another interesting aspect is that using the dissociated effects on the GR, SEGRAs might constitute a valuable tool to further dissect the effects of GCs on the inner ear and could thereby extend the understanding of the molecular pathways involved.

The histological evaluation of the ears showed alterations in the tympanic membrane, the auditory ossicles and the RWM, all of which are able to cause conductive hearing loss. Since the outer and inner hair cells as well as the spiral ganglion cells appeared unaffected, we believe that the described hearing loss is at least partially conductive in nature. However, there seems to be some impact of CpdA on the inner ear. The pronounced hearing loss occurring immediately after the IT application of 1 mM and 10 mM of CpdA cannot be sufficiently explained by the described morphological changes, as they need more time to develop. The lack of differentiation between sensorineural and conductive hearing loss in the audiometric measurements may be regarded as a shortcoming of this study. Regardless of the exact nature of the threshold shifts, IT application of CpdA appears to be ototoxic and should therefore not be used in future studies.

Interestingly, the systemic application of CpdA did not result in such a hearing loss or morphological changes to the middle ears and could therefore still be used for proof of principle studies. We believe that SEGRAs should also be applied in *in-vitro* studies to distinguish between GR-dimerization-dependent and independent effects of GCs on the inner ear.

Conclusion

In conclusion, SEGRAs could be a helpful tool, both in basic science, where they could help to decipher the molecular pathways involved in the otoprotective effects of GCs, and in clinics where they could supplement the therapy of inner ear disorders. Nevertheless, given the ototoxic effects of IT CpdA described in this study, a careful evaluation of these novel compounds is warranted prior to their consideration for clinical studies.

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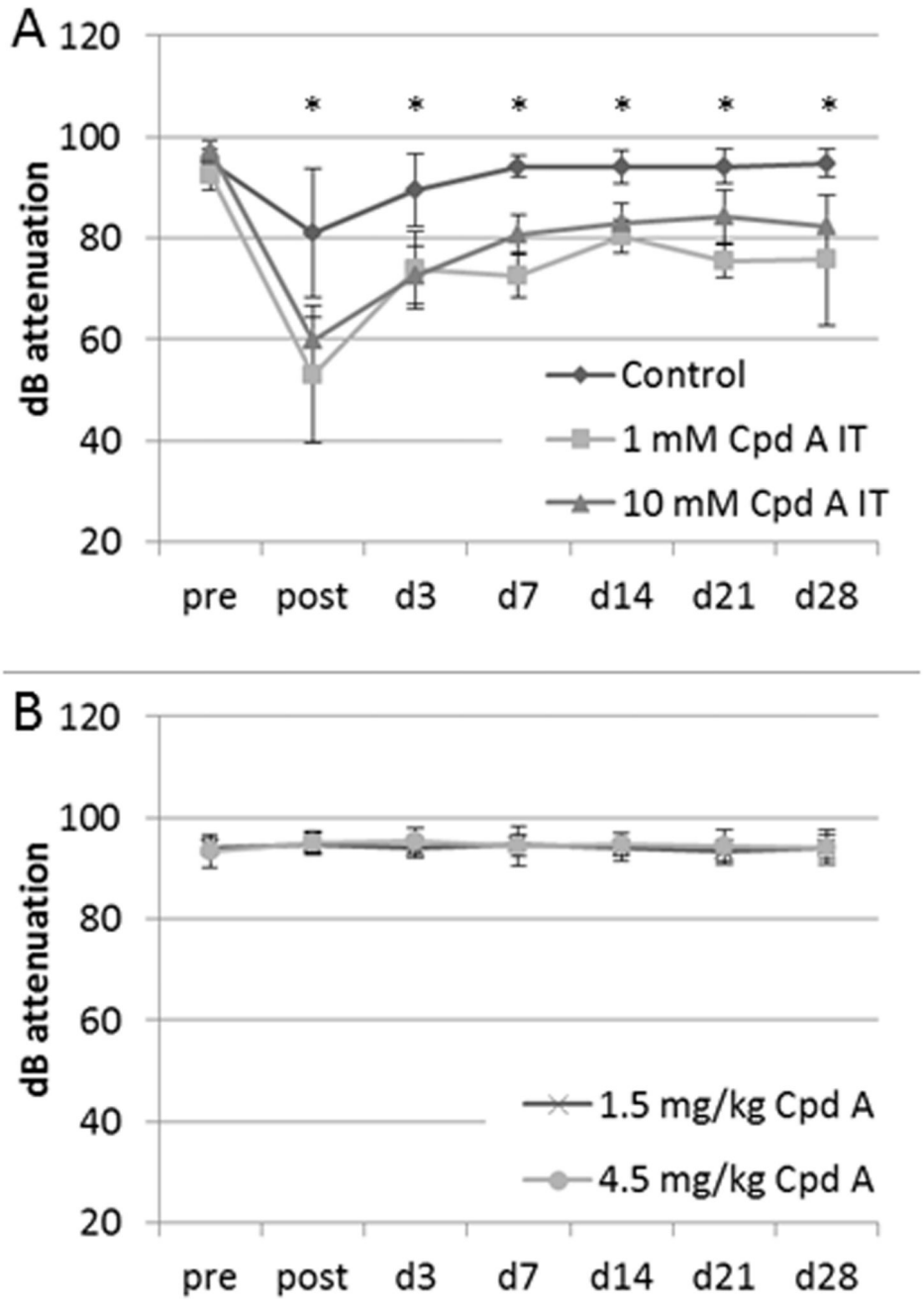

Fig. 1.

A) Click-ABR thresholds after the IT application of H_2O (diamonds), 1 mM CpdA (squares) and 10 mM CpdA (triangles). B) Click-ABR thresholds after the systemic application of 1.5 mg/kg (crosses) and 4.5 mg/kg (filled circles) of CpdA. Error-bars represent SD; *p<0.05 for both groups.

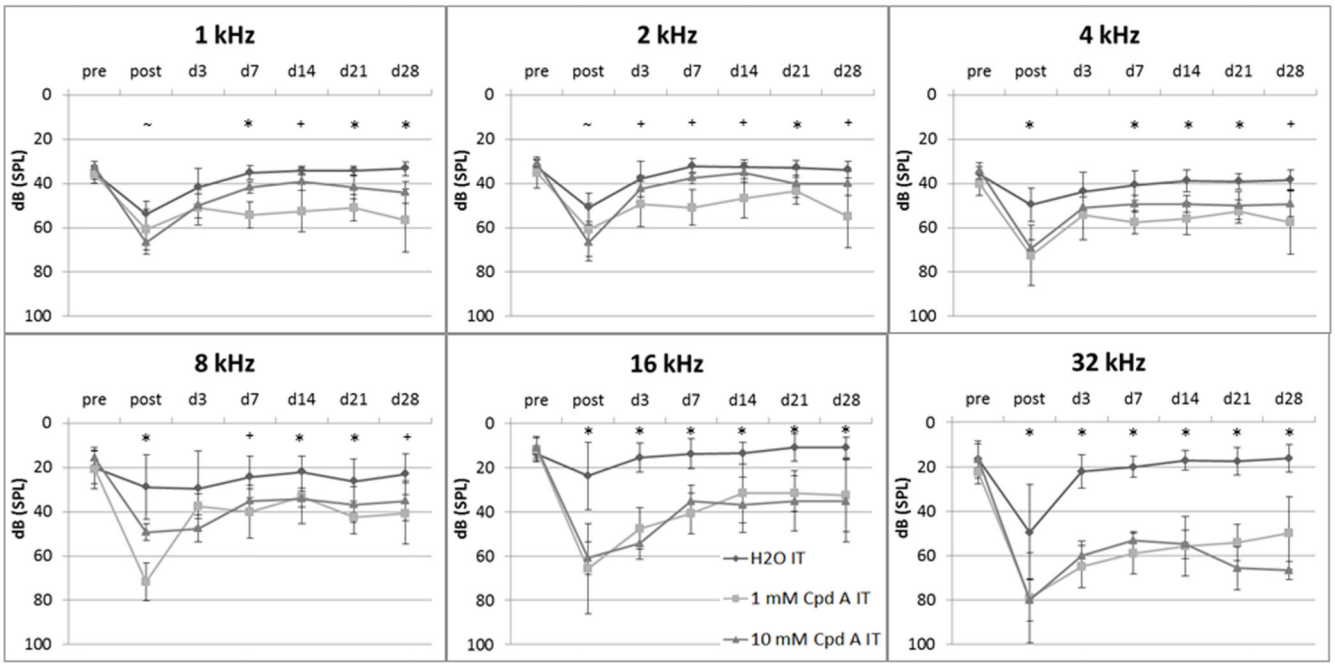

Fig. 2.

Pure-tone thresholds of guinea pigs treated intratympanically with CpdA. Controls (diamonds), animals treated with 1 mM CpdA (squares) and with 10 mM CpdA IT (triangles). Error-bars represent SD, * $p<0.05$ for both groups; + $p<0.05$ for 1 mM; ˜ $p<0.05$ for 10 mM.

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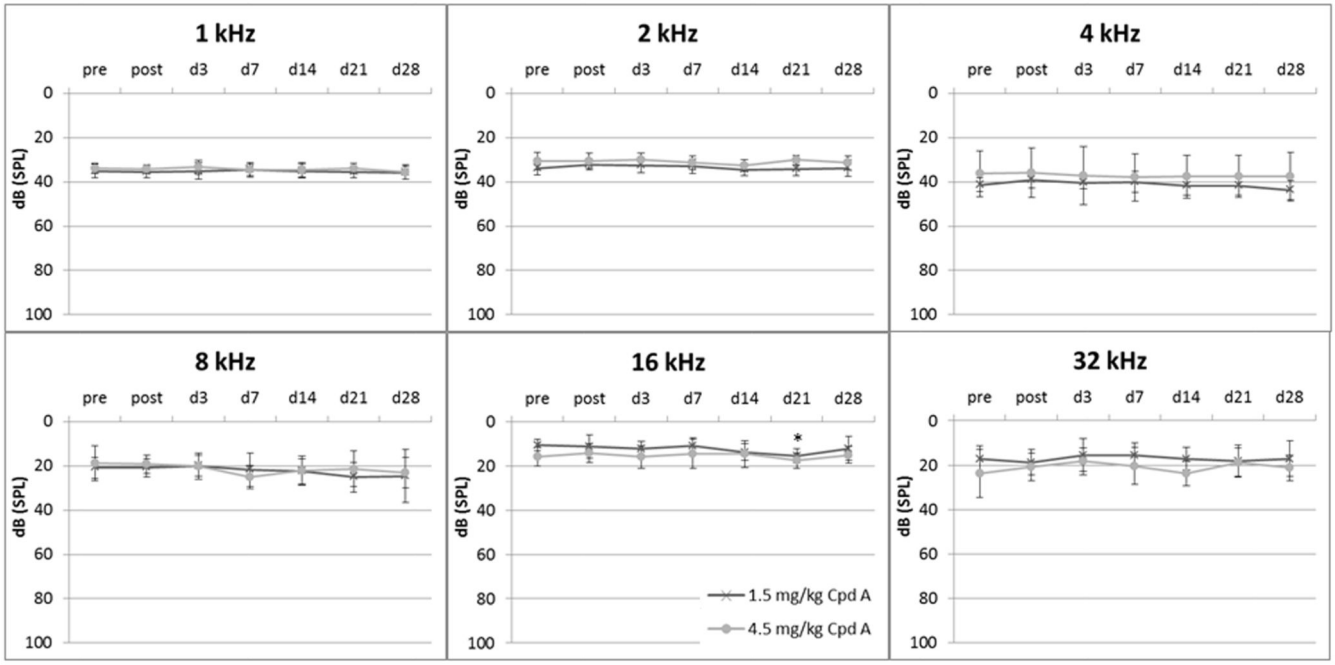

Fig. 3.

Pure-tone thresholds of guinea pigs treated systemically with 1.5 mg/kg CpdA (crosses) and 4.5 mg/kg CpdA (filled circles). Error bars represent SD, *p<0.05 for 1.5 mg/kg CpdA.

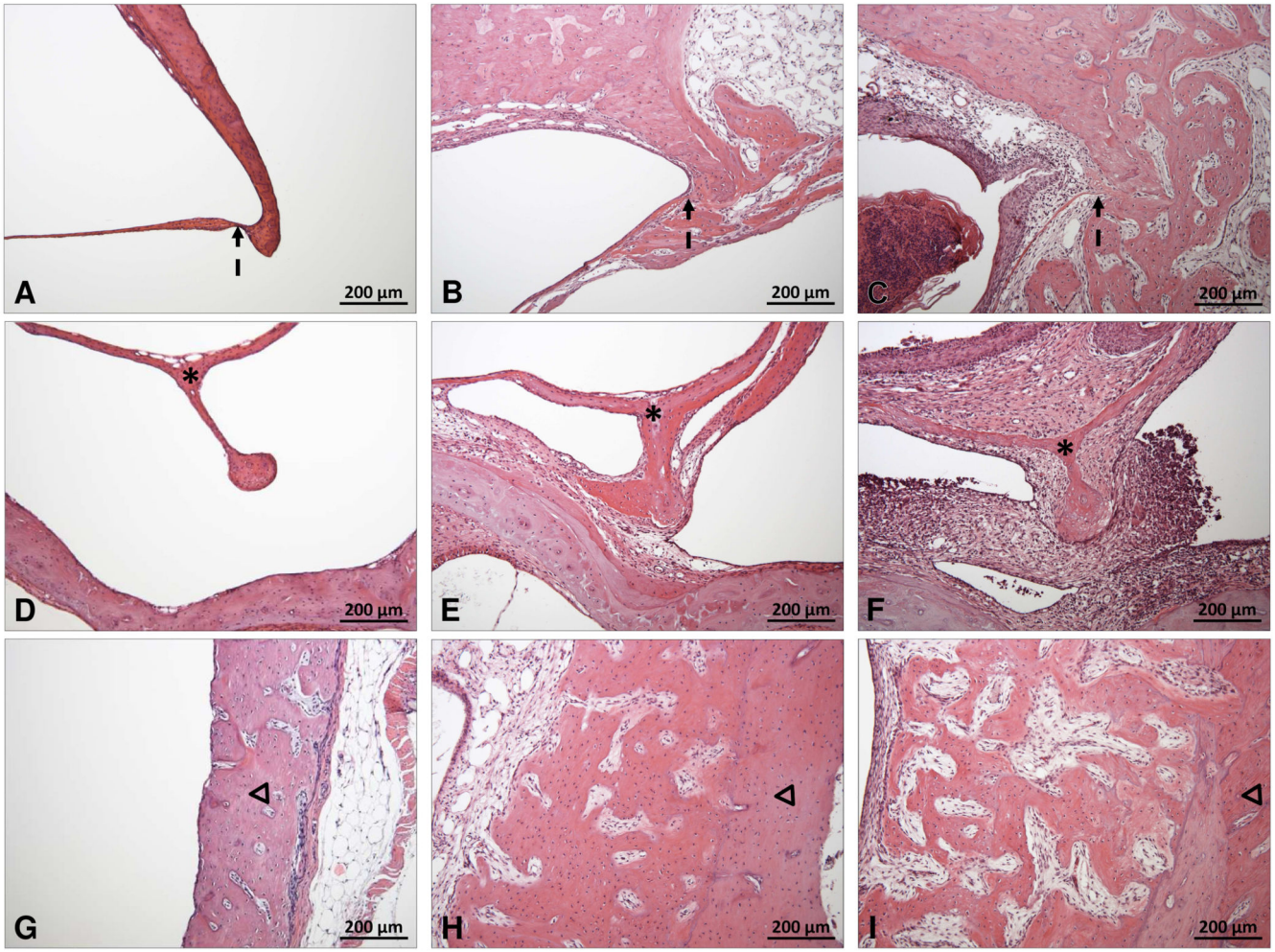

Fig. 4.

Images of representative paraffin sections (H&E staining) of guinea pig middle and inner ears. *A, D, G*: control; *B, E, H*: treated intratympanically (IT) with 1mM CpdA; *C, F, I*: treated intratympanically with 10mM CpdA. Tympanic membranes and their attachment to the tympanic annulus (*A, B, C* →). *A*, image of a physiological tympanic membrane. *B,* metaplasia of inner epithelium of tympanic membrane in a guinea pig ear treated with 1mM IT CpdA; partial osseus overgrowth from the tympanic annulus and increased amount of connective tissue within the tympanic bulla. *C,* metaplasia of both, internal and external epithelial layers of tympanic membrane of a 10mM IT CpdA treated animal. Massive growth of new bone and fibrous tissue is visible around the tympanic annulus, as well as detritus and fibrin in the external acoustic canal in this image. Aspect of the manubrium mallei (*) in close relation to the cochlea (*D, E, F*). *D,* image of a physiological manubrium mallei and cochlear wall. *E,* excessive fibrous and osseus tissue on manubrium mallei and cochlea connecting both structures of a 1mM IT CpdA treated animal. *F,* metaplasia of epithelium towards the external auditory canal with massive increase in connective tissue in a 10 mM IT CpdA treated animal; infiltration of excess connective tissue connecting manubrium mallei and the cochlea. Bulla wall (*G, H, I,* △). *G,* image of a physiological

bulla wall lined with a simple squamous epithelium on a thin layer of connective tissue. *H,* intratympanic osteoneogenesis of the bulla wall with additional increase of connective tissue of the mucosa of 1 mM IT CpdA treated guinea pig. *I,* massive bone and fibrous tissue growth of the bulla wall of an animal treated with 10 mM IT CpdA. Scale bar = 200 μm.

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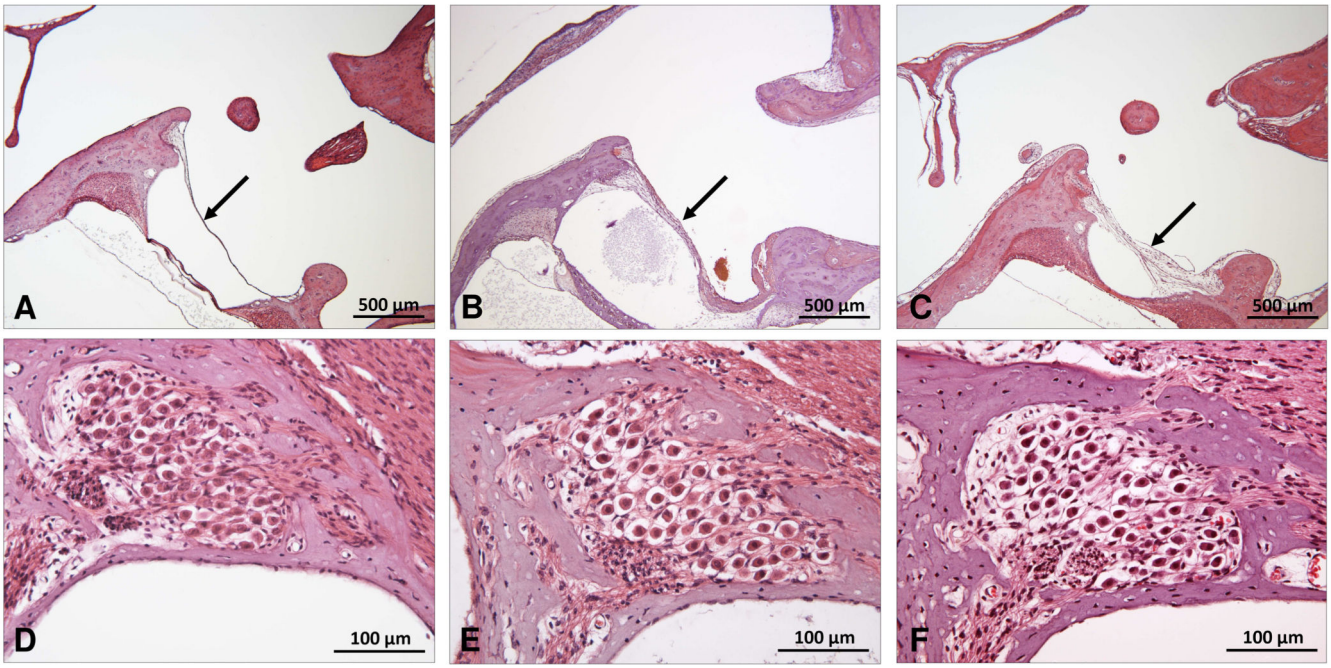

Fig. 5.

Images of representative paraffin sections (H&E staining) of guinea pig middle and inner ears. *A, D*: control; *B, E*: treated intratympanically (IT) with 1mM CpA; *C, F*: treated intratympanically with 10mM CpdA. Round window membrane (*A, B, C* →, Scale bar = 500 µm). *A*, image of a physiologically thin round window membrane and inconspicuous neighboring structures like scala tympani, tympanic membrane with manubrium mallei and first aspect of stapes and stapedius muscle. B, image of a thickened round window membrane with osteoid incorporation from a treated guinea pig with 1mM IT CpdA. Note the aspect of thickened tympanic membrane. *C*, image of the round window membrane of a guinea pig treated with 10mM IT CpdA. Layers of connective tissue are enlarged in round window membrane, on the bulla and cochlea wall as well as in the tympanic membrane. Interestingly the aspect of stapes appears inconspicuous. Spiral ganglion cells (*D, E, F*, Scale bar = 100 µm). *D*, image of spiral ganglion cells of a control animal.*E, F*, Spiral ganglion cells of guinea pigs treated with 1mM or 10 mM IT CpdA without any obvious alterations related to treatment.

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Table I

Outer hair cell counts (%)

	1 mM IT	10 mM IT	1.5 mg/kg	4.5 mg/kg	Control
Basal turn	99.5 ± 1.4	99.6 ± 1.3	99.6 ± 1.2	99.9 ± 0.6	99.4 ± 1.4
2nd turn	99.5 ± 1.3	98.4 ± 2.4	99.8 ± 1.3	99.7 ± 1.3	98.0 ± 4.1
3rd turn	99.0 ± 3.5	98.8 ± 3.3	97.7 ± 6.4	98.5 ± 3.5	97.4 ± 4.6
Apex	95.2 ± 7.5	97.7 ± 4.2	93.8 ± 9.3	98.5 ± 3.0	96.3 ± 6.7

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Table II

Inner hair cell counts (%)

	1 mM IT	10 mM IT	1.5 mg/kg	4.5 mg/kg	Control
Basal turn	100 ± 0	100 ± 0	100 ± 0	100 ± 0	99.6 ± 1.3
2nd turn	100 ± 0	100 ± 0	99.6 ± 1.3	100 ± 0	100 ± 0
3rd turn	100 ± 0	100 ± 0	99.7 ± 1.2	100 ± 0	100 ± 0
Apex	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

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Table III

Histological evaluation of middle and inner ears

	1 mM IT	10 mM IT	1.5 mg/kg	4.5 mg/kg	H_2O IT
Tympanic membrane thickened	3/3	3/3	0/3	0/3	0/3
Bony alterations	3/3	3/3	0/3	0/3	0/3
RWM thickened	3/3	3/3	0/3	0/3	0/3
Ossicular chain altered	3/3	3/3	0/3	0/3	0/3
SGCs inhomogenous/reduced	0/3	0/3	0/3	0/3	0/3