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Prophylactic and therapeutic functions of drug combinations against noise-induced hearing loss

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Abstract

Noise is the most common occupational and environmental hazard. Noise-induced hearing loss (NIHL) is the second most common form of sensorineural hearing deficit, after age-related hearing loss (presbycusis). Although promising approaches have been identified for reducing NIHL, currently there are no effective medications to prevent NIHL. Development of an efficacious treatment has been hampered by the complex array of cellular and molecular pathways involved in NIHL. We turned this difficulty into an advantage by asking whether NIHL could be effectively prevented by targeting multiple signaling pathways with a combination of drugs already approved by U.S. Food and Drug Administration (FDA). We previously found that antiepileptic drugs blocking T-type calcium channels had both prophylactic and therapeutic effects for NIHL. NIHL can also be reduced by an up-regulation of glucocorticoid (GC) signaling pathways. Based on these findings, we tested a combination therapy for NIHL that included ethosuximide and zonisamide (anticonvulsants) and dexamethasone and methylprednisolone (synthetic GCs) in mice under exposure conditions typically associated with dramatic permanent threshold shifts (PTS). We first examined possible prophylactic effects for each drug when administered alone two hours before noise, and calculated the median effective dose (ED₅₀). We then tested for synergistic effects of two-drug combinations (anticonvulsant + GC), and identified combinations with the strongest synergy against NIHL, based on a previously established combination index (CI) metric. We repeated similar tests to determine their therapeutic effects when administered the same drugs 24 hours after the noise exposure. Our study shows the feasibility of developing pharmacological intervention in multiple pathways, and discovering drug combinations with optimal synergistic effects in preventing permanent NIHL.

Keywords

Noise-induced hearing loss; Mouse; Antiepileptic drugs; Glucocorticoids; Cochlea; Hair cells

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1. Introduction

Noise-induced hearing loss (NIHL) is the single predominant health hazard posed by occupational and recreational settings (Bohnker et al., 2002; Henderson et al., 2003; Seixas et al., 2005). Although promising approaches have been identified for reducing NIHL mainly based on free radical pathways (Campbell et al., 2007; Kopke et al., 2005; Le Prell and Bao, 2012; Lynch and Kil, 2005), currently no effective pharmacologic agents are approved by FDA to diminish permanent threshold shifts (PTS). Development of an efficacious treatment has been hampered by the complex array of cellular and molecular pathways involved in NIHL.

One major mechanism underlying NIHL is mitochondrial free radical formation due to noise-induced intense metabolic activity in the cochlea (Darrat et al., 2007; Henderson et al., 2006; Le Prell et al., 2003). The involvement of this pathway in NIHL is strongly supported by three main lines of evidence: (1) Noise-induced increase of free radicals is observed in stria vascularis, outer hair cells (OHCs), supporting cells of the organ of Corti, and spiral ganglion (Ohinata et al., 2000; Ohlemiller, 2006; Yamane et al., 1995). This elevation may continue up to at least 14 days post-exposure (Yamashita et al., 2004); (2) Depletion of endogenous antioxidants such as superoxide dismutase and glutathione peroxidase results in increased susceptibility to NIHL (McFadden et al., 2001; Ohlemiller et al., 1999; 2000); (3) Enhancement of antioxidants attenuates NIHL (Lynch et al., 2004; McFadden et al., 2005; Ohinata et al., 2003). Thus, it is not surprising that attempts to prevent NIHL with antioxidants have become the focus of much research (Bielefeld et al., 2007; Hight et al., 2003; McFadden et al., 2005; Seidman et al., 1993; Yamashita et al., 2005). Nevertheless, most interventions with single agents are only partially effective in preventing NIHL. A few studies have sought to intervene at multiple sites in free radical pathways, or in combination with other pathways. Synergic effects have been observed in some, but not all, studies (Le Prell et al., 2007a; 2007b; Yamasoba et al., 1999).

Two additional pathways that have emerged as major contributors to NIHL involve calcium and glucocorticoid (GC) signaling. Disturbance in calcium homeostasis has long been suspected to contribute to trauma-induced neuronal injury (Hansen et al., 2003; Nikonenko et al., 2005; Park et al., 2008; Werling et al., 2007). Calcium homeostasis in the cochlea is maintained in part by several types of calcium channels, which include voltage-gated calcium channels (VGCCs) (Adamson et al., 2002; Errington et al., 2005; Fuchs, 2002; Rodrigues-Contreras and Yamoah, 2001; Schnee and Ricci, 2003). VGCCs can be divided into two groups: high-voltage-activated (L-type) and low-voltage-activated calcium channels (T-type) (Lacinova et al., 2000; Perez-Reyes, 1998; Triggle, 2006; Yunker and McEnery, 2003). Blockers of L-type channels have been found to attenuate NIHL in some studies (Heinrich et al., 1997; Uemaetomari et al., 2009), but not others (Boettcher, 1996; Boettcher et al., 1998; Ison et al., 1997). We reported that NIHL can be reduced by the administration of anticonvulsant drugs blocking T-type calcium channels, applied either before or after noise exposure, and these channels are present in the organ of Corti and spiral ganglion neurons (SGNs) (Shen et al., 2007). Inhibition of T-type calcium channels also protects neurons after stroke (Nikonenko et al., 2005). Thus, it is possible that anticonvulsant drugs blocking T-type calcium channels can prevent injury-induced alterations of calcium homeostasis that contribute to NIHL.

Another major molecular mechanism in NIHL involves glucocorticoid signaling. Synthetic GCs are already used clinically to treat hearing loss in a variety of cochlear disorders such as autoimmune inner ear disease, tinnitus and Menière's disease (Dodson et al., 2004; MacArthur et al., 2008; Trune and Canlon, 2012). Extensive evidence also suggests an important role of GC pathways in NIHL. First, glucocorticoid receptors (GRs) are present in

the cells of the organ of Corti, spiral ligament, spiral limbus, and SGNs (Shimazaki et al., 2002; ten Cate et al., 1993; Zuo et al., 1995). Second, stressful ‘preconditioning’ events such as restraint, heat stress, and even low-level sound—all of which are likely to engage GC signaling—have been found to be protective against NIHL in animals (Paz et al., 2004; Wang and Liberman, 2002; Yoshida et al., 1999). Third, because the noise exposure itself is a stressful event, pretreatment with blockers of GC signaling accordingly makes animals more susceptible to NIHL (Tahera et al., 2006a). Fourth, synthetic GCs such as dexamethasone and methylprednisolone can protect against NIHL (Canlon et al., 2007; Sendowski et al., 2006; Tabuchi et al., 2006; Tahera et al., 2006b; Tahera et al., 2006c). Fifth, although GCs can bind to both GR and mineralocorticoid receptors, antagonists against mineralocorticoid receptors have no effect on NIHL (Tahera et al., 2006a). Finally, a series of studies have systematically revealed the role of GR signaling pathways in NIHL (Canlon et al., 2007; Lang et al., 2006; Peppi et al., 2011; Tahera et al., 2006b; Tahera et al., 2006c; Shen et al., 2011).

Interventions based on synthetic GC drugs or anticonvulsants blocking T-type calcium channels show limited success in preventing NIHL (Canlon et al., 2007; Shen et al., 2007). Since these agents are from completely different drug families, and likely act on different molecular pathways underlying NIHL, identifying optimal combinations of these that may act in a synergistic manner seemed to us a logical next step. Here we describe methods for identifying and quantifying synergistic drug interactions against NIHL in a mouse model.

2. Materials and Methods

2.1. Animals

All animal procedures were approved by the Animal Studies Committee at Washington University in St. Louis. The study included a total 297 C57BL/6J mice aged two months (150 males and 147 females), purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed three to five per cage in a noise-controlled environment on a 12 hr light/dark cycle with light onset at 6:00 a.m. Except as noted (Fig. 5) all experimental groups contained equal numbers of male and female animals.

2.2. Drug application

Animals were subject to one of two protocols, a ‘prevention’ protocol under which drugs were administered two hours prior to a single noise exposure, and a ‘treatment’ protocol wherein drugs were administered 24 hours after noise. Two drugs from each family were chosen based on their favorable clinical usage and low side effects. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The dosage range for each drug was decided in two steps. We began with the known median effective dose (ED₅₀) from previous studies. We then expanded the dosage around the ED₅₀ by adding at least two concentrations to determine the ED₅₀ against NIHL. Each drug was dissolved in either physiological saline solution (ethosuximide and zonisamide) or vegetable oil (dexamethasone and methylprednisolone), and then administered intraperitoneally. Control animals were injected with physiological saline solution or vegetable oil. The groups exploring drug combinations received two injections (one for each drug). Since one of our main research goals was to reduce side-effects of these drugs by reducing their dosages, we focused on possible synergies against NIHL from each drug's ED₅ to ED₂₀.

2.3. Noise Exposure

All animals were exposed the noise at two months old. As described previously (e.g., Bao et al., 2004), noise exposures were performed in a foam-lined, double-walled soundproof room (Industrial Acoustics). The noise exposure apparatus consisted of a 21×21×11 cm wire cage

mounted on a pedestal inserted into turntable. The cage was rotated at 1 revolution/80 s. A Motorola KSN1020A piezo ceramic speaker (four totals) was attached to each side of a metal frame surrounding the cage. Opposing speakers were driven by independent channels of a Crown D150A power amplifier. Noise was generated by two General Radio 1310 generators and filtered to 4.0-45.0 kHz by Krohn-Hite 3550 filters. The overall noise level was measured at the center of the cage using a B&K 4135 ¼ inch microphone in a combination with a B&K 2231 sound level meter set to broadband (0.2 Hz-70 kHz). Mice were exposed one time in pairs at 110 dB sound pressure level (SPL) for 30 min.

2.4. Auditory brainstem recording (ABR)

ABR testing was performed prior to treatment, then two weeks after the noise exposure to estimate PTS. ABR thresholds were obtained as described previously (Ohlemiller et al., 2000; Bao et al., 2005). For ABR recording, we used Tucker-Davis Technologies (TDT) System II hardware and software. Animals were anesthetized with a solution of ketamine and xylazine (80/15 mg/kg, i.p.) and positioned dorsally in a custom headholder. Subdermal platinum needle electrodes (Grass) were placed in the mid-back (ground), behind the right pinna (active), and at the vertex (reference). Body temperature was monitored throughout testing using a rectal probe, and maintained at $37.5 \pm 1.0^{\circ}\text{C}$ using a DC current-based isothermal pad (FHC). The right ear of each mouse was stimulated in freefield using a TDT ES-1 speaker placed at 7 cm distance along the interaural axis. Stimuli were 5 ms tonebursts (1000 repetitions, 20/s, 1.0 ms rise/fall time) at frequencies of 5, 10, 20, 28.3, and 40 kHz. To eliminate contributions to the ABR by the unstimulated ear, the left external meatus was compressed using a spring-loaded clip. Responses were amplified x100,000 and filtered at 100-10,000 Hz.

2.5. Histological analysis

After ABR recording, mice were overdosed and perfused transcardially with cold 2% paraformaldehyde and 2% glutaraldehyde in PBS. Each cochlea was rapidly isolated, immersed in the same fixative, and the stapes immediately removed. Complete infiltration of the cochlea by fixative was ensured by making a small hole at the apex of the cochlear capsule and gently circulating the fixative over the cochlea using a transfer pipet. After decalcification in sodium EDTA, cochleae were post-fixed in buffered 1% osmium tetroxide, dehydrated in an ascending acetone series, and embedded in Epon.

Hair cells were counted using a 60x oil immersion objective lens under Nomarski optics. To facilitate the comparison among groups, inner and outer hair counts were averaged by group for discrete non-overlapping segments each constituting 10% of the apical-basal length of the basilar membrane. To count SGNs, we used the optical fractionator method to sample cell numbers in a defined fraction of the cochlear neural volume by optical dissection. Optical dissection was achieved using a 60x oil immersion lens to view along the Z-axis of sections having a thickness at least a few micrometers greater than the diameter of SGN cell bodies. The rationale for this requirement was that a few micrometers at the surface and bottom of the sections are needed to avoid the unevenness and artifacts at the surfaces of the section. Based on our preliminary experiments, we cut a series of 50 μm cochlea sections for this approach. In order to count cells, a sampling grid was placed over the contour in a random fashion using Stereological Investigator software. Each box of the sampling grid contained an unbiased counting frame, representing a defined fraction of SGNs. The specific feature chosen for counting was the nucleolus of each cell.

2.6. Data analysis

For ABR threshold shift data, we used three-way mixed model analysis of variance (ANOVA) taking consideration of drug concentration, gender, and frequency, two-way

ANOVA taking consideration of drug concentration and frequencies, and one-way ANOVA taking consideration of ABR thresholds at each frequency. To identify synergistic interactions between drugs, we applied CompuSyn software (ComboSyn, Inc.) to carry out an analysis based on the multi-drug effect equation of Chou-Talalay (Chou, 2006). This allowed us to derive the median effective dose (ED₅₀) for each drug, as well as a combination index (CI) whereby CI<1, CI=1, and CI>1 respectively indicate synergy, additivity, or antagonism of two-drug combinations.

3. Results

3.1. Pre-exposure application of anticonvulsant drugs

Ethosuximide doses of 0, 60, 90, 130, 190, or 260 mg/kg were used to determine the ED₅₀ of this drug in preventing NIHL. Figure 1A shows ABR threshold shifts two weeks after noise exposure in animals given different doses of ethosuximide. Three-way ANOVA analysis indicated a significant main effect of drug concentration ($F_5 = 24.85$, $p < 2.0 \times 10^{-16}$), but not gender ($F_1 = 0.21$, $p = 0.65$). However, the ethosuximide dose-response pattern was complicated. At 60 mg/kg, ethosuximide significantly enhanced NIHL by about 8 dB ($p < 0.002$, Tukey Post-hoc pair-wise comparisons); the average PTS across the five frequencies tested was 44.6 dB for the control group and 52.6 dB for the group treated with 60 mg/kg of ethosuximide. At 90 mg/kg, ethosuximide significantly protected against NIHL (average PTS 30.8 dB; $p < 0.001$). For the next three higher doses (from 130 to 260 mg/kg), protection was reduced to non-significance, and the average PTS was 36.5, 36.7, and 47.6 dB respectively. Due to this nonlinear dose-response, ED₅₀s of ethosuximide against NIHL could not be obtained using the Chou-Talalay equation (Table 1).

Zonisamide showed clear dose-dependent prevention of NIHL (Table 2 and Fig. 1B). For this drug, the average PTS was 35.7 dB at 80 mg/kg (about 9 dB protection), and 23.8 dB at 120 mg/kg (about 21 dB protection). There were significant dose effects ($F_2 = 16.51$, $p < 1.29 \times 10^{-6}$), but again gender had no effect ($F_1 = 3.54$, $p = 0.06$). The calculated ED₅₀ for zonisamide against NIHL was 125 mg/kg (Table 1).

3.2. Pre-exposure application of synthetic GCs

Figure 2 shows reduction of NIHL by synthetic GCs. For methylprednisolone (Table 2 and Fig. 2A), the average PTS across all test frequencies was 43.4 dB for the control group, 35.5 dB at 30 mg/kg (about 8 dB protection), and 34.0 dB at 45 mg/kg (about 9 dB protection). Two-way ANOVA showed a significant effect of drug concentration ($F_2 = 3.32$, $p = 0.04$) with no effect of frequency. Tukey post-hoc tests indicated ABR thresholds were significantly different between the drug-treated group (45 mg/kg) and the control group. Three-way ANOVA with gender added as a factor also supported significant dose effects ($F_2 = 3.04$, $p < 0.05$) but no gender effects ($F_1 = 0.32$, $p = 0.58$). The calculated ED₅₀ for methylprednisolone was 525 mg/kg (Table 1).

For dexamethasone (Table 2 and Fig. 2B), the average PTS was 39.2 dB at 5 mg/kg (about 4 dB protection), and 35.4 dB at 10 mg/kg (about 8 dB protection). Two and three-way ANOVA showed no significant prophylactic effects ($F_2 = 2.61$, $p = 0.09$), or gender effects ($F_1 = 0.04$, $p = 0.85$). However, based on the Chou-Talalay equation, dose-dependent PTS reduction was still present for this drug. We calculated an ED₅₀ against NIHL for dexamethasone at 39.4 mg/kg (Table 1).

3.3. Synergistic effect against NIHL by zonisamide and synthetic GCs

Because no ED₅₀ could be obtained for ethosuximide, to uncover synergistic drug effects against NIHL, we focused on combinations of zonisamide and two GCs. Significant

protective effects were observed for the combination of zonisamide and methylprednisolone at their ED₁₀s (Fig. 3; $p < 0.005$). Synergy was also found for this combination (CI = 0.97). No synergy was found for the combination of zonisamide and dexamethasone at their ED₅s (CI = 1.19) or ED₁₀s (CI = 3.22).

3.4. Post-exposure application

We next examined possible therapeutic effects of individual drugs against NIHL when administered 24 hours after noise exposure. For ethosuximide the average PTS across test frequencies was 44.1 dB for the control group, 43.8 dB at 130 mg/kg (about 0.2 dB protection), and 39.3 dB at 190 mg/kg (about 5 dB protection) (Table 2 and Fig. 4A). Three-way ANOVA showed significant dose effects ($F_2 = 3.11$, $p < 0.05$), and gender effects ($F_1 = 3.97$, $p < 0.05$).

For zonisamide, the average PTS was 41.9 dB at 120 mg/kg (about 2 dB protection), and 41.3 dB at 160 mg/kg (about 3 dB protection) (Table 2 and Fig. 4B). Three-way ANOVA indicated neither significant drug effects ($F_2 = 1.29$, $p = 0.28$) nor gender effects ($F_1 = 2.01$, $p = 0.16$). However, one-way ANOVA at different frequencies supported a significant difference at 40 kHz for the three groups ($p = 0.02$). At this frequency, Tukey post-hoc tests showed a significant difference between controls and the group treated with zonisamide at 120 mg/kg ($p = 0.017$).

For methylprednisolone, the average PTS across test frequencies was 45.9 dB for the control group, 40.0 dB at 30 mg/kg (about 7 dB protection), and 30.9 dB at 60 mg/kg (about 15 dB protection) (Table 2 and Fig. 5A). Three-way ANOVA showed significant dose effects ($F_2 = 8.28$, $p < 0.001$), but no gender effects ($F_1 = 1.43$, $p = 0.24$). One-way ANOVA supported a significant difference at 10 kHz ($p = 0.01$). Follow-up Tukey post-hoc tests showed that the drug-treated group at 60 mg/kg was significantly different from the control ($p = 0.005$).

For dexamethasone, the average PTS across test frequencies was 44.8 dB at 30 mg/kg (about 1 dB protection), and 37.6 dB at 60 mg/kg (about 8 dB protection) (Table 2 and Fig. 5B). Two-way ANOVA analysis showed significant differences by dose ($F = 5.06$, $p = 0.0078$), and Tukey post-hoc tests showed a significant difference for the drug group treated at 60 mg/kg from the control group, and from the drug group at 30 mg/kg ($p = 0.0183$ and 0.019 , respectively). Three-way ANOVA detected no gender effects.

We calculated an ED₅₀ for ethosuximide at 243 mg/kg, methylprednisolone at 95.6 mg/kg, and dexamethasone at 96.3 mg/kg, respectively (Table 1). No synergistic effects against NIHL by two-drug combinations were ever observed between ethosuximide and methylprednisolone, or ethosuximide and dexamethasone when applied at doses from their ED₅s to ED₂₀s.

3.5. Histological findings

To address the anatomic bases of our functional observations, we repeated a set of experiments with animal treated (i.p.) with vegetable oil (Control), zonisamide (120 mg/kg), methylprednisolone (45 mg/kg), and both drugs (120 and 45 mg/kg respectively) before the noise exposure. Two weeks after the noise exposure, we performed hair cell counts for both inner hair cells (IHCs) and outer hair cells by row (OHC1, OHC2, and OHC3; see Fig. 6A). Consistent with previous studies (e.g., Ohlemiller et al., 2000a,b), moderate PTS was not associated with hair cell loss, except for the most basal 10-20%, or hook region. For IHCs (Fig. 6B), two-way ANOVA showed no significant difference among different treatments ($F_3 = 0.28$; $p = 0.84$). Likewise, for OHC1 and OHC2 (Fig. 6C and 6D), no significant difference by treatment ($F_3 = 0.56$ and $p = 0.642$ for OHC1; $F_3 = 0.312$ and $p = 0.817$ for OHC2). For OHC3 (Fig. 6E). Two-way ANOVA showed no significant difference among

different treatments ($F_3 = 0.595$; $p = 0.619$). However, one-way ANOVA restricted to the two most basal segments supported a significant difference by treatment ($p = 0.0032$). Post hoc Tukey tests indicated that row OHC3 was protected by the drug combination of zonisamide and methylprednisolone.

We also estimated a total number of surviving SGNs. One-way ANOVA analysis showed no significant difference among the same four groups featured in Figure 5 ($F_3 = 1.87$, $p = 0.188$), although the drug combination group had the most surviving SGNs (15,752/mouse vs 13,084/mouse controls, 14,096/mouse for the zonisamide-treated group, and 13,749/mouse for the methylprednisolone-treated group).

4. Discussion

Building on previous studies, we tested a combination therapy against NIHL that included anticonvulsants ethosuximide and zonisamide, and synthetic GCs dexamethasone and methylprednisolone. ED_{50} s for these drugs could be determined in most cases. A synergistic effect was observed for the combination of methylprednisolone and zonisamide. Three issues raised by this study are discussed below.

4.1. Prophylactic and therapeutic functions of T-type calcium blockers

Our previous work demonstrated both prophylactic and therapeutic effects against NIHL from trimethadione and ethosuximide, two drugs from the same anticonvulsant family blocking T-type calcium channels (Shen et al., 2007). A subsequent study found no prophylactic function against NIHL for two similar blockers for T-type calcium channels, mibefradil and flunarizine (Uemaetomari et al., 2009), but numerous methodologic differences (different mouse strains, different noise exposures, different drug regimens) could account for this. We fed drugs to mice via drinking water for three weeks before noise exposure while intraperitoneal injections were used by Uemaetomari et al. (2009). When we adopted their injection method in the current study, protection could be observed for ethosuximide and zonisamide. However, the dose-response pattern for ethosuximide was non-monotonic (Fig. 1A), suggesting that different effective doses may explain the differences between these two studies.

In our previous work (Shen et al, 2007), a significant therapeutic effect of trimethadione against NIHL was observed only in male mice. In the current study, gender differences were also observed for therapeutic application (after noise) of ethosuximide, although neither ethosuximide nor zonisamide showed gender effects in their NIHL prophylactic (before noise) efficacy. In sum, these data raise the possibility that gender effects could impact, clinical efficacy of these agents, and that different molecular mechanisms may underlie their prophylactic and therapeutic functions against NIHL.

Currently, molecular mechanisms underlying prophylactic and therapeutic effects of T-type anticonvulsants are unknown. Previously, we reported strong expression of the α_1H subunit in cochlear spiral ganglion neurons, which showed no enhanced survival in treated animals (Shen et al., 2007). We also have observed no differences in severity of NIHL between control mice and mice with reduced expression of the α_1H subunit (unpublished), indicating that this subunit is not involved in protection by these drugs against NIHL. By contrast, hair cells and supporting cells appear to express both α_1G and α_1I calcium channel subunits, and protection of OHCs by trimethadione was observed in our previous study (Shen et al., 2007). Thus, these drugs could act on α_1G , α_1I , or both subunits located on OHCs or supporting cells in reducing NIHL. The present histological data did not show a compelling difference among control and drug-treated groups in the number of surviving hair cells or SGNs. One main reason could be due the noise condition (110 dB SPL for 30 min.) used in this study.

Although this established condition with its high PTS level is ideal for our pharmacodynamic studies, mice do not generally show close correspondence between frequencies showing NIHL and cochlear regions exhibiting marked OHC loss because this noise exposure does not promote rupture of the reticular lamina, (Wang et al., 2002). Most NIHL—including that inflicted by our noise exposure—appears best explained by unrepaired OHC injury and possible conformation changes to the organ of Corti. The protection we found may therefore reflect reduced non-lethal injury to OHCs and supporting cells via other molecular mechanisms (So et al., 2005; Le Prell and Bao, 2012).

4.2 Prophylactic and therapeutic functions of GCs

The ability of GCs to reduce NIHL has been demonstrated in various animal models (for recent reviews see Jin et al., 2009; Trune and Canlon, 2012). However, GCs are known to cause serious dose-dependent side effects, including psychosis, gastritis, hypertension, insulin resistance, sleep disturbances, and aseptic necrosis of the hip (Hanaoka et al., 2012). Therefore, we sought to identify low prophylactic and therapeutic effective doses for two widely used GCs, dexamethasone and methylprednisolone. Previous studies showed that dexamethasone had no protective effects against NIHL at 1 mg/kg in rats (Bas et al., 2008). In mice, methylprednisolone exhibited protective effects against NIHL at doses ranging from 10-100 mg/kg, while its therapeutic effects at 30 mg/kg were present only if it was administered within three hours after noise exposure (Tabuchi et al 2006). In the current study, a therapeutic effect of methylprednisolone against NIHL at 60 mg/kg was found, even 24 hours after noise (Fig. 5A). One implication is that detailed pharmacodynamic studies are needed for each model before any agents are labeled ineffective. More importantly, we discovered synergistic effects for combinations of methylprednisolone and zonisamide against NIHL that allowed the dose of methylprednisolone to be reduced as low as 8 mg/kg. This finding reinforces of the potential benefits of simultaneously interdicting different molecular pathways as a strategy for reducing NIHL prior to damaging noise. On the other hand, no synergy found for the two drug families when applied after noise exposure. This may again indicate that somewhat different pathways were involved in protection associated with drug application before versus after noise.

Prophylactic and therapeutic effects of GCs can act through both genomic and nongenomic pathways (Jin et al., 2009, Shen et al., 2011). Possible protective actions of GCs could include: (a) Initiation of downstream pathways by nuclear factor κ B (NF κ B; Lang et al., 2006), STAT5 (Xu et al., 2009), and PLZF, a corticosteroid-responsive transcription factor (Peppi et al., 2011), (b) Stabilization of cellular membranes, (c) Inhibition of phospholipase A2, and (d) Scavenging of free radicals and inhibition of lipid peroxidation. The latter suggests overlap with antioxidant pathways.

4.3. Consideration of the Chou-Talalay model

Drug combination therapies have been highly successful in treating diseases such as HIV infections (Richman, 2001). They have demonstrated advantages over single drug therapies such as achieving clear therapeutic effects at lower doses, and with fewer side effects. However, uncovering optimal drug combinations by direct screening without any computational analysis is challenging, due to the large number of potential combinations. Pharmacodynamic analysis in this study was based on the Chou-Talalay median-effect equation (Chou and Talalay, 1984). This equation was derived from the mass-action law principle, which provides the common link between single and multiple drug-target interactions, and first order and higher order dynamics. The Chou-Talalay equation has in fact the same mathematical form as the Hill function. Besides its usefulness in dose-response trends of single drugs, the resulting CI from this equation offers quantitative definition for additive effect ($CI = 1$), synergism ($CI < 1$), and antagonism ($CI > 1$) in drug

combinations. Thus, this equation has been widely used in the discovery of drug combinations (Chou, 2006; Lee and Kong, 2009). Although the Chou-Talalay equation is independent of drug mechanisms of action, and does not require knowledge of conventional kinetic constants (Chou 2006), its assumption that underlying mechanisms for two drugs are independent has led to underestimation of synergistic effects for drug with overlapping mechanisms (Lee, 2010). This possible underestimation could be the cause for lack of synergy found for therapeutic effects of drug combinations between antiepileptic and GC drug families.

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In the paper, we have demonstrated

1. Prophylactic effects for NIHL from antiepileptic and glucocorticoid drugs
2. Therapeutic effects for NIHL from these same two drug families
3. A synergistic prophylactic effect for NIHL from these two drug families.

Fig. 1. Prophylactic function of ethosuximide and zonisamide. (A) ABR threshold shifts (Mean-S.D) for mice treated with ethosuximide at indicated doses, two hours before noise exposure (n=8/group). (B) ABR threshold shifts for mice treated with zonisamide at indicated doses, two hours before the noise exposure (n=8/group). Four mice per gender were used in each group.

Fig. 2. Prophylactic function of methylprednisolone and dexamethasone. (A) ABR threshold shifts (Mean-S.D) for mice treated with methylprednisolone two hour before the noise exposure (n=8/group). (B) ABR threshold shifts for mice treated dexamethasone two hours before the noise exposure (n=8/group). Four mice per gender were used in each group.

Fig. 3. Synergistic function of methylprednisolone and zonisamide. ABR threshold shifts (Mean-S.D) for control mice (n=16) and mice treated with both methylprednisolone and zonisamide two hours before noise exposure (n=6).

Fig. 4. Therapeutic function of ethosuximide and zonisamide. (A) ABR threshold shifts (Mean-S.D) for mice treated with ethosuximide 24 hours after noise exposure (n=8/group). (B) ABR threshold shifts for mice treated with zonisamide 24 hours after the noise exposure (n=8 each for controls and zonisamide at 160 mg/kg; n=10 zonisamide at 120 mg/kg).

Fig. 5. Therapeutic function of methylprednisolone and dexamethasone. (A) ABR threshold shifts (Mean-S.D.) for mice treated with methylprednisolone 24 hours after noise (n=8/group). (B) ABR threshold shifts for mice treated with dexamethasone 24 hours after noise exposure (n=8 for controls, n=10 dexamethasone at 30 mg/kg, n=9 for dexamethasone at 60 mg/kg with four males and five females).

Fig. 6. Quantitative comparison of hair cell survival (Mean $\pm$ S.D.) in control and drug-treated mice (see legend). (A) Schematic organ of Corti depicts IHC, OHC1, OHC2, and OHC3. (B, C, D, E) Hair cell density by cell type (IHC, OHC1, OHC2, and OHC3) and treatment group, (n=4/group).

Table 1

Summary of two modes of drug protection against NIHL. ED₅₀ of each drug for its prophylactic (two hours before the noise exposure) and therapeutic effects (24 hours after the noise exposure)

Drug	Prophylactic Effect ED ₅₀	Therapeutic Effect ED ₅₀
Ethosuximide	None	243 mg/kg
Zonisamide	125 mg/kg	None
Methylprednisolone	525 mg/kg	95.6 mg/kg
Dexamethasone	39.4 mg/kg	96.3 mg/kg

Table 2

Dose-response data for each drug based on PTS averaged across five frequencies (5, 10, 20, 28.3, and 40 kHz).

Drug	Prophylactic Effects		Therapeutic Effect	
	Dose (mg/kg)	PTS (dB)	Dose (mg/kg)	PTS (dB)
Ethosuximide	0	44.56	0	44.05
	60	52.55	130	43.84
	90	30.83	190	39.25
	120	36.48		
	190	36.7		
	260	47.55		
Zonisamide	0	44.56	0	44.05
	80	35.7	120	41.9
	120	23.8	160	41.25
Methylprednisolone	0	43.39	0	45.88
	30	35.45	30	39.35
	45	33.98	60	30.88
Dexamethasone	0	43.39	0	45.88
	5	39.2	30	44.78
	10	35.38	60	37.56
Zonisamide Methylprednisolone	60	39.05		
	8	40.11		
Zonisamide/Methylprednisolone	60/8	35.33		