Potassium ion channel openers, Maxipost and Retigabine, protect against peripheral salicylate ototoxicity in rats

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1. Introduction

High-dose sodium salicylate (SS), the active ingredient in aspirin, reliably induces transient sensorineural hearing loss, tinnitus (Bauer et al., 1999; Jastreboff et al., 1988; Lobarinas et al., 2004; Yang et al., 2007) and hyperacusis (Chen et al., 2014). Consequently, SS is frequently used to investigate peripheral and central mechanisms of hearing loss, tinnitus and hyperacusis in animal models. However, despite being used in auditory research for more than 50 years SS’s mechanisms of action are still not fully understood.

SS has a profound influence on the cochlea, including a reduction in OHC electromotility and potassium currents. A significant reduction in distortion product otoacoustic emissions (DPOAEs) accompanies a high-dose SS injection in rats (Chen et al., 2010; Sheppard et al., 2014; Stolzberg et al., 2011). This reduction has been reported as frequency-specific (Stolzberg et al., 2011). DPOAEs arise from prestin-mediated outer hair cell (OHC) electromotility, supported in part by the +80 mV endocochlear potential and high potassium concentration in endolymph (Liberman et al., 2002; Schmiedt et al., 2002). SS competitively suppresses the binding of chloride to anion binding sites on the motor protein prestin (Oliver et al., 2001; Rybalchenko and Santos-Sacchi, 2003), thereby reducing the electromotility of OHCs. SS also blocks potassium (K+) currents on cochlear sensory cells. Within inner hair cells (IHCs), SS blocks the outward K+ current (I_{K,1}), but does not affect the inward potassium current (I_{K,n}) in guinea-pigs (Kimitsuki et al., 2011). Based on its sensitivity to iberiotoxin and charybdotoxin (BK blockers), the K+ current I_{K,n} represents the large conductance Ca2+- and voltage-activated BK channels (Kros and Crawford, 1990; Kros et al., 1998). Within OHCs, SS blocks the K+ channel KCNQ4 (Kv7.4) in guinea-pig, which has been suggested as the molecular correlate of the potassium current I_{K,n} (Housley and Ashmore, 1992; Wu et al., 2010). K+ channels have different expression levels along the tonotopic gradient of the mouse and rat cochlea (Beisel et al., 2000; Engel et al., 2006; Hafidi et al., 2005; Kharkovets et al., 2000; Wersinger et al., 2010). Therefore, SS blockage of BK or Kv7.4 could contribute to a frequency-dependent reduction in compound action potentials (CAPs) or DPOAEs.

The K+ ion channel openers BMS-204352 (Maxipost) and Retigabine (RTG) were originally developed for stroke (Grikhoff et al., 2001a, b) and epilepsy (Rostock et al., 1996), respectively. However, they also have anxiolytic (Korsgaard et al., 2005), antinociceptive (Blackburn-Munro and Jensen, 2003; Nodera et al., 2011), and neuroprotective properties (Jensen, 2002; Rundfeldt, 1997), resulting from Kv7 and BK K+ channel activation. These

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potassium channels are present in the CNS (Brenner et al., 2005; Hansen et al., 2006; Korsgaard et al., 2005; Sausbier et al., 2006) and on cochlear hair cells of mice and rats with varying expression along the tonotopic gradient (Beisel et al., 2000; Hafidi et al., 2005; Kharkovets et al., 2006). Mutations in four of five known Kv7 potassium channel subunits cause human-inherited diseases, some of which are characterized by central hyperactivity and peripheral deafness (Maljevic et al., 2010). Since tinnitus has been hypothesized as a central hyperactivity disorder, potassium channel openers have been evaluated for their efficacy in suppressing SS- and noise-induced tinnitus in both mice and rats (Li et al., 2013; Lobaranis et al., 2011). Maxipost eliminates SS-induced tinnitus in rats (Lobaranis et al., 2011), while RTG prevents noise-induced tinnitus in mice (Li et al., 2013). However, these mechanisms are not well understood.

In the present study, we characterize the influence that Maxipost and RTG have on the peripheral auditory system alone and in combination with SS using a rat animal model. Since Kv7 and BK channels are abundantly expressed in the peripheral system of murine species, and are blocked by SS, we hypothesized that Maxipost and/or RTG would enhance the gross cochlear potentials alone or in combination with SS. Alterations occurring in the periphery as a result of these drugs can elucidate the role of potassium channels in cochlear function and SS-induced ototoxicity.

### 2. Materials and methods

#### 2.1. Subjects

Seventy-seven male Sprague–Dawley rats (250–500 g, Charles River) between the ages of 2–5 months were used in this study. Animals were housed two per cage, provided free access to food and water, and kept on a 12/12 h light–dark cycle. For CAP measurements (n = 48), animals were randomly assigned into eight groups: Control (n = 6), vehicles (Saline n = 6), or dimethyl sulfoxide (DMSO, n = 6), SS (n = 6), Maxipost (n = 6), RTG (n = 6), Maxipost + SS (n = 6), and RTG + SS (n = 6). For DPOAE measurements (n = 29), animals were randomly assigned to seven groups: vehicles (Saline n = 4, or DMSO n = 6), SS (n = 3), Maxipost (n = 4), RTG (n = 4), Maxipost + SS (n = 4), and RTG + SS (n = 4).

#### 2.2. Compound action potentials

Rats were anesthetized with a ketamine (60 mg/kg) and xylazine (6 mg/kg) injection intraperitoneally (i.p.). Body temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus, Holliston, MA). The animal was placed in a custom head-holder, the right bulla was exposed and a small opens were performed 3 h post SS.

#### 2.3. Distortion product otoacoustic emissions

Rats were anesthetized with a ketamine (60 mg/kg) and xylazine (6 mg/kg) injection (i.p.). Using a commercial instrument (Intelligent Hearing Systems; Miami, FL), DPOAEs were obtained with two primary tones (f1 and f2) presented at an f2/f1 ratio of 1.2. The intensity of f1 (11) was presented 10 dB higher than the intensity of f2 (12). I/O functions for distortion products f1–f2 were plotted for f2 frequencies of 4, 6, 8, 6.13, 12, 16, 24, 30 and 35 kHz using L1 intensities of 25–80 dB SPL (5 dB steps). I/O functions were analyzed for significance using two-way ANOVA (Prism GraphPad v5).

#### 2.4. Experimental conditions

##### 2.4.1. Experiment 1: vehicles (DMSO & saline)

Saline was used to dissolve SS and DMSO (Sigma–Aldrich, St. Louis, MO) was used to dissolve Maxipost and RTG. One vehicle group was treated with saline alone and the other vehicle group was treated with DMSO alone. CAPs and DPOAEs were acquired 3 h later.

##### 2.4.2. Experiment 2: SS

SS (Sigma–Aldrich) was dissolved in saline (50 mg/ml), and administered via i.p. injection (200 mg/kg). This dose reliably induces tinnitus and hearing loss (Chen et al., 2013; Lobaranis et al., 2004; Sheppard et al., 2014; Stolzberg et al., 2011). For DPOAEs, a within-subject experiment, measurements were made at baseline and 3 h post SS. For CAPs, a between-group experiment, recordings were performed 3 h post SS.

##### 2.4.3. Experiment 3: Maxipost or RTG alone

Maxipost (Axon Medchem, 9713 GZ Groningen, The Netherlands) was dissolved in DMSO (25 mg/ml) and administered via i.p. injection (10 mg/kg). Previous reports indicate this dose eliminated behavioral evidence of SS-induced tinnitus in rats (Lobaranis et al., 2011). RTG (Alomone Labs, Har Hotzvim Hi-Tech Park, P.O. Box 4287 Jerusalem 9104201, Israel) was dissolved in DMSO (50 mg/ml) and administered via i.p. injection (10 mg/kg). Previous reports indicate this dose prevents noise-induced tinnitus in mice (Li et al., 2013). DPOAEs were obtained at baseline and 2 h post Maxipost or RTG. CAPs were obtained approximately 3 h post drug administration.

##### 2.4.4. Experiment 4: Maxipost or RTG + SS

Maxipost or RTG (10 mg/kg i.p.) was administered 10 min prior to SS (200 mg/kg i.p.). DPOAEs or CAPs were obtained 3 h later.

#### 2.5. Compliance

The research project was performed in accordance with regulations approved by the Institutional Animal Care and Use Committee (IACUC, HERO058080Y) at the University of Buffalo and was carried out in accordance with National Institute of Health guidelines.
3. Results

3.1. Vehicles

Fig. 1A illustrates CAP amplitudes as a function of tone bursts presented at 80 dB SPL for control, saline, and DMSO groups. Fig. 1B illustrates DPOAEs as function of f2 frequencies with L1 intensity set at 70 dB SPL for control, saline, and DMSO groups. There is no significant difference between control and vehicle (saline and DMSO) treated animals. This indicates that the vehicles used had no significant impact on peripheral hearing.

3.2. SS

It is well-established that SS is ototoxic (Chen et al., 2010, 2013; Stolzberg et al., 2012). Fig. 2A illustrates that mean (+/− SEM) CAP thresholds were significantly higher than saline vehicles by approximately 30 dB SPL across the tested frequency range (two-way ANOVA, p = 0.0001, F1,10 = 205). Fig. 2B illustrates mean (+/− SEM) CAP I/O functions at 6, 20 and 35 kHz. The average CAP amplitudes decreased significantly at all frequencies 3 h post SS (two-way ANOVA, 4 kHz p < 0.001, F1,2 = 13.17; 6–40 kHz p < 0.0001, F1,4 = 13–37). Fig. 2C illustrates mean (+/− SEM) DPOAE I/O functions 3 h post SS. DPOAEs were significantly reduced at all tested frequencies (two-way ANOVA, 4–24 kHz p < 0.0001, F1,12 = 18–40; 30 kHz p < 0.02, F1,7 = 5.948; 35 kHz p < 0.04, F1,11 = 4.212). These results indicate that SS induced a mild-to-moderate CAP threshold shift, moderately decreased CAP amplitudes, and mildly reduced DPOAEs, indicative of OHC dysfunction.

3.3. Maxipost and RTG alone

Fig. 3A illustrates CAPs at 80 dB SPL as a function of tone bursts and Fig. 3B illustrates DPOAEs at L1 = 70 dB SPL as a function of f2 frequencies for rats treated with Maxipost, RTG, or the DMSO vehicle. CAPs and DPOAEs for Maxipost or RTG alone were not significantly different from the DMSO vehicle. Similar results were obtained at all other measured intensities. These results indicate that Maxipost or RTG alone did not have a significant effect on peripheral hearing.

3.4. Maxipost and SS

Fig. 5A illustrates mean (+/− SEM) CAP I/O functions at a low (6 kHz), mid (16 kHz), and high (35 kHz) frequency for Maxipost + SS and SS alone groups. For reference, data are shown for saline and DMSO vehicles. Compared to SS alone, Maxipost + SS significantly enhanced mean CAPs in low frequencies <8 kHz (two-way ANOVA, 2 kHz: p = 0.05, F1,3 = 6.215; 4 kHz: p < 0.05, F1,2 = 4.84; 6 kHz: p < 0.0001, F1,14 = 18.92; 8 kHz: p < 0.01, F1,3 = 9.796), but not in mid or high frequencies ≥12 kHz. Fig. 5B illustrates mean DPOAE I/O functions for Maxipost + SS and SS alone. There was no significant difference between Maxipost + SS and SS alone (two-way ANOVA, p > 0.05). Since Maxipost reversed SS-reduced CAP amplitudes, but not DPOAEs, these data suggest that Maxipost inhibits SS-induced K+ channel blockage of IHCs in the basal high-frequency region of the cochlea.

3.5. RTG and SS

Fig. 6A illustrates mean (+/− SEM) CAP I/O functions at a low (6 kHz), mid (16 kHz), and high (35 kHz) frequency for RTG + SS and SS alone groups. For reference, data are shown for saline and DMSO vehicles. Compared to SS alone, RTG + SS significantly enhanced mean CAPs in low frequencies ≥100 kHz (two-way ANOVA, F3,4 = 17.64; 4 kHz: p < 0.05, F1,3 = 5.48; 6 kHz: p < 0.0001, F1,14 = 18.92; 8 kHz: p < 0.01, F1,3 = 9.796), but not in mid or high frequencies ≥12 kHz. Fig. 6B illustrates that CAP thresholds in the low frequencies were significantly reduced in RTG + SS compared to SS alone (one-way ANOVA, 2 kHz: p < 0.01, F1,4 = 8.899; 4 kHz: p < 0.05, F1,4 = 5.462; 6 kHz: p < 0.05, F1,4 = 10.42; 8 kHz: p < 0.01, F1,4 = 20.22). Additionally, CAP thresholds were not significantly different between saline and RTG + SS (two-way ANOVA, p > 0.05: 2 kHz, 4 kHz, 6 kHz, 8 kHz, indicating that RTG completely reversed the SS-induced threshold shift in low frequencies. Fig. 6B illustrates mean DPOAE I/O functions for RTG + SS and SS alone. Results indicate no significant difference between RTG + SS and SS alone in all tested frequencies (two-way ANOVA, p > 0.05). Since RTG reversed SS-reduced CAP amplitudes, but not DPOAEs, these data suggest that RTG inhibits SS K+ channel blockage in the apical low-frequency region of the cochlea.

4. Discussion

4.1. Salicylate pathophysiology of Kv7.4 and BK K+ channels

Previous research using knockout mouse models have determined that BK and Kv7.4 channel function is essential for normal hearing (Kharkovets et al., 2006; Ruttiger et al., 2004), although

![Image](HEARES6932_proof_4_May_2015_3_8.png)
their direct mechanisms are not well understood. It is speculated that Kv7.4 modulates the resting potential, and therefore excitation of sensory cells through removal of intracellular K\(^+\) at the basal pole of OHCs (Kubisch et al., 1999). This theory is supported by the basal pole location of Kv7.4 on OHCs (Kharkovets et al., 2000). Salicylate blocks Kv7.4, reduces K\(^+\) conductance, and subsequently depolarizes guinea-pig OHCs (Wu et al., 2010). This depolarization could reduce the net driving force for K\(^+\) influx, and consequently reduce electromotility of OHCs (Wu et al., 2010), resulting in increased thresholds. BK channel function in the mammalian

Fig. 2. SS significantly reduces peripheral hearing. (A) CAP thresholds (smallest detectable response above 4 \(\mu\)V) 3 h after saline and SS. CAP thresholds are significantly increased in the SS group across the frequency spectrum. (B) CAP amplitudes at a low- mid- and high-frequency. All amplitudes were significantly decreased in the SS group. (C) DPOAEs at a low- mid- and high-frequency. All DPOAEs were significantly decreased in the SS group. Circles represent saline group, X's represent SS group, and dotted lines in DPOAEs (C) represent noise floors. (**p < 0.01, *p < 0.05).

Fig. 3. Maxipost or RTG alone have no impact on CAPs or DPOAEs. (A) CAP amplitudes for DMSO vehicle, Maxipost, and RTG alone groups acquired at 80 dB SPL (6, 8, 12, 16, 20, 24, 30, 35, 40 kHz). (B) DPOAEs obtained with L1 = 70 dB SPL (f2 = 5.3, 6, 12, 16, 20, 24, 30, 35 kHz). (ns = not significant).
cochlea is unclear but they may play a role in modulating neurotransmitter release (Skinner et al., 2003). This theory is supported by the observation that specific BK channel blockers, charybdotoxin (ChTX) and iberiotoxin (IbTX), significantly reduce sound-evoked cochlear CAPs in guinea pigs (Skinner et al., 2003). Salicylate blocks the IHC BK sensitive K^+ current I_{K,f} (Kimitsuki et al., 2011), which plays a role in cellular high frequency transduction by accelerating membrane repolarization (Kros and Crawford, 1990).

This suggests that salicylate-induced high-frequency hearing loss is due to blocking BK channels, causing impaired IHC repolarization and therefore reduced transmitter release.

4.2. High frequency Maxipost protection at BK channels

Our results suggest that Maxipost protection from SS results from an opening action on BK channels in the basal region of the cochlea.

![Graph A](image1.png)

**Fig. 4.** RTG but not Maxipost significantly reduces SS-enhanced thresholds. (A) Illustrates CAP threshold shift (DMSO thresholds = 0) for Maxipost + SS and SS. Maxipost did not significantly reduce SS enhanced thresholds. (B) Illustrates CAP threshold shift (DMSO thresholds = 0) for RTG + SS and SS. RTG + SS thresholds were significantly reduced in the low frequencies (2, 4, 6, 8 kHz) when compared to the SS group (one-way ANOVA, 2 kHz: p < 0.01, 4 kHz: p < 0.05, 6 kHz: p < 0.05, 8 kHz: p < 0.01) and were not significantly different from vehicle CAP thresholds (two-way ANOVA, p > 0.05, 2 kHz, 6 kHz, and 8 kHz).

![Graph B](image2.png)

**Fig. 5.** Maxipost protects against SS-induced high-frequency amplitude reductions in CAPs, but not DPOAEs. (A) CAP amplitudes of Maxipost + SS and SS at 6 kHz. Maxipost + SS significantly protected against SS reduced CAP amplitudes in high frequencies (two-way ANOVA, *** p < 0.001, ns = not significant). (B) DPOAE amplitudes from Maxipost + SS and SS at 8 kHz. There was no significant difference in DPOAEs (two-way ANOVA, p > 0.05, ns = not significant). Dashed lines represent saline vehicle and dotted lines represent DMSO vehicle for reference.
cochlea. BK channels are likely the primary contributor since they are blocked by salicylate (Kimitsu et al., 2011), opened by Maxipost, but not RTG (Jensen, 2002; Korsgaard et al., 2005; Tatulian et al., 2001), and are primarily expressed in the high frequency region of the cochlea (Kimitsu et al., 2003; Wersinger et al., 2010). Previous studies demonstrate BKα−/− mice develop a progressive high-frequency hearing loss (Ruttiiger et al., 2004). SS-induced BK channel blockage could impose a similar hearing loss and be reversed by treatment with a BK channel opener, such as Maxipost.

Fig. 7A speculative the antagonistic influences that SS and Maxipost have on BK channels in the basal region of cochlea and illustrates mean CAP waveforms obtained from saline, SS alone, and Maxipost + SS to a high frequency stimuli (35 kHz).

4.3. Low frequency Retigabine protection at Kv7.4 channels

Our results suggest that RTG protection from SS results from an opening action on Kv7.4 channels in the apical region of the cochlea. Both Maxipost and RTG open Kv7.4 channels (Schroder et al., 2001); however, RTG has a higher affinity for Kv7.4 than Maxipost (Jensen, 2002; Korsgaard et al., 2005; Schroder et al., 2001). Salicylate blocks OHC Kv7.4 currents (Wu et al., 2010), which are primarily expressed on OHCs in the apical region of the cochlea (Beisel et al., 2000). Previous studies indicate that Kv7.4−/− mice develop hearing loss with elevated ABR thresholds and reduced DPOAEs (Kharkovets et al., 2006), suggestive of OHC dysfunction. Therefore, SS-induced Kv7.4 blockage could impose a similar hearing loss and be reversed by treatment with a Kv7.4 channel opener, such as RTG. In contradiction, in the present study, RTG failed to reverse SS-reduced DPOAEs, suggesting that RTG protection was not mediated at the level of OHCs. Further studies using more sensitive measures such as whole-cell patch clamping are needed for further clarification. One of these shall be the antagonistic influence that SS and RTG have on Kv7.4 channels in the apical region of the cochlea and demonstrate mean CAP waveforms obtained from saline, SS alone, and RTG + SS to a low frequency stimuli (6 kHz).

4.4. Tinnitus and potassium channel openers

While chronic tinnitus is generated, at least partially, within the CNS (House and Brackmann, 1981), the peripheral auditory system plays a role in precipitating tinnitus onset (Bartels et al., 2007; Salvi et al., 2000). Therefore, considering the results of the current study, Maxipost or RTG might prevent tinnitus generation by reducing peripheral damage. RTG would suppress low-frequency tinnitus, while Maxipost, in contrast, would suppress high frequency tinnitus. Since SS is believed to induce a high pitched tinnitus (Jastreboff and Sasaki, 1994; Lobainas et al., 2004; Yang et al., 2007), these results explain why Maxipost was effective at preventing SS-induced tinnitus (Lobarinas et al., 2011).

Maxipost and RTG also influence the CNS (Brenner et al., 2005; Gribkoff et al., 2001a, b; Li et al., 2013). Maxipost has cortical
neuroprotective properties (Gribkoff et al., 2001a, b; Jensen, 2002) and regulates neurotransmitters important for excitation (Jensen, 2002). RTG suppresses noise-induced spontaneous hyperactivity in high frequency fusiform cells of the dorsal cochlear nucleus in vitro (Li et al., 2013). Therefore, more detailed research is needed to characterize the effects that Maxipost and RTG have on central auditory structures involved with tinnitus perception.

5. Summary

Consistent with previous studies, our results indicate that CAP amplitudes are suppressed and thresholds increased 3 h after a high dose of SS (Chen et al., 2010; Stolzberg et al., 2011; Sun et al., 2009). Recent studies suggest that this observation results in part by SS blockage of BK and Kv7.4 K+ channels (Kimitsuki et al., 2011; Wu et al., 2010). Combined treatment of Maxipost or RTG with SS partially or completely reversed SS-reduced CAP amplitudes and thresholds. Maxipost protection was observed in the basal high frequency region (>20 kHz), and RTG protection was observed in the apical low frequency region (<8 kHz). These results are likely due to (1) the tonotopic dependent expression of K+ channels in the cochlea, and (2) the antagonistic influence of SS (blocking K+ channels) and Maxipost or RTG (opening K+ channels).

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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