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Inhibition of Aminoglycoside Acetyltransferase Resistance Enzymes by Metal Salts

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Aminoglycosides (AGs) are clinically relevant antibiotics used to treat infections caused by both Gram-negative and Gram-positive bacteria, as well as Mycobacteria. As with all current antibacterial agents, resistance to AGs is an increasing problem. The most common mechanism of resistance to AGs is the presence of AG-modifying enzymes (AMEs) in bacterial cells, with AG acetyltransferases (AACs) being the most prevalent. Recently, it was discovered that Zn$^{2+}$ metal ions displayed an inhibitory effect on the resistance enzyme AAC(6$\beta$)-Ib in Acinetobacter baumannii and Escherichia coli. In this study, we explore a wide array of metal salts (Mg$^{2+}$, Cr$^{3+}$, Cr$^{6+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, and Au$^{+}$ with different counter ions) and their inhibitory effect on a large repertoire of AACs [AAC(2$\beta$)-Ic, AAC(3)-Ia, AAC(3)-Ib, AAC(3)-IV, AAC(6$\beta$)-Ib', AAC(6$\beta$)-Ie, AAC(6$\beta$)-IId, and Eis]. In addition, we determine the MIC values for amikacin and tobramycin in combination with a zinc pyrithione complex in clinical isolates of various bacterial strains (two strains of A. baumannii, three of Enterobacter cloacae, and four of Klebsiella pneumoniae) and one representative of each species purchased from the American Type Culture Collection.

Aminoglycosides (AGs) are broad-spectrum bactericidal anti-biotics that are used clinically for the treatment of serious bacterial infections (1, 2). These antibiotics were originally isolated from Streptomyces and Micromonospora (3) and display activity against Gram-positive bacteria, aerobic Gram-negative pathogenic bacteria, and Mycobacteria. Since the discovery of streptomycin, the first-in-class AG isolated, many AGs have been discovered and developed with improved efficacy. This property has kept them clinically relevant despite their inherent ototoxicity and nephrotoxicity. Currently, amikacin (AMK), gentamicin (GEN), and tobramycin (TOB) are the most commonly prescribed AGs for systemic administration in the United States against bacterial infections (4). Another AG, kanamycin A (KAN), is also used systemically but only to treat resistant strains of Mycobacterium tuberculosis in patients who show no response to first-line antituberculosis treatments. Other AGs, such as neomycin B (NEO), are found in antibiotic ointment formulations for topical use (5).

The increased importance and popularity of AGs have, unfortunately, led to lapses in antimicrobial stewardship. This has accelerated the development of resistance against AGs and reduced the effective agents available for combating ever-evolving pathogens. The most common mechanism of bacterial resistance to AGs is the acquisition of AG-modifying enzymes (AMEs) (6, 7). Based on the reactions that they catalyze, AMEs can be classified as AG N-acetyltransferases (AACs), AG O-phosphotransferases (APHs), or AG O-nucleotidytransferases (ANTs), among which AACs are responsible for the majority of resistant infections. AACs modify AG substrates and disrupt their binding to the ribosome by transferring the acetyl group from acetyl coenzyme A (AcCoA) onto amine moieties of the AG scaffolds. Although most AACs are regiospecific and modify only a single amino group, the unique enhanced intracellular survival (Eis) protein upregulated in resistant M. tuberculosis strains is capable of acetylated AG substrates at several different positions (8–10).

In an effort to overcome bacterial resistance, a large amount of time and money has been invested toward the development of inhibitors of these resistance enzymes. Most commonly, small organoglycic molecules have been explored for this purpose. For example, in silico and high-throughput screening was used to identify small organic molecules capable of inhibiting AAC(6$\beta$)-Ib (11) and Eis (12), respectively. Traditional synthesis was utilized to generate a library of 45 noncarbohydrate molecules containing a 1,3-di-amine scaffold commonly found in AGs, which were found to be competitive inhibitors of APH(3$\beta$)-IIIa (13). Bisubstrate inhibitors comprised of an AG (GEN or neamine) linked to an AcCoA-like molecule were also found to inhibit various AAC(3) and AAC(6$\beta$) resistance enzymes (14, 15).

Interestingly, Zn$^{2+}$ metal ions were recently reported to inhibit AAC(6$\beta$)-Ib and reduce AMK resistance in Acinetobacter baumannii and Escherichia coli (16). Inspired by this study, we aimed to decipher whether metal ions could be used to inhibit a larger repertoire of AACs in a variety of bacterial strains. Here, we explore the possibility of using Zn$^{2+}$ and other ions (Mg$^{2+}$, Cr$^{3+}$, Cr$^{6+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, and Au$^{+}$ in different salt forms) to inhibit various AACs, including AAC(2$\beta$)-Ic (from M. tuberculosis) (17, 18), AAC(3)-Ia (from Serratia marcescens) (19), AAC(3)-Ib from the bifunctional AAC(3)-Ib/AAC(6$\beta$)-Ib' (from Pseudomonas aeruginosa) (20, 21), AAC(3)-IV (from E. coli) (22), AAC(6$\beta$)-Ib' from the bifunctional AAC(3)-Ib/AAC(6$\beta$)-Ib' (from P. aeruginosa) (20, 21), AAC(6$\beta$)-Ie from the bifunctional enzyme AAC(6$\beta$)-Ie/APH(2$\beta$)-Ia (from Staphylococcus aureus) (23–25), AAC(6$\beta$)-IId from the bifunctional ANT(3$\beta$)-Ib/TM resistance enzymes.


**TABLE 1** Resistance profiles of clinical isolates against the three AGs used for treatment of systemic infections in the United States as well as other clinically relevant antibiotics.

<table>
<thead>
<tr>
<th>Species and isolate designation</th>
<th>MIC (µg/ml) of drug&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AMK</td>
</tr>
<tr>
<td><strong>A. baumannii</strong></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>&gt;128</td>
</tr>
<tr>
<td>81</td>
<td>4</td>
</tr>
<tr>
<td><strong>E. cloacae</strong></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td>52</td>
<td>1–2</td>
</tr>
<tr>
<td>61</td>
<td>4–8</td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td></td>
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<tr>
<td>22</td>
<td>32</td>
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<td>24</td>
<td>16</td>
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<tr>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>44</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviations: AZA, aztreonam; CFPM, cefepime; CFX, cefoxitin; CFZ, ceftazidime; CEFX, ceftriaxone; CIP, ciprofloxacin; LEV, levofloxacin; MER, meropenem; NTF, nitrofurantoin; TET, tetracycline.
determined by calculating the initial reaction rates using the first 2 to 5 min of the reaction (Fig. 1; also see Fig. S3 and Table S1 in the supplemental material). NEO and TOB are not substrates of AAC(3)-Ia and AAC(3)-Ib. In addition, NET is not a substrate of AAC(3)-Ib. Therefore, these AGs were not tested with the two AAC(3)s listed above. All experiments were performed in triplicate.

TLC. Some metals in this study can have high affinities for free thiols that are detected by the UV-Vis assay as indicative of acetylation of AGs. Therefore, we performed thin-layer chromatography (TLC) experiments to further confirm that metal salts would exert inhibition directly on the conversion from parent to acetylated AGs and not only react with the CoASH that would be generated during this conversion, which would nullify our results. Reaction mixtures (20 μl) contained 50 mM buffer [HEPES (pH 7.5) for AAC(6’)-Ib and AAC(6’)-IId and MES (pH 6.6) for other enzymes, pH adjusted at RT], 0.2 mM AGs, 0.24 mM AcCoA, 1 mM metal salts, and 1 μM enzyme. Reaction mixtures were incubated for 1 h at 37°C (for AAC(6’)-Ie) or 25°C (for all other enzymes). All 20 μl of reaction mixtures was loaded onto TLC plates (Millipore TLC silica gel 60 F254), dried, and eluted with different solvent systems (5:2 ratio of MeOH to NH₄OH [25% in H₂O] for AMK and TOB and 3:2, 25:1, and 15:2 ratios of MeOH to NH₄OH [25% in H₂O] for NEO, NET, and SIS, respectively). AGs were visualized by staining with a cerium molybdate

FIG 1 Inhibition of AAC enzymes by various metal salts with and without physiological concentrations of NaCl. Activities with metal ion inhibitors are presented as a percentage of the activity of each AAC with AG without metal salts. The clinically relevant AGs AMK, GEN, and TOB are presented here. Other AGs, KAN, NEO, NET, and SIS, are presented in Fig. S3 in the supplemental material. The asterisk indicates that as AuCl₃ precipitated in HEPES (pH 7.5) buffer with DTDP in the reaction mixture, it was not tested against AAC(6’)-Ib’ and AAC(6’)-IId and is therefore not presented in this figure. AAC(6’)-Ib’ and AAC(6’)-IId were also not tested with the Cu²⁺ salts in the presence of 100 mM NaCl due to solubility issues.
stain [5 g of (NH₄)₂Ce(NO₃)₆, 120 g of (NH₄)₆Mo₇O₂₄·4H₂O in 10% H₂SO₄]. A summary of all the metal-AG-enzyme combinations tested and the results is presented in Fig. 2.

Determination of potential inhibition mechanism using EDTA. To investigate whether the inhibition of various AACs by different metal ions is reversible and establish whether AAC enzyme activity could be restored by sequestering the metal ions from the reactions, we performed assays with EDTA, which served as a strong metal ion scavenger (see Fig. S4 in the supplemental material). Reaction mixtures (200 μl) contained metal salt (1 mM), AG (100 μM), AcCoA (150 μM), DTNB (2 mM), EDTA (2 mM, pH 8.0), and AAC(3)-Ib (0.5 μM) or AAC(3)-IV (0.125 μM). Reactions were carried out in 50 mM MES, pH 6.6. The AAC(3)-Ib enzyme was tested with SIS and the metal salts Cu(OAc)₂, ZnCl₂, and AuCl₃. AAC(3)-IV was tested with TOB and the salts Cu(OAc)₂, ZnCl₂, CdSO₄, and AuCl₃. After the addition of the enzyme, the absorbance of each reaction was recorded every 30 s for 20 min (see Fig. S4). All reactions were performed in triplicate. Three control reactions were performed in which (i) EDTA (which represented the inhibited reactions), (ii) EDTA and metal salts (which represented the standard N-acetylation reactions of AGs by AACs to which all other reactions were compared in this study), and (iii) EDTA and AGs (which showed that the metal salts did not interfere with the assay conditions).

Inhibition of AACs by metal salts at near-physiological salt concentration. To establish if the metal inhibition of AACs is unaffected by the salt concentration found physiologically, experiments were performed in the presence of 100 mM NaCl. Experiments with Cu²⁺ and Zn²⁺ were performed as described above for each enzyme, except for Eis, as its activity is inhibited in the presence of salt and therefore was not tested with NaCl. AAC(6')-Ib and AAC(6')-IId were not tested with the Cu²⁺ salts in the presence of NaCl due to solubility issues. Results of these control experiments are presented in Fig. 1 (see also Fig. S3 in the supplemental material).

Determination of IC₅₀s by UV-Vis assays. For selected metal salts that showed complete inhibition of the AAC activities against certain AGs,
we determined the 50% inhibitory concentrations (IC50s) in combination with the corresponding AGs. Assays were performed in 96-well plates with DTNB or DTDP as indicators for specific enzymes as described above. The metal salts were dissolved in appropriate buffers (100 mM HEPES, pH 8.0) for each enzyme, and a 5-fold dilution was performed, making the final concentration of metal salts range from 2.6 pM to 1 mM in the assay. AG and enzyme in reaction mixture containing no metal salt inhibitors. IC50s were then calculated using a Hill plot fit with Kaleidagraph 4.1. Experiments were performed in duplicate or triplicate. The data with standard errors are summarized in Table 2, and a sample graph for the IC50 of AuCl3 against Eis with NET is presented in Fig. S5 in the supplemental material.

**Determination of MIC values for combinations of metals and AGs.** MIC values were determined using the double-microdilution method. Various concentrations of AGs were combined with different concentrations of ZnPT (0 to 80 μM), and bacteria were grown for 16 h prior to MIC value determination. The AG concentrations used varied with each bacterial strain as follows: AMK at 0.06 to 32 μg/ml with A. baumannii ATCC 19606, 0.25 to 128 μg/ml with isolate 75, and 0.015 to 8 μg/ml with isolate 81; 0.063 to 16 μg/ml with E. cloacae ATCC 13047, 0.015 to 8 μg/ml with isolate 41, 0.004 to 2 μg/ml with isolate 52, and 0.015 to 8 μg/ml with isolate 61; 0.031 to 16 μg/ml with K. pneumoniae ATCC 27736, 0.125 to 64 μg/ml with isolate 22, 0.031 to 16 μg/ml with isolate 24, 0.004 to 2 μg/ml with isolate 34, and 0.031 to 16 μg/ml with isolate 44; TOB at 0.031 to 16 μg/ml with A. baumannii ATCC 19606, 0.015 to 8 μg/ml with isolate 75, and 0.004 to 2 μg/ml with isolate 81; 0.008 to 4 μg/ml with E. cloacae ATCC 13047, 0.015 to 8 μg/ml with isolate 41, 0.015 to 8 μg/ml with isolate 52, and 0.015 to 8 μg/ml with isolate 61; 0.031 to 16 μg/ml with K. pneumoniae ATCC 27736, 0.25 to 128 μg/ml with isolate 22, 0.25 to 128 μg/ml with isolate 24, 0.006 to 0.3 μg/ml with isolate 34, and 0.25 to 128 μg/ml with isolate 44. For combinational studies, the concentration of AGs was varied horizontally on the plate, while the metal concentration was varied vertically on the plate. Observed bactericidal concentrations were then compared to that of AG or ZnPT (Tables 3 and 4). For the strains that showed observable synergistic effect between ZnPT and AGs (A. baumannii ATCC 19606 and K. pneumoniae ATCC 27736, as presented in the isobolograms of Fig. 3), we further performed determination of MIC values of AGs or ZnPT alone in the presence of 25 mg/ml of bovine serum albumin (BSA; data not shown), as well as AGs in combination with ZnPT in the presence of BSA (see Tables S2 and S3 in the supplemental material).

**RESULTS**

**In vitro inhibition of AACs by metal salts.** To determine the inhibitory effect of different metal salts on the N-acetylation activity of AACs with various AGs, we first performed UV-Vis assays in 96-well plates (Fig. 1; see also Fig. S3 and Table S1 in the supplemental material). Among the metal ions (Mg2+, Cr3+, Cr6+, Mn2+, Co2+, Ni2+, Cu2+, Zn2+, Cd2+, and Au3+) selected, Cu2+ salts [Cu(OAc)2, Cu(NO3)2, and CuSO4] showed the most inhibitory activity against AACs in general. The presence of Zn2+ ions also resulted in generally lower AAC activity, with the exception of AAC(2')-Ic, which was not well inhibited by Zn2+ or Cd2+. Eis also seemed to remain uninhibited by Zn2+ salts when tested with AMK and by ZnSO4 when tested with other AGs. Ac-
explored whether different anions affected the inhibitory ability of -acetylation was not inhibited. We also noted that the reactions with metal salts that showed no inhibition in UV-Vis assays (Mg2+ and Ca2+) were determined for CdCl2 in the presence of NEO and CdSO4 in the presence of GEN and SIS where 3-N-acetylation was not inhibited. We also found that the presence of EDTA can restore enzyme activity against AG substrates, potentially by chelating the metal ions and preventing them from interfering with the enzymatic reaction between the AACs and AGs (see Fig. S4 in the supplemental material). With the exception of AAC(3)-IV in the presence of 100 mM NaCl (Fig. 1; see also Fig. S3 in the supplemental material). We observed no significant difference between the enzyme activities with and without physiological concentrations of NaCl in the presence of different metal ions. AAC(6')-Ie, as well as AAC(6')-Ib' and AAC(6')-Id (with Zn2+ salts), showed complete inhibition when tested against AMK and KAN in the presence of NaCl, indicating that under physiological conditions these metal salts could be even better inhibitors. It is important to note that AAC(6')-Ib' and AAC(6')-Id were not tested with Cu2+ salts due to solubility issues.

We next determined the half-maximum inhibition concentration (IC50) values for selected salts that showed complete inhibition in our UV-Vis assays (Table 2; see also Fig. S5 in the supplemental material). With the exception of AAC(3)-IV in the presence of NEO where the IC50 for ZnCl2, Zn(OAc)2, and ZnBr2 were 44 ± 19 μM, 34 ± 8 μM, and 17 ± 5 μM, respectively, Zn2+ salts were found to be lower IC50 ranging from 0.04 ± 0.01 μM to 7.8 ± 1.7 μM against all enzymes. For the inhibition of AAC(3)-IV by Cd2+ salts, IC50 of 7.3 ± 2.7 μM and 8.7 ± 1.7 μM were determined for CdCl2 in the presence of NEO and CdSO4 in the presence of NET, respectively. Cd2+ salts were found to be much better inhibitors of AAC(6')-Ib' and Eis with IC50 ranging from 0.41 ± 0.03 μM to 0.79 ± 0.10 μM. AuCl3 was found to display the widest range of IC50 (0.010 ± 0.003 μM to 121 ± 26 μM).

Finally, to elucidate the potential mechanism by which these metal ions inhibit the AAC activities, we tested whether the effect that metal ions exert on AAC activity is reversible. We added EDTA to the reaction mixtures to chelate the metal inhibitors and found that the presence of EDTA can restore enzyme activity against AG substrates, potentially by chelating the metal ions and preventing them from interfering with the enzymatic reaction between the AACs and AGs (see Fig. S4 in the supplemental material).

Activity of AGs in combination with ZnPT in various bacterial strains. Since Zn2+ was overall a great metal ion inhibitor in our study, we decided to further investigate Zn2+ for its effect on the MIC values of AMK or TOB against strains of A. baumannii, E. cloacae, and K. pneumoniae. After discovering that ZnCl2 had no effect on the MIC values of AMK and TOB against A. baumannii,
possibility due to poor intracellular Zn\(^{2+}\) concentration, we decided to use ZnPT, an organic zinc complex, which was previously reported to have improved membrane permeability (16). This study showed that the presence of ZnPT significantly reduced the MIC values of AMK and TOB in most of the 12 bacterial strains that we tested (Tables 3 and 4).

Against A. baumannii ATCC 19606 as well as clinical isolate 81, the MIC value of AMK was reduced from 4 \(\mu\)g/ml to 0.25 \(\mu\)g/ml in the presence of 20 \(\mu\)M ZnPT (Table 3). With the same amount of ZnPT, the MIC value of AMK was reduced from >128 \(\mu\)g/ml to 0.25 \(\mu\)g/ml against A. baumannii clinical isolate 75. The same trend held true against the E. cloacae and K. pneumoniae strains. Against E. cloacae clinical isolate 41, the MIC value of AMK was reduced from 8 \(\mu\)g/ml to 1 \(\mu\)g/ml in the presence of 10 \(\mu\)M ZnPT. Against the other strains (E. cloacae ATCC 13047, isolate 52, and isolate 61), the AMK MIC values were reduced from 2 \(\mu\)g/ml to 0.5 \(\mu\)g/ml, from >2 \(\mu\)g/ml to 1 \(\mu\)g/ml, and from >8 \(\mu\)g/ml to 4 \(\mu\)g/ml in the presence of 5 to 10 \(\mu\)M ZnPT, respectively. Against K. pneumoniae strains, 4- to 32-fold reductions in the MIC values of AMK were observed in the presence of 5 to 20 \(\mu\)M ZnPT.

The reduction in MIC values in the presence of ZnPT was also observed for TOB against most strains tested (Table 4). Against the different A. baumannii strains, the MIC values of TOB were reduced from 1 \(\mu\)g/ml to ≤0.0313 \(\mu\)g/ml (ATCC 19606), and from 2 \(\mu\)g/ml to 1 \(\mu\)g/ml (clinical isolate 81) in the presence of 10 \(\mu\)M and 1.25 \(\mu\)M ZnPT, respectively. Yet, against clinical isolate 75, the MIC value of TOB went from >8 \(\mu\)g/ml to no growth in the control wells, suggesting that ZnPT had no effect on the MIC of TOB in this strain. This was similarly observed against E. cloacae ATCC 13047 and clinical isolates 41 and 61. Against the E. cloacae clinical isolate 52, the MIC value of TOB was reduced from 8 \(\mu\)g/ml to 4 \(\mu\)g/ml with 5 \(\mu\)M ZnPT. Against K. pneumoniae ATCC 27736 and clinical isolates 22, 24, and 44, 10 \(\mu\)M ZnPT effectively reduced the MIC values of TOB from 2 \(\mu\)g/ml to ≤0.0313 \(\mu\)g/ml, from >128 \(\mu\)g/ml to 8 \(\mu\)g/ml, from 128 \(\mu\)g/ml to 4 \(\mu\)g/ml, and from >128 \(\mu\)g/ml to 8 \(\mu\)g/ml, respectively. Against K. pneumoniae clinical isolate 34, the MIC value of TOB was reduced from 0.038 \(\mu\)g/ml to 0.0094 \(\mu\)g/ml in the presence of 5 \(\mu\)M ZnPT. With the two strains that showed synergy between AGs and ZnPT (A. baumannii ATCC 19606 and K. pneumoniae ATCC 27736 [Fig. 3]), we supplemented the medium with 25 mg/ml BSA and found that the presence of BSA did not change the MIC of AMK, TOB, and ZnPT alone (data not shown). When testing the MIC of AG in combination with ZnPT with supplemented BSA, we found that ZnPT still decreased the MIC of AGs in K. pneumoniae ATCC 27736 from 16 \(\mu\)g/ml to 0.25 \(\mu\)g/ml for AMK (see Table S2 in the supplemental material) and 8 \(\mu\)g/ml to 0.06 \(\mu\)g/ml for TOB. However, the same effect was not observed in A. baumannii ATCC 19606 (see Table S3).

**DISCUSSION**

To investigate their inhibitory effect on eight AACs, we tested a panel of nine metal ions (in different salt forms) and studied their interference in modifying seven AGs (AMK, GEN, KAN, NEO, NET, SIS, and TOB) (Fig. 1; see also Fig. S3 and Table S1 in the supplemental material). By UV-Vis assays, we demonstrated that Cu\(^{2+}\) was overall the best at inhibiting the different AACs studied. We found Zn\(^{2+}\) and Cd\(^{2+}\), which also showed significant inhibition of most enzymes, to be the second-best candidates as AAC inhibitors. Inhibition of AAC activity by Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Ni\(^{2+}\) with AAC(6\(^{\prime}\))s only, and Au\(^{3+}\) was also confirmed by TLC studies (Fig. 2). Some negative controls were also performed during the TLC experiments.

Although metal salts were found to be good inhibitors of AACs, the inherent toxicity of some of these metals can render them less desirable regardless of their high inhibitory effect. Despite Zn\(^{2+}\) salts falling short in their inhibitory ability compared to Cu\(^{2+}\) salts, Zn\(^{2+}\) salts remain more desirable for their potential development into clinical AAC inhibitors as they are among the least toxic metal salts based on 50% lethal dose (LD\(_{50}\)) values (e.g., 794 mg/kg of body weight in rats by oral intake for Zn(OAc)$_2$ com-
pared to 501 mg/kg for Cu(OAc)₂ and 1,710 mg/kg in rats by oral intake for ZnSO₄ compared to 561 mg/kg for NiSO₄, 482 mg/kg for CuSO₄, and 280 mg/kg for CdSO₄. Even though Zn²⁺ and Cd²⁺ showed similar inhibitory activities in vitro and displayed similar ranges of IC₅₀ (0.04 ± 0.01 μM to 44 ± 19 μM for Zn²⁺ salts compared to 0.41 ± 0.03 μM to 8.7 ± 1.7 μM for Cd²⁺ salts [Table 2]), Zn²⁺ salts remain more clinically relevant, as Cd²⁺ salts display higher toxicity (LD₅₀ values of 107 mg/kg and 280 mg/kg for CdCl₂ and CdSO₄, respectively, in rats by oral intake).

It is known that Cl⁻ salts are inherently more toxic than some other salt forms. Within the Zn²⁺ salt family, the toxicity of ZnCl₂ and Zn(OAc)₂ [LD₅₀ values of 359 mg/kg, 794 mg/kg, 1,447 mg/kg, and 1,710 mg/kg for ZnCl₂, Zn(OAc)₂, ZnBr₂, and ZnSO₄, respectively, in rats by oral intake] and the failure of the less toxic ZnSO₄ at inhibiting some AACs render ZnBr₂ the most desirable among the Zn²⁺ salts studied. Within the Cu²⁺ salts, NO₃⁻ seems to be a safer counter ion than SO₄²⁻ and OAc⁻ [LD₅₀ values of 482 mg/kg, 501 mg/kg, and 940 mg/kg for CuSO₄, Cu(OAc)₂, and Cu(NO₃)₂, respectively]. As for gold salts, even though gold is generally known to be toxic and expensive, it is currently part of therapies for diseases such as rheumatoid arthritis (31). Given that no other metal ions worked as well as gold at inhibiting the AAC(2’)-Ic enzyme, gold should not be dismissed as a potential candidate to combat the resistance associated with this enzyme.

Having established Zn²⁺ salts as top candidates for AAC inhibition, we set to determine their effect on the activity of AGs in various bacterial strains of A. baumannii, E. cloacae, and K. pneumoniae. Prior to working in bacterial cells, we demonstrated that the presence of NaCl, at a concentration mimicking that under physiological conditions, did not interfere with the inhibitory effect of the metal ion salts on AACs (Fig. 1; see also Fig. S3 in the supplemental material). We also showed that in the presence of EDTA, the inhibitory effect of the metal ions on the AACs could be reversed as EDTA, a strong metal-chelating agent, sequestered the metal ions from the enzymatic reactions (see Fig. S4).

Within each bacterial species selected for our studies with Zn²⁺ salts, we included one commercially purchased ATCC strain as well as a few clinical isolates. We chose some clinical isolates displaying susceptibility to the three commonly prescribed AGs (AMK, GEN, and TOB) and some displaying resistance to these drugs (Table 1). Overall, all clinical isolates selected displayed a complex resistance profile to a variety of clinically relevant antibiotics, including different classes and generations of β-lactams, fluoroquinolones, tetracycline, etc.

When we tried to determine the MIC values of AMK and TOB against A. baumannii in the presence of ZnCl₂, we quickly realized that the Zn²⁺ salts studied had no effect on the MIC values of these AGs. We postulated that this could possibly be due to poor intracellular Zn²⁺ concentration resulting from the poor membrane permeation of these salts. We therefore decided to use ZnPT, a membrane-permeant organic zinc complex. We found ZnPT to significantly reduce the MIC values for both AGs in most strains tested. Of the A. baumannii strains, ATCC 19606 and clinical isolate 81 are both susceptible to AMK. Cotreatment with 40 μM ZnPT reduced the MIC value of AMK by 67-fold against the ATCC 19606 strain, whereas using 20 μM ZnPT reduced the MIC value of AMK by 16-fold against clinical isolate 81. The most significant effect was observed against clinical isolate 75, which was resistant to AMK (>128 μg/ml) and became highly sensitive to this AG (≤0.25 μg/ml) in the presence of 20 μM ZnPT. Against E. cloacae and K. pneumoniae strains, which were all sensitive or displayed intermediate levels of resistance to AMK to start with, the MIC values of AMK showed as much as a 32-fold reduction with 20 μM ZnPT. The resistance profile to TOB is different from that to AMK in the strains that we studied. Most of the K. pneumoniae strains (isolates 22, 24, and 44) are resistant to TOB while ATCC 27736 and clinical isolate 34, as well as all A. baumannii and E. cloacae strains, are susceptible to this AG. Although not as significant as those observed in AMK, the reductions in the MIC values of TOB were still observed against most of these strains. We looked for potential synergistic effects between AGs and ZnPT and observed strong synergy between AMK or TOB and ZnPT in A. baumannii ATCC 19606 and K. pneumoniae ATCC 27736 as suggested by the isobolograms presented in Fig. 3. Since AGs are given intravenously when used to treat systemic infections, we further supplemented the medium with BSA in our MIC studies in order to see whether the ZnPT can still enhance AG actions in the two strains above in a more complex environment that mimics the blood. With this more complex system, ZnPT still significantly reduced the MIC of AMK and TOB in K. pneumoniae ATCC 27736. However, this effect was not retained in A. baumannii ATCC 19606 (see Tables S2 and S3 in the supplemental material).

In sum, we showed that Zn²⁺ and other metal ions are capable of inhibiting the AG N-acetyltransferase activity of a variety of AACs in vitro. Developing metal salts into AAC inhibitors for combinatorial therapies with AGs is potentially a promising avenue for improving the clinical outcomes of AGs in treatment of resistant bacterial infections resulting from the action of AAC enzymes. The mechanism(s) by which these metal ions inhibit AACs is still unknown. Future studies, outside the scope of this article, aimed at elucidating the potential mechanism(s) underlying this inhibition would further enhance our understanding and help combat bacterial resistance in clinical therapeutic regimens.

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