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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′)-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^{3+} with different counter ions) and their inhibitory effect on a large repertoire of AACs [AAC(2′)-Ic, AAC(3)-Ia, AAC(3)-Ib, AAC(3)-IV, AAC(6′)-Ib′, AAC(6′)-Ie, AAC(6′)-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 antibiotics 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 oto- 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 (see Fig. S1 in the supplemental material) (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*-nucleotidyltransferases (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 acetylating 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 organic 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′)-Ib (11) and Eis (12), respectively. Traditional synthesis was utilized to generate a library of 45 noncarbohydrate molecules containing a 1,3-diamine scaffold commonly found in AGs, which were found to be competitive inhibitors of APH(3′)-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′) resistance enzymes (14, 15).

Interestingly, Zn^{2+} metal ions were recently reported to inhibit AAC(6′)-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^{3+} in different salt forms) to inhibit various AACs, including AAC(2′)-Ic (from *M. tuberculosis*) (17, 18), AAC(3)-Ia (from *Serratia marcescens*) (19), AAC(3)-Ib from the bifunctional AAC(3)-Ib/AAC(6′)-Ib′ (from *Pseudomonas aeruginosa*) (20, 21), AAC(3)-IV (from *E. coli*) (22), AAC(6′)-Ib′ from the bifunctional AAC(3)-Ib/AAC(6′)-Ib′ (from *P. aeruginosa*) (20, 21), AAC(6′)-Ie from the bifunctional enzyme AAC(6′)-Ie/APH(2″)-Ia (from *Staphylococcus aureus*) (23–25), AAC(6′)-IId from the bifunctional ANT(3″)-Ii/

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

Species and isolate designation	MIC (µg/ml) of drug[a]:												
	AMK	GEN	TOB	AZA	CFPM	CFX	CFZ	CEFX	CIP	LEV	MER	NTF	TET
A. baumannii													
75	>128	>8	>8	>16	>16	>16	>16	>32	0.2	>4	>8	>64	>8
81	4	8	≤2	>16	>16	>16	>16	>32	>2	>4	>8	>64	>8
E. cloacae													
41	8	>8	>8	>16	>16	>16	>16	>32	1	≤1	>8	64	>8
52	1–2	8	>8	>16	16	>16	8	>32	>2	>4	>8	32	8
61	4–8	>8	8	>16	≤1	>16	>16	16	>2	>4	≤1	>64	8
K. pneumoniae													
22	32	>8	>8	>16	>16	>16	>16	>32	>2	>4	≤1	>64	>8
24	16	8	>8	>16	16	>16	>16	>32	>2	>4	2	>64	4
34	1	≤2	≤2	≤2	≤1	≤4	2	>32	≤0.5	≤1	≤1	64	≤2
44	16	>8	>8	>16	≤1	>16	>16	>32	<2	>4	>8	>64	>8

[a] Abbreviations: AZA, aztreonam; CFPM, cefepime; CFX, cefoxitin; CFZ, ceftazidime; CEFX, ceftriaxone; CIP, ciprofloxacin; LEV, levofloxacin; MER, meropenem; NTF, nitrofurantoin; TET, tetracycline.

AAC(6′)-IId (from *S. marcescens*) (26, 27), and Eis (from *M. tuberculosis*) (8). We also present MIC data for AMK and TOB in combination with a zinc complex, zinc pyrithione (ZnPT).

MATERIALS AND METHODS

Materials and instrumentation. With the exception of AAC(3)-Ia, for which the cloning, overexpression, and purification are described below, all of the enzymes involved in this study, AAC(2′)-Ic (8), AAC(3)-Ib (28), AAC(3)-IV (29), AAC(6′)-Ie/APH(2″)-Ia (29), AAC(6′)-Ib′ (28), AAC(6′)-IId (30), and Eis (8), were expressed and purified as previously described. Here, AAC(6′)-Ie/APH(2″)-Ia was studied solely for its AAC(6′) activity and is referred to as AAC(6′)-Ie throughout the manuscript. The *S. marcescens* genomic DNA used to clone AAC(3)-Ia was a gift from Paul H. Roy (Université Laval, Québec, Canada). Primers for PCR were purchased from IDT (Coralville, IA). Ladders for DNA agarose gels as well as cloning enzymes, including Phusion DNA polymerase, restriction endonucleases, and T4 DNA ligase, were purchased from New England BioLabs (NEB; Ipswich, MA). The pET28a vector was purchased from Novagen (Gibbstown, NJ). TOP10 and BL21(DE3) chemically competent *E. coli* cells were purchased from Invitrogen (Carlsbad, CA). The Ni[II]-nitrilotriacetic acid (NTA) resin used for affinity chromatography was purchased from Qiagen (Valencia, CA). The AcCoA; 4,4′-dithiodipyridine (DTDP); 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB); all metal salts [$MgCl_2$, $CrCl_3$, CrO_3, $MnSO_4$, $CoCl_2$, $NiCl_2$, $NiSO_4$, $Cu(OAc)_2$, $Cu(NO_3)_2$, $CuSO_4$, $Zn(OAc)_2$, $ZnCl_2$, $ZnBr_2$, $ZnSO_4$, $CdCl_2$, $CdSO_4$, and $AuCl_3$]; and the AGs AMK, GEN, and sisomicin (SIS) were purchased from Sigma-Aldrich (Milwaukee, WI). The AGs KAN, NEO, netilmicin (NET), and TOB were purchased from AK Scientific (Mountain View, CA) (see Fig. S1 in the supplemental material). Ninety-six-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). UV-visible (UV-Vis) assays were monitored on a multimode SpectraMax M5 plate reader.

Previously identified bacterial strains were obtained from the American Type Culture Collection (ATCC), including *A. baumannii* ATCC 19606, *Enterobacter cloacae* ATCC 13047, and *Klebsiella pneumoniae* ATCC 27736. Clinical isolates of *A. baumannii* (isolates 75 and 81), *E. cloacae* (isolates 41, 52, and 61), and *K. pneumoniae* (isolates 22, 24, 34, and 44) were obtained from the microbiology laboratory at the University of Kentucky Hospital System. Known resistance profiles of the clinical isolates are presented in Table 1.

Cloning, overexpression, and purification of AAC(3)-Ia (NHis). The *aac(3)-Ia* gene was PCR amplified from *S. marcescens* genomic DNA by using forward primer 5′-CGATAC<u>CATATG</u>TTACGCAGCAGCAACGA TG-3′ and reverse primer 5′-TTGAAC<u>CTCGAG</u>TTAGGTGGCGGTAC-3′. The amplified fragment was digested by using the NdeI and XhoI restriction enzymes (cut sites are underlined in the primer sequences) and ligated into the linearized pET28a vector. The ligated pAAC(3)-Ia-pET28a plasmid was transformed into *E. coli* TOP10 chemically competent cells for plasmid replication and then into *E. coli* BL21(DE3) chemically competent cells for protein overexpression. After confirmation of its DNA sequence, a fresh transformant of the pAAC(3)-Ia-pET28a plasmid was grown in LB broth (KAN, 50 µg/ml) (3 liters) at 37°C with shaking at 200 rpm until the optical density at an attenuance of 600 nm reached 0.6. The protein expression was induced with 1 mM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside). After induction, bacteria were kept growing overnight at 20°C. The desired AAC(3)-Ia protein was then purified using Ni[II]-NTA affinity column chromatography in 25 mM Tris-HCl (pH 8.0) (adjusted at room temperature [RT]), 200 mM NaCl, and 10% glycerol with a gradient of imidazole (10 ml of 5 mM imidazole and 3 times with 5 ml each of 20 mM, 40 mM, and 200 mM imidazole). Pure fractions, as determined by SDS-PAGE (see Fig. S2 in the supplemental material), were dialyzed in Tris-HCl (50 mM, pH 8.0), NaCl (300 mM), and glycerol (10%); flash-frozen in liquid nitrogen; and stored at −80°C after concentrating using an Amicon (10,000-molecular-weight-cutoff [MWCO]) ultracentrifugal filter cellulose protein concentrator. Protein purification yielded 0.5 mg of AAC(3)-Ia per liter of culture.

Determination of inhibition of AAC enzymes by various metal salts. To determine the inhibition of various AACs by different metal salts, we performed UV-Vis assays in 96-well plates. Each reaction mixture (200 µl) contained metal salts (1 mM), AGs (100 µM), AcCoA (500 µM for Eis and 150 µM for all other AACs), indicator (DTNB or DTDP; 2 mM), and enzyme [0.5 µM for AAC(6′) enzymes, AAC(3)-Ib, AAC(3)-Ia, and Eis or 0.125 µM for AAC(2′)-Ic and AAC(3)-IV] in the appropriate buffer [100 mM K_2HPO_4 for AAC(2′)-Ic; 50 mM Tris-HCl (pH 8.0) for Eis; 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.6) for AAC(3)-Ia, AAC(3)-Ib, AAC(3)-IV, and AAC(6′)-Ie; and 50 mM HEPES (pH 7.5) for AAC(6′)-Ib′ and AAC(6′)-IId]. The pH of all buffers was adjusted at RT. Reactions were carried out at 25°C for all enzymes except for AAC(6′)-Ie, where reactions were performed at 37°C. DTNB was used to monitor reaction progress with all enzymes, except with AAC(6′)-Ib′ and AAC(6′)-IId, where DTDP was used. Reaction mixtures containing DTDP were monitored at 324 nm ($\varepsilon = 19,800$ M^{-1} cm^{-1}), and those containing DTNB were monitored at 412 nm ($\varepsilon = 14,150$ M^{-1} cm^{-1}). Absorbance was recorded every 30 s for 20 min. Enzyme activities were
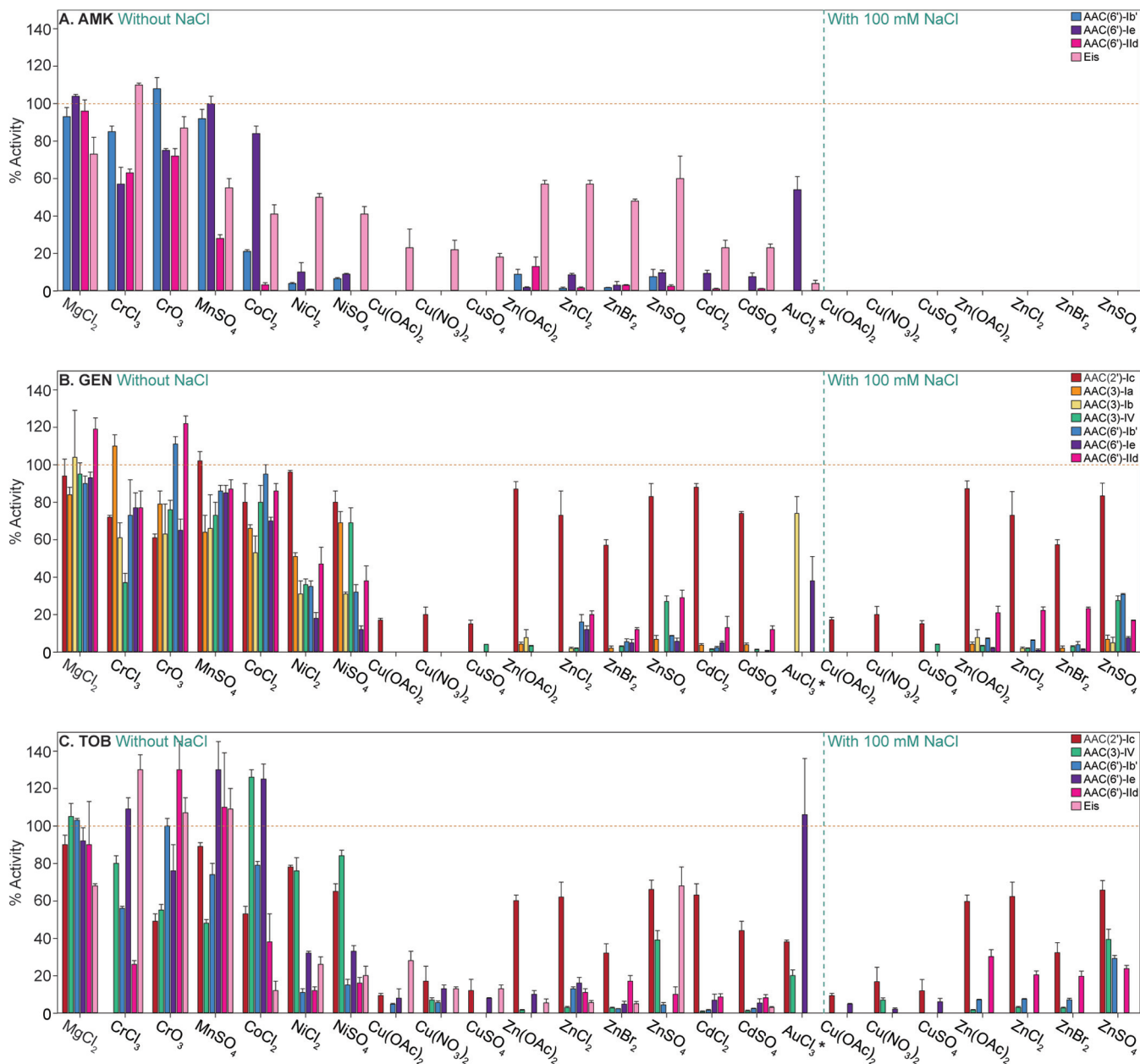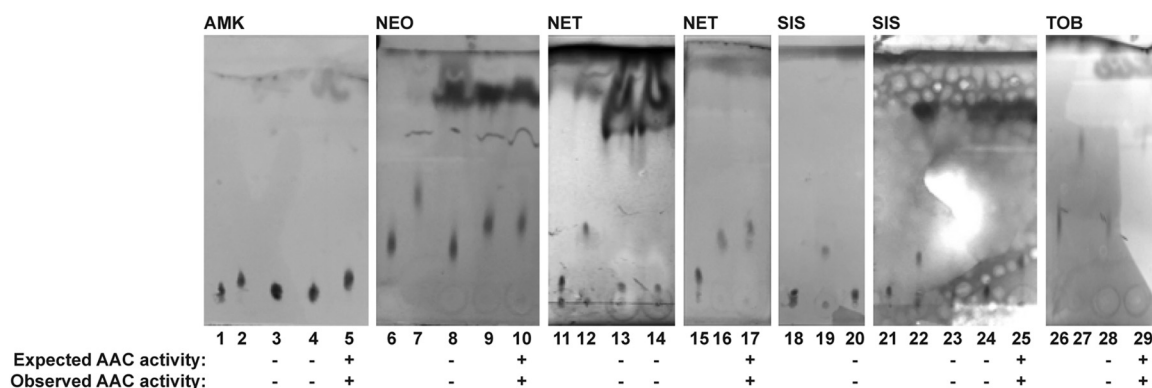
FIG 1 Inhibition of AAC enzymes by various metal salts with and without physiological concentrations of NaCl. Activities with metal ion inhibitors are presented as a percentage of the activity of each AAC with AG without metal salts. The clinically relevant AGs AMK, GEN, and TOB are presented here. Other AGs, KAN, NEO, NET, and SIS, are presented in Fig. S3 in the supplemental material. The asterisk indicates that as AuCl$_3$ 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 Cu^{2+} salts in the presence of 100 mM NaCl due to solubility issues.

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 F$_{254}$), dried, and eluted with different solvent systems (5:2 ratio of MeOH to NH$_4$OH [~25% in H$_2$O] for AMK and TOB and 3:2, 25:1, and 15:2 ratios of MeOH to NH$_4$OH [~25% in H$_2$O] for NEO, NET, and SIS, respectively). AGs were visualized by staining with a cerium molybdate

FIG 2 TLCs monitoring the acetylation of various AGs (AMK, NEO, NET, SIS, and TOB) by different AACs in the presence of metal salts. Note that details about AG, AAC, metal salt, and R_f values are provided for each line on the TLCs. The expected and observed enzyme activities are also provided.

The TLC lanes and activity rows:

	1 2	3	4	5	6 7	8	9	10	11 12	13	14	15 16	17	18 19	20	21 22	23	24	25	26 27	28	29
Expected AAC activity:		-	-	+		-		+		-	-		+		-			-	-	+		-	+
Observed AAC activity:		-	-	+		-		+		-	-		+		-			-	-	+		-	+

AG	TLC line #	AAC	Metal salt	R_f	Expected enzyme activity[a]	Observed enzyme activity[a]
AMK	1	--	--	0.04		
	2	AAC(6')-Ie	--	0.09		
	3	AAC(6')-Ib	CdSO$_4$	0.04	-	-
	4	AAC(6')-Ie	CuSO$_4$	0.04	-	-
	5	AAC(6')-IId	MgCl$_2$	0.09	+	+
NEO	6	--	--	0.22		
	7	AAC(6')-Ie	--	0.42		
	8	AAC(6')-IId	Cu(NO$_3$)$_2$	0.22	-	-
	9	AAC(3)-IV	--	0.31		
	10	AAC(3)-IV	MnSO$_4$	0.31	+	+
NET	11	--	--	0.08		
	12	AAC(6')-Ie	--	0.30		
	13	AAC(6')-Ie	ZnSO$_4$	0.08	-	-
	14	AAC(6')-Ie	NiSO$_4$	0.08	-	-
	15	--	--	0.08		
	16	AAC(3)-Ia	--	0.24		
	17	AAC(3)-Ia	CrCl$_3$	0.25	+	+
SIS	18	--	--	0.04		
	19	AAC(6')-Ie	--	0.21		
	20	AAC(6')-Ie	ZnBr$_2$	0.04	-	-
	21	--	--	0.04		
	22	AAC(3)-Ib	--	0.19		
	23	AAC(3)-Ia	AuCl$_3$	0.04	-	-
	24	AAC(3)-Ib	ZnCl$_2$	0.04	-	-
	25	AAC(3)-Ib	CrO$_3$	0.19	+	+
TOB	26	--	--	0.30		
	27	AAC(6')-Ie	--	0.60		
	28	AAC(6')-Ib'	Zn(OAc)$_2$	0.30	-	-
	29	AAC(6')-Ib'	CoCl$_2$	0.60	+	+

[a] + indicates that the metal salt is not inhibiting the reaction tested and the enzyme should be active. - indicates that the metal salt is inhibiting the reaction tested and the enzyme should be inactive.

stain [5 g of (NH$_4$)$_2$Ce(NO$_3$)$_6$, 120 g of (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O in 10% H$_2$SO$_4$]. 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 μM, 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)$_2$, ZnCl$_2$, and AuCl$_3$. AAC(3)-IV was tested with TOB and the salts Cu(OAc)$_2$, ZnCl$_2$, CdSO$_4$, and AuCl$_3$. 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

the following components were lacking: (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 Cu^{2+} and Zn^{2+} 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 Cu^{2+} 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 IC$_{50}$s by UV-Vis assays. For selected metal salts that showed complete inhibition of the AAC activities against certain AGs,

TABLE 2 IC$_{50}$s for the various metal ions that have shown complete inhibition in UV-Vis assays against various AACs in combination with different AGs

Enzyme	AG	Metal ion	IC$_{50}$ (μM)
AAC(2')-Ic[a]	GEN	AuCl$_3$	12 ± 3
AAC(3)-Ia[a]	GEN	ZnCl$_2$	1.4 ± 0.3
AAC(3)-Ib[a]	SIS	ZnCl$_2$	0.13 ± 0.02
		ZnSO$_4$	1.4 ± 0.2
AAC(3)-IV[a]	NEO	ZnCl$_2$	44 ± 19
		Zn(OAc)$_2$	34 ± 8
		ZnBr$_2$	17 ± 5
		CdCl$_2$	7.3 ± 2.7
	NET	CdSO$_4$	8.7 ± 1.7
	TOB	CuSO$_4$	0.35 ± 0.14
AAC(6')-Ib'[b]	AMK	CdSO$_4$	0.41 ± 0.08
		AuCl$_3$	0.41 ± 0.07
	SIS	Zn(OAc)$_2$	7.8 ± 1.7
AAC(6')-Ie[a]	SIS	ZnBr$_2$	0.41 ± 0.08
	TOB	ZnSO$_4$	5.2 ± 1.3
Eis[a]	KAN	AuCl$_3$	7.7 ± 2.3
	NET	AuCl$_3$	18 ± 1
	SIS	CdCl$_2$	0.78 ± 0.09
		CdSO$_4$	0.79 ± 0.10
		AuCl$_3$	121 ± 26
	TOB	Zn(OAc)$_2$	0.04 ± 0.01
		CdCl$_2$	0.41 ± 0.03
		AuCl$_3$	0.010 ± 0.003

[a] Reactions were monitored by recording the absorbance at 412 nm with DTNB.
[b] Reactions were monitored by recording the absorbance at 324 nm with DTDP.

we determined the 50% inhibitory concentrations (IC$_{50}$s) 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 μl) for each enzyme, and a 5-fold dilution was performed, making the final concentration of the metal salts range from 2.6 pM to 1 mM in the assay. AG and enzyme in buffer (50 μl) were then added into each well and incubated for 10 min to allow potential positioning or binding of the substrate and/or inhibitor into the enzyme active site. Reactions were then initiated by the addition of AcCoA and indicator (50 μl). Reactions with AAC(6')-Ie were performed at 37°C. All other enzymatic reactions were performed at 25°C. Absorbance at 412 nm (for DTNB) or 324 nm (for DTDP) was recorded every 30 s for 20 min. Initial rates of reaction were calculated using the first 10 min of the reaction and expressed as a percentage of the rate of the reaction mixture containing no metal salt inhibitors. IC$_{50}$s were then calculated using a Hill plot fit with Kaleidagraph 4.1. Experiments were per-

formed in duplicate or triplicate. The data with standard errors are summarized in Table 2, and a sample graph for the IC$_{50}$ of AuCl$_3$ 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, 0.0006 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*-acetylating 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 (Mg^{2+}, Cr^{3+}, Cr^{6+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Au^{3+}) selected, Cu^{2+} salts [Cu(OAc)$_2$, Cu(NO$_3$)$_2$, and CuSO$_4$] showed the most inhibitory activity against AACs in general. The presence of Zn^{2+} and Cd^{2+} ions also resulted in generally lower AAC activity, with the exception of AAC(2')-Ic, which was not well inhibited by Zn^{2+} or Cd^{2+}. Eis also seemed to remain uninhibited by Zn^{2+} salts when tested with AMK and by ZnSO$_4$ when tested with other AGs. Ac-

TABLE 3 MICs of AMK (μg/ml) in the presence of various concentrations of ZnPT against various Gram-negative bacterial strains

ZnPT concn (μM)	MIC of AMK (μg/ml) against strain:											
	A. baumannii			*E. cloacae*				*K. pneumoniae*				
	ATCC 19606	Isolate 75	Isolate 81	ATCC 13047	Isolate 41	Isolate 52	Isolate 61	ATCC 27736	Isolate 22	Isolate 24	Isolate 34	Isolate 44
80	≤0.06	NG[a]	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
40	≤0.06	NG	NG	NG	NG	NG	NG	0.5	NG	NG	NG	NG
20	0.25	≤0.25	0.25	NG	NG	NG	4	0.5	NG	4	NG	NG
10	0.5	>128	1	0.5	1	NG	4	2	8	4	NG	NG
5	2	>128	2	2	8	1	4	4	8	4	0.25	1
2.5	2	>128	1	2	8	>2	8	8	32	8	0.5	16
1.25	2	>128	1	2	8	>2	>8	8	32	16	0.5	16
0	4	>128	4	2	8	>2	>8	16	32	16	2	>16

[a] NG, no growth.

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TABLE 4 MICs of TOB (µg/ml) in the presence of various concentrations of ZnPT against various Gram-negative bacterial strains

| | MIC of TOB (µg/ml) against strain: | | | | | | | | | | | |
| | *A. baumannii* | | | *E. cloacae* | | | | *K. pneumoniae* | | | | |
ZnPT concn (µM)	ATCC 19606	Isolate 75	Isolate 81	ATCC 13047	Isolate 41	Isolate 52	Isolate 61	ATCC 27736	Isolate 22	Isolate 24	Isolate 34	Isolate 44
80	NG[a]	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
40	≤0.0313	NG	NG	NG	NG	NG	8	≤0.0313	NG	NG	NG	NG
20	≤0.0313	NG	NG	NG	>8	NG	8	≤0.0313	NG	NG	NG	NG
10	≤0.0313	NG	NG	1	>8	4	>8	≤0.0313	8	4	NG	8
5	0.5	>8	1	1	>8	4	>8	0.5	128	128	0.0094	128
2.5	0.5	>8	1	1	>8	8	>8	0.5	>128	128	0.038	128
1.25	1	>8	1	1	>8	8	>8	0.5	>128	128	0.075	128
0	1	>8	2	1	>8	8	>8	2	>128	128	0.038	>128

[a] NG, no growth.

tually, AAC(2′)-Ic was, in general, effectively inhibited only by Cu^{2+} and Au^{3+}. Interestingly, we noted that in general Ni^{2+} ($NiCl_2$ and $NiSO_4$) exerted moderate inhibition against the AAC(6′) enzymes but not AAC(6′)-Ie (when tested with KAN or NEO) or other AACs. On the other hand, Au^{3+} inhibited all AACs tested, with the exceptions of AAC(6′)-Ie where only 6′-*N*-acetylation of KAN was inhibited and AAC(3)-Ib in the presence of GEN and SIS where 3-*N*-acetylation was not inhibited. We also explored whether different anions affected the inhibitory ability of the same metal cation, yet with the Ni^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} ions that we tested in different salt forms, we did not observe a noticeable difference in the enzyme activities among the different anions. Additionally, with few exceptions, we did not observe a difference in the metals' inhibitory effect among various AGs across all enzymes studied. In addition, Eis showed the most reduction in activity in response to Au^{3+}, which made Au^{3+} more effective as an inhibitor for Eis than Cd^{2+} and some Zn^{2+} salts. It was also noticeable that $ZnSO_4$ did not inhibit Eis activity as much as the other Zn^{2+} salts did when tested with other AGs. This trend was also observed with AAC(3)-IV, suggesting that the SO_4^{2-} ion may deteriorate the inhibitory effect of Zn^{2+}. For the different AAC(3) enzymes, Zn^{2+} and Cd^{2+} showed the second-best inhibitory effect after Cu^{2+}, even though the AAC(3)-IV activity was higher when tested with $ZnSO_4$ than when tested with other Zn^{2+} salts. Au^{3+} was also found to exert decent inhibition of the AAC(3) enzymes with the exception of AAC(3)-Ib when tested with GEN and SIS.

Some metals involved in this study may have high affinity for free thiols and might potentially bind to the released CoASH faster than the indicator (DTNB) to form a metal-thiol complex, giving false-positive results for inhibition in the UV-Vis assays. Thus, we performed TLC experiments to confirm that the inhibition observed in UV-Vis assays was real inhibition of the acetyltransferase activity. We selected one metal-AG-enzyme combination as a representative for each metal salt. For the metals that we tested with different counter ions, we tested all different salt forms for Zn^{2+} salts and SO_4^{2-} salts for other metals [as well as $Cu(NO_3)_2$]. As expected, we found that the reactions with metal salts that showed good inhibition in UV-Vis assays [Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} with AAC(6′)s only, and Au^{3+}] showed no generation of acetylated AGs, whereas the reactions with other metals that showed no inhibition in UV-Vis assays (Mg^{2+}, Mn^{2+}, Cr^{3+}, Cr^{6+}, and Co^{2+}) also suggested no inhibition of the AAC activities (Fig. 2).

To investigate if the metal salts would react similarly under physiological conditions, we tested our best overall inhibitor candidates, Cu^{2+} and Zn^{2+} salts, 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′)-IId (with Zn^{2+} 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′)-IId were not tested with Cu^{2+} salts due to solubility issues.

We next determined the half-maximum inhibitory concentration (IC_{50}) 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 IC_{50}s for $ZnCl_2$, $Zn(OAc)_2$, and $ZnBr_2$ were 44 ± 19 µM, 34 ± 8 µM, and 17 ± 5 µM, respectively, Zn^{2+} salts were found to display low IC_{50}s ranging from 0.04 ± 0.01 µM to 7.8 ± 1.7 µM against all enzymes. For the inhibition of AAC(3)-IV by Cd^{2+} salts, IC_{50}s of 7.3 ± 2.7 µM and 8.7 ± 1.7 µM were determined for $CdCl_2$ in the presence of NEO and $CdSO_4$ in the presence of NET, respectively. Cd^{2+} salts were found to be much better inhibitors of AAC(6′)-Ib′ and Eis with IC_{50}s ranging from 0.41 ± 0.03 µM to 0.79 ± 0.10 µM. $AuCl_3$ was found to display the widest range of IC_{50}s (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 Zn^{2+} was overall a great metal ion inhibitor in our study, we decided to further investigate Zn^{2+} for its effect on the MIC values of AMK or TOB against strains of *A. baumannii*, *E. cloacae*, and *K. pneumoniae*. After discovering that $ZnCl_2$ had no effect on the MIC values of AMK and TOB against *A. baumannii*,
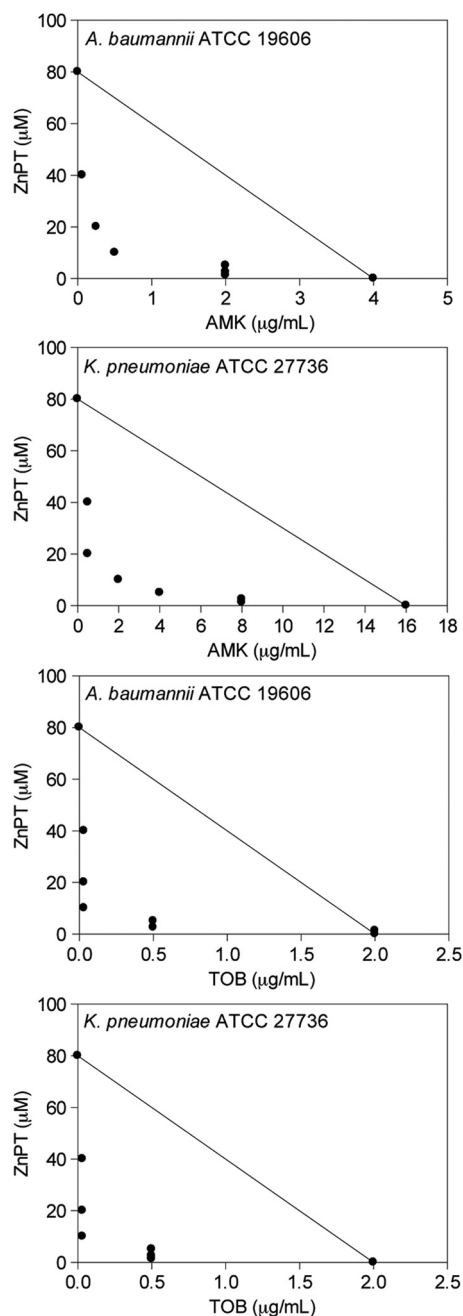
FIG 3 Isobolograms showing the synergistic effect between ZnPT and AMK or TOB in *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 27736.

possibly 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 μg/ml to 0.25 μg/ml in the presence of 20 μM ZnPT (Table 3). With the same amount of ZnPT, the MIC value of AMK was reduced from >128 μg/ml to ≤0.25 μg/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 μg/ml to 1 μg/ml in the presence of 10 μM ZnPT. Against the other strains (*E. cloacae* ATCC 13047, isolate 52, and isolate 61), the AMK MIC values were reduced from 2 μg/ml to 0.5 μg/ml, from >2 μg/ml to 1 μg/ml, and from >8 μg/ml to 4 μg/ml in the presence of 5 to 10 μM 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 μM 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 μg/ml to ≤0.0313 μg/ml (ATCC 19606), and from 2 μg/ml to 1 μg/ml (clinical isolate 81) in the presence of 10 μM and 1.25 μM ZnPT, respectively. Yet, against clinical isolate 75, the MIC value of TOB went from >8 μg/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 μg/ml to 4 μg/ml with 5 μM ZnPT. Against *K. pneumoniae* ATCC 27736 and clinical isolates 22, 24, and 44, 10 μM ZnPT effectively reduced the MIC values of TOB from 2 μg/ml to ≤0.0313 μg/ml, from >128 μg/ml to 8 μg/ml, from 128 μg/ml to 4 μg/ml, and from >128 μg/ml to 8 μg/ml, respectively. Against *K. pneumoniae* clinical isolate 34, the MIC value of TOB was reduced from 0.038 μg/ml to 0.0094 μg/ml in the presence of 5 μM 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 μg/ml to 0.25 μg/ml for AMK (see Table S2 in the supplemental material) and 8 μg/ml to 0.06 μg/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+} is 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′)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 $ZnSO_4$ compared to 361 mg/kg for $NiSO_4$, 482 mg/kg for $CuSO_4$, and 280 mg/kg for $CdSO_4$]. Even though Zn^{2+} and Cd^{2+} showed similar inhibitory activities *in vitro* and displayed similar ranges of IC_{50}s (0.04 ± 0.01 μM to 44 ± 19 μM for Zn^{2+} salts compared to 0.41 ± 0.03 μM to 8.7 ± 1.7 μM for Cd^{2+} salts [Table 2]), Zn^{2+} salts remain more clinically relevant, as Cd^{2+} salts display higher toxicity (LD_{50} values of 107 mg/kg and 280 mg/kg for $CdCl_2$ and $CdSO_4$, respectively, in rats by oral intake).

It is known that Cl^- salts are inherently more toxic than some other salt forms. Within the Zn^{2+} salt family, the toxicity of $ZnCl_2$ and $Zn(OAc)_2$ [LD_{50} values of 359 mg/kg, 794 mg/kg, 1,447 mg/kg, and 1,710 mg/kg for $ZnCl_2$, $Zn(OAc)_2$, $ZnBr_2$, and $ZnSO_4$, respectively, in rats by oral intake] and the failure of the less toxic $ZnSO_4$ at inhibiting some AACs render $ZnBr_2$ the most desirable among the Zn^{2+} salts studied. Within the Cu^{2+} salts, NO_3^- seems to be a safer counter ion than SO_4^{2-} and OAc^- [LD_{50} values of 482 mg/kg, 501 mg/kg, and 940 mg/kg for $CuSO_4$, $Cu(OAc)_2$, and $Cu(NO_3)_2$, 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 Zn^{2+} 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 Zn^{2+} 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 $ZnCl_2$, we quickly realized that the Zn^{2+} salts studied had no effect on the MIC values of these AGs. We postulated that this could possibly be due to poor intracellular Zn^{2+} 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 Zn^{2+} 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.

ACKNOWLEDGMENTS

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