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In Vitro Apramycin Activity Against Multidrug-Resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

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ABSTRACT

The *in vitro* activity of apramycin was compared to amikacin, gentamicin, and tobramycin against multidrug-resistant, extensively drug-resistant, and pandrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Apramycin demonstrated an MIC_{50}/MIC_{90} of 8/32 μg/ml for *A. baumannii* and 16/32 μg/ml for *P. aeruginosa*. Only 2% of *A. baumannii* and *P. aeruginosa* had an MIC greater than an epidemiological cutoff value of 64 μg/ml. In contrast, the MIC_{50}/MIC_{90} for amikacin, gentamicin, and tobramycin were ≥64/>256 μg/ml for *A. baumannii* with 57%, 95%, and 74% of isolates demonstrating resistance, respectively, and the MIC_{50}/MIC_{90} were ≥8/256 μg/ml for *P. aeruginosa* with 27%, 50%, and 57% of strains demonstrating resistance, respectively. Apramycin appears to offer promising *in vitro* activity against highly resistant pathogens. It therefore may warrant further pre-clinical study to assess potential for repurposing as a human therapeutic and relevance as a scaffold for further medicinal chemistry exploration.

Keywords: apramycin; aminoglycoside; activity spectrum; *Acinetobacter*; *Pseudomonas aeruginosa*; repurposing

Abbreviations: Multidrug-resistant (MDR); extensively drug-resistant (XDR); pandrug-resistant (PDR); CLSI (Clinical and Laboratory Standards Institute); carbapenem-resistant *Enterobacteriaceae* (CRE)

INTRODUCTION

Acinetobacter baumannii and *Pseudomonas aeruginosa* are two prominent members of the ESKAPE pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) for which emerging multidrug-resistance is of pressing concern (1, 2). In addition to causing severe disease in hospitalized patients, *A. baumannii* and *P. aeruginosa* are also the most frequently isolated pathogens from combat-related injuries (1, 3-5). Unfortunately, treatment options for these pathogens are increasingly limited, and aminoglycosides in particular have become among the drugs of last resort (6, 7). However, clinically-approved aminoglycosides have a narrow therapeutic index due to nephrotoxic and irreversible ototoxic side effects (8). Moreover, many *Acinetobacter* and *Pseudomonas* isolates are now also resistant to these aminoglycosides (6, 9).

Apramycin is a veterinary aminocyclitol aminoglycoside used to treat colibacillosis, salmonellosis and enteritis in farm animals (10, 11). Its structure, a bicyclic sugar moiety with a mono-substituted deoxystreptamine, is distinct from other aminoglycosides (12, 13). This distinct structure may help account for two of its unique attributes. First, most aminoglycoside modifying enzymes that confer resistance to clinically-approved aminoglycosides do not inactivate apramycin (13-17). Second, apramycin appears to offer higher selectivity for bacterial over mitochrondrial ribosomes and, therefore, is presumably associated with fewer ototoxic and nephrotoxic side effects (10, 18-20). Therefore, based on these favorable characteristics, apramycin or apramycin analogues developed through future medicinal chemistry efforts may be worthy of consideration for repurposing as a human therapeutic. However, demonstration of a compelling activity spectrum against multidrug-resistant human clinical isolates is a prerequisite to justify further translational efforts.

Previous data from our lab and others have shown broad-spectrum apramycin activity against carbapenem-susceptible and -resistant *Enterobacteriaceae* (CRE) strains from the US and the UK (10, 21, 22). However, there is sparse to no available data for contemporary human multidrug-resistant *A. baumannii* and *P. aeruginosa* isolates. Therefore, here we sought to investigate the *in vitro* activity spectrum of apramycin as compared to aminoglycosides approved for human clinical use in the United States. Testing was performed against a diverse strain set of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) *A. baumannii* and *P. aeruginosa* clinical isolates, inclusive of strains isolated from US combat-related infections.

MATERIALS AND METHODS

Bacterial strains and antimicrobials.

Amikacin disulfate salt was from Sigma-Aldrich (St. Louis, MO); apramycin sulfate and gentamicin sulfate were from Alfa Aesar (Tewksbury, MA); and tobramycin sulfate was from Research Products International (Mt. Prospect, IL).

Forty-four *P. aeruginosa* and fifty-four *A. baumannii* strains were obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank (https://www.cdc.gov/drugresistance/resistance-bank/). Fifty additional *A. baumannii* strains, , confirmed to be clonally-distinct based on whole genome sequencing and representing a diversity of multilocus sequence types were obtained from the Multidrug Resistant Organism Repository and Surveillance Network (MRSN) at the Walter Reed Army Institute of Research (WRAIR). These strains were isolated predominantly from wounded soldiers and were from two

hospitals: the Walter Reed Army Medical Center (WRAMC) in Washington, DC, and the Walter Reed National Military Medical Center (WRNMMC), Bethesda, MD.

Based on antimicrobial susceptibility provided by the FDA-CDC and WRAIR for respective isolates, 82% of the *P. aeruginosa* isolate collection and 89% of the *A. baumannii* isolate collection were non-susceptible to meropenem and/or imipenem. Among the *P. aeruginosa* isolate collection, 29.5%, 34.1%, and 2.3% of the isolates were MDR, XDR, and PDR strains, respectively, per definitions of an international expert panel consensus (23). One-third of the *P. aeruginosa* XDR isolates were only susceptible to polymyxin B or colistin and otherwise PDR. Among the *A. baumannii* isolates, 3.8%, 41.3%, and 6.7% were MDR, XDR, and PDR, respectively. An additional 48.1% were, based on more limited testing information, at least MDR with a mean resistance to 4.5 of 5 drug classes tested (aminoglycosides, imipenem, anti-pseudomonal cephalosporins, tetracycline, and fluoroquinolones). Among *A. baumannii* XDR isolates, 9% were only susceptible to polymyxin B or colistin and otherwise PDR.

Aminoglycoside susceptibility testing.

The Clinical Laboratory and Standards Institute (CLSI) broth microdilution reference method (24) was used for MIC testing of aminoglycosides. In brief, MIC panels were created by diluting apramycin, amikacin, gentamicin and tobramycin into round-bottom, 96-well plates (Evergreen Scientific, Los Angeles, CA) at 2× concentration in 50 µl well volumes for final doubling dilution concentrations ranging from 0.125 to 256 µg/ml with the addition of an equal volume of bacterial inoculum. Bacterial inocula were prepared by passaging previously frozen bacterial strains on trypicase soy agar containing 5% sheep blood, culturing for 18-24 hours at 37°C, and suspending isolated colonies to 0.5 McFarland (~$1x10^8$ CFU mL^{-1}) in sterile 0.9%

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NaCl using a DensiChek plus handheld colorimeter (bioMérieux, Durham, NC). This suspension was diluted 1:150 into Mueller-Hinton II Broth (Cation-Adjusted) (BD Diagnostics, Franklin Lakes, NJ) to ~1x10^6 CFU/mL to achieve a final inoculum concentration of ~5x10^5 CFU mL^{-1} in microwells. Per the manufacturer's certificate of analysis (BD Diagnostics, Catalogue Number 212322, Batch Number 5257869), final cation concentrations of Calcium and Magnesium in Mueller-Hinton II Broth (Cation-Adjusted) prepared according to the manufacturer's directions are 20-25 mg/L and 10-12.5 mg/L, respectively. MIC values were determined visually after incubation for 16-20 hours. Results were considered valid if *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) tested in each experiment fell within the CLSI-designated and/or veterinary quality control ranges for all aminoglycosides tested, as was consistently the case (25-27). Categorical interpretations for amikacin, gentamicin, and tobramycin were based on the most recent CLSI interpretive guidelines (25).

RESULTS

The MIC_{50}, MIC_{90}, MIC range and percent susceptibility for tested aminoglycosides are listed in table 1. The strain set was notable for a high degree of resistance to gentamicin tobramycin and amikacin, ranging from 57 to 95% for *A. baumannii* and 27 to 57% for *P. aeruginosa.* Amikacin was the most active of the aminoglycosides approved for human clinical use.

For apramycin, there are no established veterinary or clinical breakpoints for *A. baumanii* or *P. aeruginosa*. Therefore, apramycin percent susceptibility was not similarly calculated. However, for *A. baumannii*, apramycin MIC_{50} and MIC_{90} values were at least 8-fold lower than

for other aminoglycosides. For *P. aeruginosa*, the MIC_{50} for apramycin and other aminoglycosides were similar. However, the MIC_{90} for apramycin was 8-fold lower than for these other aminoglycosides. Importantly, *P. aeruginosa* ATCC 27853 quality control strain results for apramycin were consistently in range for all experiments at 8 µg/ml, comparable to values from a prior multi-center study (results evenly divided at 4 and 8 µg/ml) (26), supporting reliability of apramycin MIC determinations. Quality control results for other aminoglycosides were likewise consistently in range.

Apramycin MIC distributions for *A. baumannii* and *P. aeruginosa* are shown in Fig 1. Based on visual inspection of these distributions (28), an apramycin epidemiological cutoff value of 64 µg/ml was assigned for both species. Impressively, only 2% of *A. baumannii* (n=2) and *P. aeruginosa* (n=1) strains had an MIC above this cutoff. These findings contrasted with the frequent occurrence of strains with an MIC ≥ 256 µg/ml for other aminoglycosides.

DISCUSSION

Apramycin is currently used as an orally-dosed, non-absorbed veterinary antibiotic to treat diarrheal diseases in poultry and livestock (10). It is also used as an injectable treatment for pneumonia in calves (29), and mastitis in cows, goats and sheep (30). Veterinary pharmacodynamic data are unavailable. Human studies appear not to have been performed.

As such, despite some understanding of pharmacokinetics in farm animals (29-31), no apramycin breakpoints exist for *Acinetobacter* spp. and *P. aeruginosa*. However, application of an epidemiological cutoff value of 64 µg/ml suggested at least rarity of apramycin modifying enzymes (16, 32) in this highly aminoglycoside resistant *A. baumannii* and *P. aeruginosa* strain set.

Of note, the modal MIC for MDR *A. baumannii* (8 μg/ml) and *P. aeruginosa* (32 μg/ml) strains examined was greater than the modal MIC for the CRE strain set (4 μg/ml) that we described previously (22), suggesting generally greater potency for CRE. However, overall there was a much larger percentage (30%) of frankly apramycin resistant (MIC > 256 μg/ml) CRE isolates. Furthermore, *in vitro* apramycin MIC distribution for *P. aeruginosa* and *A. baumannii* was comparable to recent reports for plazomicin, a new semi-synthetic aminoglycoside in stage 3 clinical trials (33, 34). This was despite a much more highly aminoglycoside resistant *P. aeruginosa* strain set tested in our study. Of interest, a prior study also indicated that apramycin activity, in contrast to plazomicin, may not be undermined by 16S rRNA methylases examined (10). The relevance of these findings awaits further pharmacokinetic and pharmacodynamic comparisons.

Despite somewhat higher apramycin MIC values in *A. baumannii* and *P. aeruginosa* than in CRE, pharmacodynamic modeling may confirm that targeted dosing could achieve therapeutic effect in a fraction of isolates not effectively treatable with other antimicrobials. This may be especially true if typical aminoglycoside associated toxicities prove not to be limiting for apramycin, based on reported enhanced selectivity for bacterial ribosomes (19). In this case, exposure could be increased opening up possibilities for efficacy against strains with a higher MIC. It is also possible that apramycin could be combined with other agents such as a carbapenem (where a strain is carbapenem susceptible or relatively carbapenem susceptible) to achieve synergistic effects. Therefore, based on broad-spectrum activity against highly multidrug-resistant *A. baumannii, P. aeruginosa* and CRE strains, we suggest that further pre-clinical exploration of apramycin is warranted.

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FIGURE LEGEND

Fig 1. Apramycin MIC distribution for *A. baumannii* and *P. aeruginosa* clinical isolates. Vertical bars designate an epidemiological cutoff value of 64 μg/ml, and highlight rarity of overt apramycin resistance among these species.

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Table 1. Activity spectrum of apramycin and clinically-approved aminoglycosides against *A. baumannii* and *P. aeruginosa* clinical isolates

		MIC (μg/ml)			Susceptibility		
Bacterial species (No. Isolates)	**Antibiotic**	**50%**	**90%**	**Range**	**S**	**I**	**R**
A. baumannii (104)	Apramycin[a]	8	32	2 to 256	-	-	-
	Amikacin	64	>256	0.5 to >256	27%	16%	57%
	Gentamicin	>256	>256	2 to >256	3%	2%	95%
	Tobramycin	128	>256	0.125 to >256	23%	3%	74%
P. aeruginosa (44)	Apramycin[a]	16	32	2 to >256	-	-	-
	Amikacin	8	256	0.5 to >256	61%	11%	27%
	Gentamicin	8	256	0.5 to 256	46%	5%	50%
	Tobramycin	64	256	0.25 to >256	43%	0%	57%

[a]No official veterinary or clinical breakpoints exist for *A. baumannii* and *P. aeruginosa*, and therefore categorical susceptibility percentages were not determined.

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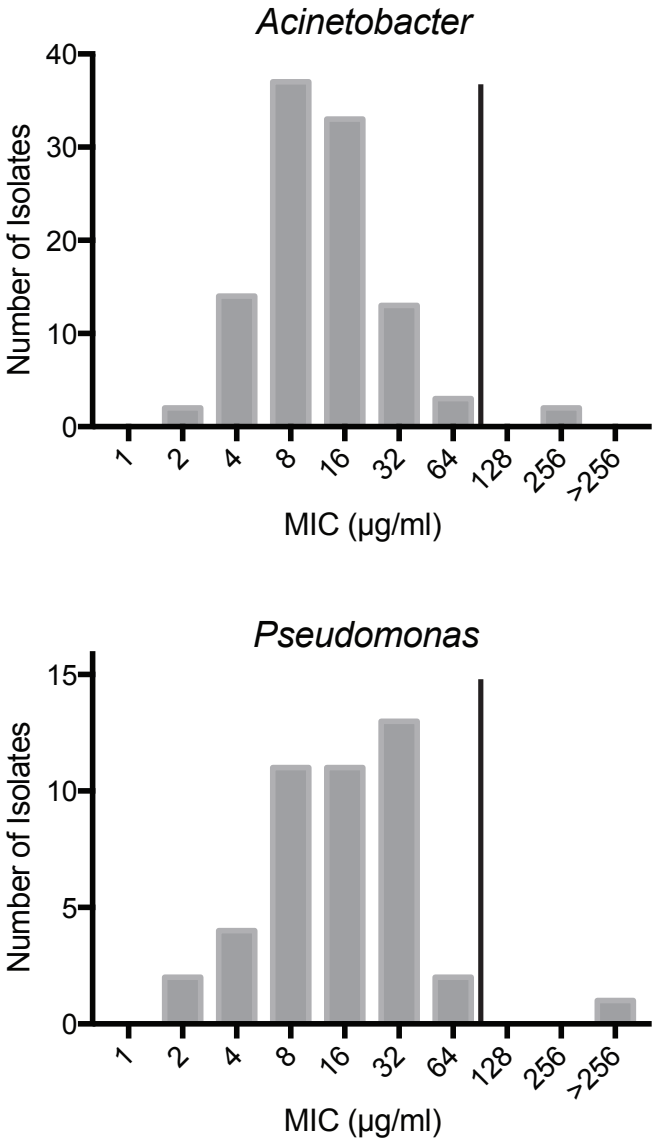

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