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Evaluation of Apramycin Activity Against Methicillin-Resistant, Methicillin-Sensitive, and Vancomycin-Intermediate *Staphylococcus aureus* Clinical Isolates

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Abstract

We evaluated the *in vitro* activity of apramycin against clinical strains of vancomycin-intermediate and methicillin-resistant and -suseptible *Staphylococcus aureus*. Apramycin demonstrated an MIC_{50}/MIC_{90} of 8/16 μg/mL. No strains had an MIC above the epidemiological cutoff value of 32 μg/mL, suggesting apramycin resistance mechanisms are rare in this strain population. The mounting evidence for broad-spectrum *in vitro* activity of apramycin against *S. aureus* and other bacterial species suggests that further exploration of apramycin or derivatives as repurposed human therapeutics is warranted.

Keywords

apramycin; *Staphylococcus aureus*; activity spectrum; repurposing; antibiotic; natural product; synergy; aminoglycoside; gentamicin

1. Introduction

Staphylococcus aureus is both a human skin commensal and an opportunistic pathogen. It is the leading cause of bacteremia and infective endocarditis, as well skin and soft tissue, osteoarticular, and surgical site infections (Akhi, et al., 2017; Deleo, et al., 2010; Tong, et al., 2015). Strains resistant to methicillin and by proxy, all β-lactams (so called methicillin resistant *S. aureus* or MRSA), are common in the United States (Tong, et al., 2015). Vancomycin is the first-line treatment for methicillin-resistant *S. aureus* (MRSA) infection. However, limitations of vancomycin include relatively lower bactericidal activity compared with β-lactams; nephrotoxicity, associated with high dosages and underlying risk factors;

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Disclosure Statement

The HP D300 digital dispenser was provided by Tecan (Morrisville, NC). Tecan had no role in study design, data collection/ interpretation, manuscript preparation, or decision to publish.

and a requirement for routine monitoring of drug levels to ensure adequate dosing (Hazlewood, et al., 2010). Furthermore, vancomycin tolerant and heteroresistant populations may emerge during treatment (Bamberger & Boyd, 2005; Dombrowski & Winston, 2008; Hawkins, et al., 2007; Lodise, et al., 2008; Steinkraus, et al., 2007).

Therefore, additional treatment options for *S. aureus* would be welcome, especially for those strains with resistance to newer agents such as daptomycin and linezolid. minoglycosides are not currently used to treat *S. aureus* infections as single agents. In particular, renal and ototoxic side effects are of significant concern (Jose, et al., 2010; Matt, et al., 2012). Therefore aminoglycoside therapy is generally reserved for treatment of Gram-negative infection, where pharmacodynamic considerations are considered more favorable (Tam, et al., 2006). Currently, gentamicin treamtent of *S. aureus* is advocated only in low dose (1mg/kg q8 h or 3–5mg/kg q 24h) in combination with vancomycin or a β-lactam and rifampin, during therapy of staphylococcal endocarditis where prosthetic material is present and in combination with daptomycin for persistent bactermia (Liu, et al., 2011). However, the relative benefit of gentamicin adjunctive therapy versus risk of kidney damage has been a subject of extensive debate (Bruss, 2009; Buchholtz, et al., 2009; Buchholtz, et al., 2011; Cosgrove, et al., 2009; Frippiat, et al., 2009). Therefore, a non-toxic aminoglycoside with predictable activity against *S. aureus* would therefore be especially welcome.

Apramycin is a structurally unique aminoglycoside used in veterinary medicine and is characterized by a bicyclic sugar moiety and a 4-monosubsituted 2-deoxystreptamine ring (Meyer, et al., 2014). In contrast to aminoglycosides currently used in human therapy, apramycin appears highly selective for bacterial ribosomes and on this basis thought devoid of significant ototoxic and renal toxic side effects (Matt, et al., 2012). Previous data from our group and others have shown broad-spectrum *in vitro* activity of apramycin against carbapenem-resistant Enterobacteriaceae and multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Kang, et al., 2017; Livermore, et al., 2011; K.P. Smith & J.E. Kirby, 2016a, 2016b). Furthermore, we recently demonstrated potent *in vivo* bactericidal activity against *Acinetobacter baumannii* in a murine thigh infection model (Kang, et al., 2018).

However, activity spectrum data for *S. aureus* is limited. Therefore, the goal of this study was to explore *in vitro* activity of apramycin against contemporary clinical isolates of *S. aureus*.

2. Materials and Methods

2.1 Bacterial Strains and Antimicrobials

We evaluated a collection of 109 strains of *S. aureus* for their susceptibility to apramycin. Fourteen *S. aureus* strains obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank (https://www.cdc.gov/drugresistance/resistance-bank/) were part of the vancomycin intermediate *S. aureus* panel with vancomycin MIC values of 4–8 μg/mL. 95 additional strains of de-identified *S. aureus* clinical isolates were obtained from the Beth Israel Deaconess Medical Center (Boston, MA) clinical microbiology laboratory. Our strain collection was comprised of 38.5% methicillin-resistant *S. aureus* (MRSA), 48.6%

methicillin-suseptible *S. aureus* (MSSA) and 12.9% vancomycin-intermediate *S. aureus* (VISA). All strains were stored frozen at −80°C in a stock solution of 50% glycerol and 50% cation-adjusted Mueller-Hinton broth (BD Diagnostics, Franklin Lakes, NJ) until use in experiments.

Apramycin sulfate was obtained from Alfa Aesar (Tewksbury, MA) and was dissolved in deionized water at 32 mg/ml and stored at −20°C in aliquots that were used only once. For synergy studies, apramycin, gentamicin, daptomycin, and vancomycin were dissolved at 100, 100, 10, and 10 mg/ml in water supplemented with 0.3% polysorbate 20 (P-20; Sigma–Aldrich), respectively. Linezolid was dissolved at 30 mg/ml in DMSO (Signma-Aldrich). Solvents used reflect requirements for liquid handling by the HP D300 Digital Dispensing System (HP Inc, Palo Alto, CA) (Brennan-Krohn, et al., 2017; K. P. Smith & J. E. Kirby, 2016).

2.2 Suseptibility Testing

The Clinical Laboratory and Standards Institute (CLSI) broth microdilution reference method was used for MIC testing of apramycin (CLSI, 2015). MIC panels were created by serial dilution of stock apramycin with cation-adjusted Mueller-Hinton broth in round bottom, 96-well plates (Evergreen Scientific, Los Angeles, CA). Dilutions were prepared at 2× the final concentration in volumes of 50 μL with final concentrations ranging from 1–256 μg/mL after an equal volume of bacterial inoculum (5×10^5 cfu/ml final concentration) was added. Stock solutions were quality controlled against *S. aureus* ATCC 29213 on three separate days. All MIC values for ATCC 29213 were consistently 4 μg/ml, which was in the middle of the 2–8 μg/mL acceptable quality control range suggested by CLSI. Bacterial inocula were prepared by passaging previously frozen bacterial strains on trypticase soy agar containing 5% sheep's blood at 37°C. Isolated colonies were then suspended in cation-adjusted Mueller-Hinton broth for a final inoculum concentration of 5×10^5 CFU/mL. After inoculation, broth microdilution plates were incubated at 35°C in ambient air for 16–20 hours. Each experiment also included both a positive (*S. aureus* ATCC 25923) and a negative control to which no organisms were added. *S. aureus* 25923 always showed an MIC of 4 or 8 μg/ml supporting consistency of assay readout.

2.3 MIC Interpretation

The MIC of each strain from our collection was determined in duplicate on separate days. If duplicate MICs were within one doubling dilution of each other but were not the same, the higher MIC was used. If duplicate MICs were not within one doubling dilution of each other, a third replicate was performed, and the MIC was defined as the modal MIC of the three replicates. Categorical breakpoints for apramycin are not available either from the CLSI or the European Committee for Antimicrobial Susceptibility Testing and, therefore, categorical intepretation was not performed.

2.4 Time-Kill Studies

Time-kill studies were performed according to CLSI guidelines (Clinical and Laboratory Standards Institute, 1999). To prepare a starting inoculum for the time-kill studies, 100 μL of a 0.5 McFarland suspension of colonies from an overnight plate was added to 5 mL of

CAMHB and incubated on a shaker in ambient air at 35°C until it reached log phase growth. The log phase culture was then adjusted to a turbidity of 1.0 McFarland in CAMHB, and 100 μL of this suspension was added to 10 mL volumes of antimicrobial solutions in cation-adjusted Mueller-Hinton broth (BD Diagnostics, Franklin Lakes, NJ) at time 0. Antibiotic concentrations selected were based on multiples of each isolate's MIC as determined through broth microdilution assays. A growth control and a negative control were run in parallel with each experiment. Cultures were incubated on a shaker in ambient air at 35°C.

Aliquots from the culture were removed at 0, 1, 2, 4, 6, and 24 hours. A serial 10-fold dilution in 0.9% sodium chloride was prepared and a 10 μL drop from each dilution was transferred to a Mueller Hinton plate (Thermo Fisher, Waltham, MA) (Chen, et al., 2003; Herigstad, et al., 2001; Naghili, et al., 2013) and incubated overnight in ambient air at 35°C. Colonies within each drop were counted. For drops containing 3 to 30 colonies, the cell density of the sample was calculated; if more than one dilution for a given sample had a countable number of colonies, the cell density of the two dilutions was averaged. If no drops had a countable number of colonies, the two drops above and below the countable range were averaged. The limit of detection was 300 CFU mL^{-1}. Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ CFU mL^{-1} at 24 hours compared to the starting inoculum (Leber, 2016; Pillai, et al., 2005).

2.5 Synergy studies

Checkboard synergy arrays consisting of orthogonal two-fold serial dilutions of antibiotic combinations were set up using the HPD300 as previously described (Brennan-Krohn, et al., 2017). Combinatorial fractional inhibitory concentrations ≤ 0.5 were considered synergistic; > 0.5 and < 4 were considered indifferent; and ≥ 4 were considered antagonistic (Odds, 2003).

2.6 Statistical Analysis

R was used to plot MIC distributions and for statistical analysis (RStudio, 2017; Team, 2017). $P < 0.05$ was considered to be statistically significant.

3. Results

The *S. aureus* collection had a narrow apramycin MIC range of 4 to 32 μg/mL (see Fig. 1). The MIC_{50} and the MIC_{90} were 8 μg/mL and 16 μg/mL, respectively. Based on the visual inspection method, an apramycin epidemiological cutoff value of 32 μg/mL was assigned (Turnbridge & Patterson, 2007). A chi-square test of independence detected no significant relationship between MSSA, MRSA and VISA strain phenotype and apramycin MIC distribution ($P = 0.17$).

Apramycin exposure led to rapid rapid killing of clinical MSSA strain S27 and MRSA strain S19 at 1×–4× the broth microdilution MIC in time-kill experiments. However, regrowth was observed bewteen 6 and 24 hours (Fig. 2). In contrast, VISA strain, FDA-CDC AR Bank #226, showed bacteriostatic activity at 1×–4× the broth microdilution MIC at all time points examined.

During checkerboard synergy testing of the same strains use for time-kill studies, both apramycin and gentamicin demonstrated indifference when tested in combination with vancomycin, daptomycin, and linezolid.

4. Discussion

In our examination of human clinical isolates of *S. aureus*, we observed a very narrow distribution of MIC values. No isolate had an MIC above an epidemiological cutoff value of 32 μg/mL, suggesting near to complete absence of apramycin modifying enzymes in our geographic region and in the smaller number of FDA-CDC VISA strains examined. This observation contrasted with a low prevalence of apramycin resistance in MRSA strains isolated from dairy cattle with mastitis and from diseased swine (Fessler, et al., 2011; Kadlec, et al., 2009). It is possible that selective pressure led to somewhat increased prevalence of apramycin resistance in these veterinary isolates. In the current study, we also found that apramycin demonstrated rapid, early, time-kill properties against MSSA and MRSA isolates with later regrowth, similar to previous reports for gentamicin (Schafer, et al., 2006), with static activity against a VISA isolate.

Apramycin is currently used as an orally administered, non-absorbable antibiotic to treat diarrheal diseases in poultry and livestock, as well as an intravenous treatment for pneumonia in calves and mastitis in cows and sheep (Livermore, et al., 2011; Ziv, et al., 1985; Ziv, et al., 1995). *S. aureus* is the most frequently isolated pathogen in bovine mastitis specimens (Bradley, et al., 2007), suggesting potential therapeutic efficacy against one type of *S. aureus* infection in large mammals. In addition, efficacy of apramycin against a single MRSA strain was demonstrated in an immunocompromised murine septicemia model (Meyer, et al., 2014). Here, apramycin decreased bacterial burden in a dose-dependent manner by 2- to 3-$\log_{10}$ in the blood and up to 4-$\log_{10}$ in the kidneys. Therefore, there is evidence supporting therapeutic effect of apramycin aginst *Staphylococcal aureus* infection.

Notably, aminoglycosides are not used currently as single agents for treatment of *S. aureus* infections in humans based on limiting toxicities. Further, risk-benefit of short term use in combination with vancomycin and other agents for treat of persistent bacteremia and prosthetic valve endocarditis, although recommended by some guidelines (Liu, et al., 2011) has been a matter of debate (Deresinski, 2009). It is possible that apramycin, based on a putatively more compelling side effect profile, may offer an alternative treatment for *S. aureus* infections as a single agent or in combination. However, basic pharmacokinetic and pharmacodynamic parameters still need to be defined in humans. Therefore use of apramycin for treatment of human *Staphylococcus aureus* infection as yet remains speculative.

Conclusions

In this study, we found that apramycin shows consistent *in vitro* activity against contemporary *S. aureus* strains including MSSA, MRSA and VISA. Furtheremore, apramycin demonstrated rapid bactericidal activity against MSSA and MRSA. Ultimate utility against human *S. aureus* infection will depend on pharmacokinetic and

pharmacodynamic parameters that have yet to be fully established in animal models and investigated in humans. Nevertheless, the broad-spectrum *in vitro* activity of apramycin against multidrug-resistant *S. aureus* strains in combination with prior descriptions of activity against carbapenem-resistant Enterobacteriaceae, and multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, suggests that further exploration of apramycin and/or derivatives as repurposed human therapeutics may be warranted.

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Highlights

1. Apramycin is an aminoglycoside approved for veterinary use
2. Activity against highly drug resistant *S. aureus* was studied
3. Frank apramycin resistance was not found in MSSA, MRSA and VISA strains studied
4. Apramycin could potentially be repurposed against highly drug-resistant pathogens

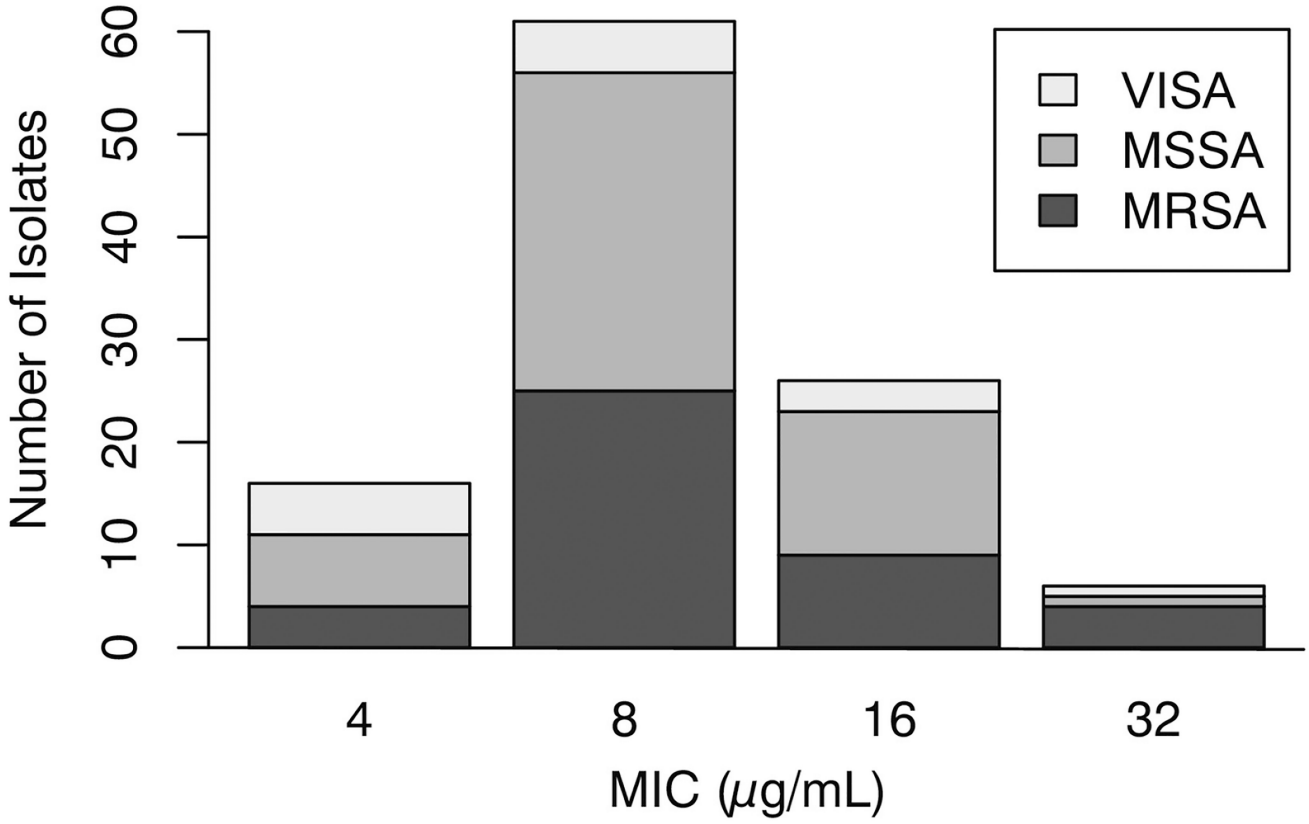

Figure 1. Apramycin MIC distribution for *S. aureus* clinical isolates

MSSA (methicillin-susceptible *S. aureus*); MRSA (methicillin-resistant *S. aureus*); VISA (vancomycin-intermediate *S. aureus*).

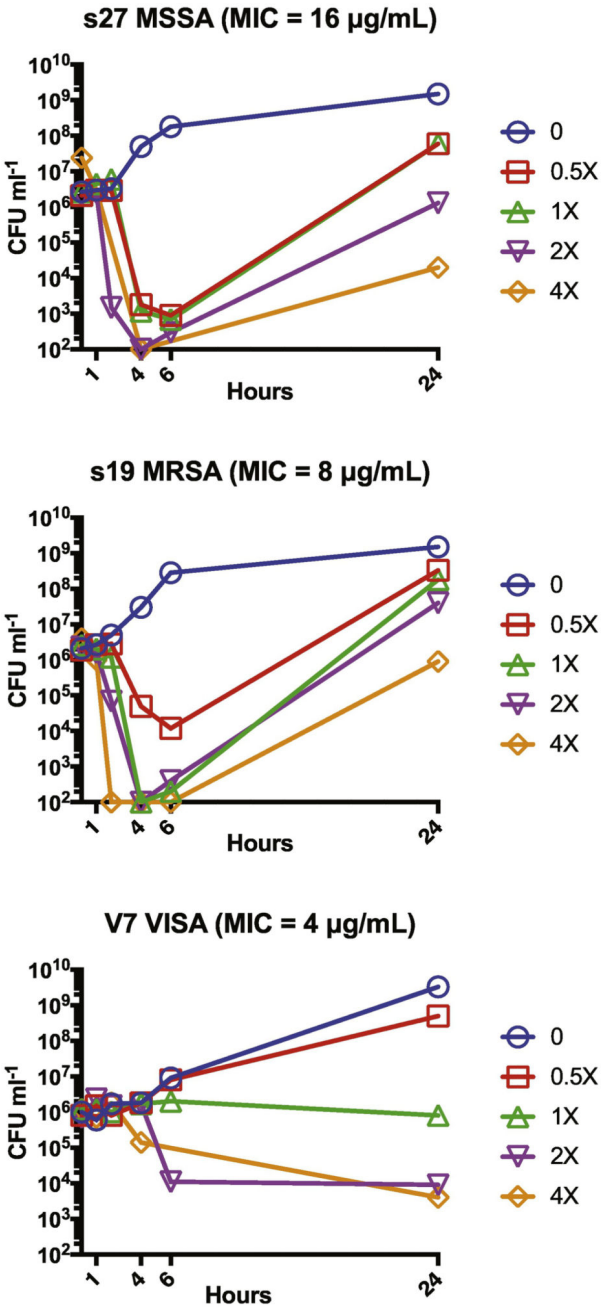

Figure 2. Time-kill experiments

Macrobroth time-kill analysis was performed against representative MSSA, MRSA, and VISA strains. Data points plotted at 10^2 cfu indicate no growth and correspond to the assay detection limit. Panel titles list the broth microdilution MIC associated with each isolate.