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Original Article

Azithromycin Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens

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ABSTRACT

Antibiotic resistance poses an increasingly grave threat to the public health. Of pressing concern, rapid spread of carbapenem-resistance among multidrug-resistant (MDR) Gram-negative rods (GNR) is associated with few treatment options and high mortality rates. Current antibiotic susceptibility testing guiding patient management is performed in a standardized manner, identifying minimum inhibitory concentrations (MIC) in bacteriologic media, but ignoring host immune factors. Lacking activity in standard MIC testing, azithromycin (AZM), the most commonly prescribed antibiotic in the U.S., is never recommended for MDR GNR infection. Here we report a potent bactericidal action of AZM against MDR carbapenem-resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. This pharmaceutical activity is associated with enhanced AZM cell penetration in eukaryotic tissue culture media and striking multi-log-fold synergies with host cathelicidin antimicrobial peptide LL-37 or the last line antibiotic colistin. Finally, AZM monotherapy exerts clear therapeutic effects in murine models of MDR GNR infection. Our results suggest that AZM, currently ignored as a treatment option, could benefit patients with MDR GNR infections, especially in combination with colistin.

1. Introduction

Hospital-acquired infections, half caused by drug-resistant bacteria (Mauldin et al., 2010), cause ~99,000 deaths annually and increase healthcare costs by $5–10 billion in the U.S. alone (Peleg and Hooper, 2010). Recent reports by the U.S. Centers for Disease Control and Prevention (CDC, 2013) and the World Health Organization (WHO, 2014) describe this ever-worsening antibiotic resistance crisis, highlighting the "urgent threat" of emerging carbapenem-resistant Gram-negative rods (GNRs) that forebode the entry of human medicine into a "post-antibiotic era". Rapid spread of carbapenem resistance in *Pseudomonas aeruginosa* (*PA*), *Klebsiella pneumoniae* (*KP*) and *Acinetobacter baumannii* (*AB*) is of particular concern as effective antibiotic candidates are currently lacking in the development pipeline (Diene and Rolain, 2014).

Unconventional approaches to infectious disease treatment are gaining more attention, including virulence factor inhibition, bacteriophage therapy, probiotics and immune boosting (Cegelski et al., 2008; Hancock et al., 2012). Along these lines, we have probed interactions of conventional antibiotics with antimicrobial effectors of the innate immune system, with encouraging results. Drugs with no direct activity in standard minimum inhibitory concentration (MIC) testing nevertheless sensitized multidrug-resistant (MDR) bacterial pathogens to human antimicrobial peptide killing in vitro, e.g., nafcillin vs. methicillin-resistant *Staphylococcus aureus* (MRSA) or ampicillin vs. vancomycin-resistant *Enterococcus* (VRE), and contributed to clinical resolution in refractory infections (Sakoulas et al., 2012, 2014).

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Our recent experiences with β-lactams and MRSA or VRE indicate that simple MIC testing overlooks potential synergies with cationic antibiotics (e.g., daptomycin) and host AMPs (e.g., human cathelicidin LL-37) that promote bactericidal activity in vitro and bacterial clearance in patients (Sakoulas et al., 2012, 2014). We asked whether similar phenomena could be identified in MDR-GNRs to challenge conventional antibiotic treatment paradigms. AZM, the most commonly prescribed antibiotic in the U.S. (51.5 million in 2010) (Hicks et al., 2013), is never recommended for inpatient treatment of serious GNR infections because of poor or absent in vitro activity by standard MIC testing in bacteriologic media. However, antibacterial activity of AZM is enhanced in mammalian tissue culture media vs. standard bacteriologic media (Buyck et al., 2012), a finding reminiscent of observations we made for LL-37 (Dorschner et al., 2006), prompting us to examine its interaction with MDR GNRs more closely.

2. Materials and Methods

2.1. Bacterial Strains

P. aeruginosa (*PA*) strain PA01, *K. pneumoniae* (*KP*) strain K700603, and *A. baumannii* (*AB*) strain AB19606 were obtained from the American Type Culture Collection (ATCC). Human clinical MDR isolates *PA* P4 (lung) and *KP* K1100 (lung) (Fair et al., 2012) were obtained from a tertiary academic hospital in the New York metropolitan area. MDR-*AB* AB5075 (bone) (Zurawski et al., 2012) was obtained from Walter Reed Army Medical Center. All three MDR strains were independently identified and subject to antibiotic susceptibility testing by the clinical microbiology laboratory at the San Diego Veterans Affairs Hospital (table S1). Additional MDR GNR clinical isolates tested were also obtained from a tertiary academic hospital in the New York metropolitan area, except for PA USCD P1, which was obtained from the UC San Diego hospital system. Bacteria were grown overnight in Luria Broth (LB), glycerol was added (30% final), and stocks stored at −80 °C. Fresh colonies were streaked onto LB plates each week for all experiments.

2.2. Antibiotics and Antimicrobial Peptides

For in vitro studies, AZM, colistin sulfate, and ciprofloxacin were purchased from Sigma-Aldrich; erythromycin and clarithromycin were purchased from Fischer Scientific. Stock solutions were prepared in phosphate buffered saline (PBS) at 2560 mg/L for the macrolide antibiotics, 1000 mg/L for colistin, and 10,000 mg/L for ciprofloxacin. Trace amounts of glacial acetic acid were used to prepare AZM, erythromycin and clarithromycin stocks for complete solubility (Barry et al., 2004). LL-37 and TAMRA-tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular quality water (Corning Cellgro) at 640 μM and 320 μM, respectively, and stored at −80 °C. For in vivo studies, AZM for human injection (Sagent Pharmaceuticals) was reconstituted per manufacturer's guidelines (AZM Package Insert, 2013). Pooled human serum was obtained from six healthy consented lab volunteers under a protocol approved by the UCSD Human Research Protection Program and immediately aliquoted and stored at −80 °C.

2.3. Reagents

Mueller–Hinton Broth (MHB, Spectrum Chemicals) was supplemented with $CaCl_2$ and $MgCl_2$ to make cation-adjusted MHB (Ca-MHB) — final cation concentrations (20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}). Luria Broth base (LB) was purchased from Hardy Diagnostics. RPMI-1640 was purchased from Invitrogen. Clear phenol free RPMI-1640 used for microscopy studies was purchased from Corning Cellgro. 1-N-phenylnaphthylamine (NPN) was purchased from Sigma-Aldrich and a fresh stock of 500 μM in acetone was made each week.

2.4. MIC Determinations

MIC values for AZM, erythromycin, clarithromycin, ciprofloxacin, colistin, and LL-37 were determined using broth microdilution in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using Ca-MHB media, the recommended bacteriologic broth, or eukaryotic cell culture media RPMI-1640 supplemented with 5% LB (Sakoulas et al., 2014).

2.5. Time-Kill Curves and Serum Survival Assays

Time-kill studies, ±20% pooled human serum, were performed as previously described (Haste et al., 2011). Bacteria were grown overnight in LB at 37 °C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS via centrifugation at 3220 ×*g* at room temperature with a final re-suspension in PBS to an $OD_{600} = 0.40$. Bacterial stocks in PBS were diluted in Ca-MHB or 5% LB-RPMI to an initial inoculum of 1×10^6 CFU/mL (standard time-kill) or 5×10^4 CFU/mL (serum survival). AZM, erythromycin, clarithromycin, colistin, and LL-37 stocks were diluted in Ca-MHB or 5% LB-RPMI to the assay concentrations indicated. For serum studies, AZM 0.5 mg/L was chosen in order to approximate human plasma concentrations upon intravenous administration of 500 mg of AZM (AZM Package Insert, 2013). Assays were conducted in triplicate in a final volume of 200 μL in 96-well round bottom plates (Costar) ±20% pooled human serum. The 96-well plates were wrapped in paraffin and placed in a shaking incubator at 37 °C. Aliquots were collected at the indicated times and serially diluted for CFU enumeration; limit of detection = 100 CFU/mL.

2.6. Electron Microscopy of Bacterial Gross Morphology

Transmission electron microscopy was performed essentially as described (Sato, 1968). MDR-*AB* was grown overnight in LB at 37 °C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS and resuspending in PBS to $OD_{600} = 0.40$. Then 2.5 mL of each bacterial stock was added to 47.5 mL of Ca-MHB or RPMI + 5% LB media pre-warmed to 37 °C. For treatment, the AZM stock was diluted to a concentration of 0.5 mg/L in the final volume of 50 mL. 50 mL cultures were placed in a shaking incubator at 37 °C for 2 h. Cultures were then centrifuged at 3220 × *g* at room temperature for 10 min. The supernatant was aspirated and bacterial pellets re-suspended in 1 mL of PBS. These 1 mL samples were immersed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 h, post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 h, and stained en bloc in 2% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50–60 nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4 k HS digital camera (FEI). Images were taken from multiple random fields at 1200×, 2900×, 23,000×; and gross morphology was analyzed in a blinded fashion.

2.7. Fluorescence Microscopy for MDR-AB Cytological Profiling

The fluorescence microscopy studies for MDR-*AB* were performed as previously described (Nonejuie et al., 2013). These microscopy studies required higher concentrations of AZM because the concentration of MDR-*AB* used was 100× higher (5×10^7 CFU/mL) compared to the concentration used in the MIC assays (5×10^5 CFU/mL). All AZM concentrations used in these studies are pharmacologically obtainable in human tissue. Single MDR-*AB* colonies were picked from LB plates and grown in LB or RPMI + 5% LB overnight. Overnight cultures were then diluted