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Author manuscript

Infect Microbes Dis. Author manuscript; available in PMC 2024 March 18.

Published in final edited form as:

Infect Microbes Dis. 2020 March ; 2(1): 10–17. doi:10.1097/im9.0000000000000014.

Azithromycin Exerts Bactericidal Activity and Enhances Innate Immune Mediated Killing of MDR *Achromobacter xylosoxidans*

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Abstract

Azithromycin (AZM), the most commonly prescribed antibiotic in the United States, is thought to have no activity against multidrug-resistant Gram-negative pathogens such as *Achromobacter xylosoxidans* (AX) per standard minimum inhibitory concentration testing in

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Author contributions: AK and MK conducted the in vitro and ex vivo experiments. ERU conducted the biofilm experiments. ERU, HT, and JP conducted and/or supervised fluorescence microscopy experiments. CL provided the brief history of source case and clinical isolate for experiments. ERU and AK wrote the manuscript with support from MK, GS, JP, JJL, and VN. The hypothesis was developed by GS, MK, and VN. All authors (ERU, AK, HT, GS, JP, CL, JJL, VN, and MK) conceived and planned experiments performed.

Editor: Stijn van der Veen

Conflicts of Interest: G. S. has received speaking honoraria from Allergan, Sunovion, and Melinta; has received consulting fees from Allergan and Paratek Pharmaceutical. J. P. has an equity interest in Linnaeus Bioscience. V.N. has received consulting fees from Cidara Therapeutics.

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cation-adjusted Mueller Hinton Broth. Here we provide the first report of AZM bactericidal activity against carbapenem-resistant isolates of AX, with a multifold decrease in minimum inhibitory concentration across 12 clinical isolates when examined under physiologic testing conditions that better recapitulate the in vivo human environment. This pharmaceutical activity, evident in eukaryotic tissue culture media, is associated with enhanced AZM intracellular penetration and synergistic killing with human whole blood, serum, and neutrophils. Additionally, AZM monotherapy inhibited preformed AX biofilm growth in a dose-dependent manner together with a reduction in viable bacteria. In an illustrative case, AZM in combination with piperacillin-tazobactam exerted clear therapeutic effects in a patient with carbapenem-resistant AX mediastinitis, sternal osteomyelitis, and aortic graft infection. Our study reinforces how current antimicrobial testing practices fail to recapitulate the host environment or host-pathogen interactions and may misleadingly declare complete resistance to useful agents, adversely affecting patient outcomes. We conclude that AZM merits further exploration in the treatment of drug-resistant AX infections. Novel approaches to antimicrobial susceptibility testing that better recapitulate the host environment should be considered, especially as infections caused by multidrug-resistant Gram-negative bacterial pathogens are expanding globally with high morbidity and mortality.

Keywords

Achromobacter xylosoxidans ; antimicrobial susceptibility testing; azithromycin; treatment; host defense; multidrug resistance

Introduction

Achromobacter xylosoxidans (AX) is a motile nonfermentative Gram-negative rod widely distributed in the environment and an important emerging multidrug-resistant (MDR) nosocomial pathogen.[1–3] AX has been implicated in a variety of infections, including pneumonia, endocarditis, urinary tract and gastrointestinal infections, prosthetic device-related infections, meningitis and ophthalmic disease, particularly in the immunocompromised, and has increasingly been isolated from the respiratory secretions of patients with cystic fibrosis.[1,4–8] However, AX is most frequently associated with causing bacteremia *via* central venous catheters.[9–12] Case mortality rates secondary to this pathogen are as high as 80% in neonates, 30% in bacteremia, and up to 65% in endocarditis, meningitis, and pneumonia.[10]

Intrinsic and acquired resistance of AX to multiple classes of antibiotics and the ability to form robust biofilms in vivo make this emerging pathogen particularly difficult to treat. MDR AX strains may harbor β-lactamases, penicillin binding proteins, aminoglycoside modifying enzymes, carbapenemases (eg, IMP, VIM, or TMB-type metallo-β-lactamases) and/or complex series of active efflux pumps (eg, AxyABM, AxyXY-OprZ, TetA) conferring resistance to a wide array of antibiotics including narrow-spectrum penicillins, cephalosporins, aztreonam, aminoglycosides, carbapenems, and tetracyclines.[1] Agents to which AX clinical isolates are most often susceptible include ticarcillin (99.5%),

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cefoperazone/sulbactam (98.7%) and pipercillin/tazobactam (TZP) (97.2%).[7] However, ticarcillin and cefoperazone/sulbactam are currently not available in the United States.

Clinicians are all too often presented with a serious MDR Gram-negative rod infection in a high-risk patient where the antimicrobial susceptibility testing (AST) profile presents few or no options. Yet, completely unaccounted for in the current AST paradigm are the many dynamic interactions that occur among the bacterial pathogen, potential antimicrobial agents and components of the host innate immune system, such as cathelicidin and other endogenous antimicrobial peptides (AMPs), serum complement and phagocytic cells including neutrophils and macrophages. Recent studies have revealed striking bactericidal activity of current FDA-approved antibiotics such as azithromycin (AZM) versus *Acinetobacter baumannii* (*Ab*), *Klebsiella pneumoniae* (*Kp), Pseudomonas aeruginosa (Pa)*, and *Stenotrophomonas maltophilia (Sm)*, or β-lactamase inhibitors (BLIs) such as tazobactam (TAZ) and avibactam versus *Kp* and *Ab,* despite the agents themselves having no activity in standard laboratory AST testing.[13–16] Rather these drugs reveal their potent antimicrobial activity in testing conditions reflecting a more physiologic environment (ie, mammalian tissue culture media). Therein, the neglected antibiotics can function to dramatically sensitize MDR pathogens to innate immune killing, as proven in checkerboard and kinetic AMP killing assays, ex vivo serum and phagocytic cell killing assays, and in vivo models of MDR infections.

Here we assess the activity of AZM against AX using multiple MDR isolates obtained from patients with cystic fibrosis, together with a clinical isolate from a patient who developed AX sternal osteomyelitis, ascending aortic graft infection and mediastinitis and whose successful treatment regimen included AZM. Efficacy of AZM against AX was evaluated under physiologic media conditions through minimum inhibitory concentration (MIC) testing, kinetic kill curves, biofilm assays, checkerboard synergy assays, and fluorescence microscopy. Additionally, ex vivo assays were conducted using human serum, neutrophils, and whole blood to evaluate the ability of AZM to sensitize AX to different components of innate immunity.

Brief history of the source case

A 66-year-old male with a history of aortic stenosis and an ascending aortic aneurysm underwent a bovine aortic valve replacement and repair of his aortic aneurysm with polyester graft placement later presented with pain, cellulitis, and purulent drainage along his sternotomy site 4 months post-procedure. He underwent incision and drainage down to the sternum with removal of four sternotomy wires. Intraoperative cultures grew β-lactam susceptible AX and he was started on once daily IV ertapenem therapy for ease of administration. Given poor sternal wound healing, the patient underwent re-exploration of his sternal wound ~1 month later with removal of all sternal wires, at which time an intact sternum was noted. Repeat intraoperative cultures again grew β-lactam susceptible AX. He was maintained on ertapenem therapy for nearly 5 months before re-presenting to the hospital with frank mediastinitis, ascending aortic graft infection and sternal osteomyelitis shortly following the discontinuation of antibiotics, and ultimately required multiple surgical debridements, and removal and replacement of the aortic graft. Debridement cultures from

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his most recent surgical interventions once again grew AX (hereafter referred to as the clinical isolate AX Florida) susceptible to TZP but now resistant to a cadre of other antibiotics including ampicillin, cefazolin, ceftriaxone, ampicillin/sulbactam, aztreonam, amikacin, gentamicin, trimethoprim/sulfamethoxazole, ertapenem and with intermediate susceptibility to ciprofloxacin, tobramycin, and meropenem. The patient was subsequently placed on IV TZP and adjunctive PO AZM for management of his sternal osteomyelitis, ascending aortic graft infection and mediastinitis attributed to highly MDR AX for which he remained on for 2 months before transitioning to solely chronic suppressive therapy with PO AZM. The patient continued to do well 1 year following his last surgical intervention and had no adverse side effects associated with chronic suppressive AZM therapy.

Results

Bactericidal activity of AZM versus AX revealed in tissue culture media by increasing drug entry

AZM inhibits protein synthesis by binding to and impeding the 50S ribosomal subunit of bacteria, and is traditionally perceived to lack activity against AX based on AST performed in cation-adjusted Mueller Hinton Broth (CA-MHB). AZM MICs for several MDR AX strains were assessed by broth micro-dilution methodology in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines using standard bacteriologic medium (CA-MHB) or supplemented mammalian tissue culture medium [Roswell Park Memorial Institute 1640 + 10% Luria Bertani Broth (RPMI + 10% LB)]. Growth curve analysis of AX in CA-MHB and RPMI + 10% LB demonstrated equivalent growth, and a >200-fold reduction in MIC was observed for nearly all AX isolates tested with CA-MHB (range: 16 to 512 mg/L) versus RPMI + 10% LB (range: 0.25 to 8 mg/L) (Figure 1A and Table 1). Additionally, a kinetic killing assay revealed 0.25 mg/L AZM yielded moderate bactericidal activity in RPMI + 10% LB but had no effect on AX Florida growth in CA-MHB (Figure 1B). Lastly, fluorescence microscopy based bacterial cytological profiling was employed demonstrating markedly enhanced entry of (fluorescently-labeled) NBD-tagged AZM into AX cells in RPMI + 10% LB thereby permitting access to the 50S ribosomal subunit (Figure 1C).

AZM decreases AX biofilm biomass and biofilm cell viability

AX like other Gram-negative bacteria has the propensity to form a thick biofilm particularly on foreign bodies. Using a 12 well plate, we assessed the activity of various concentrations of AZM on pre-formed AX Florida biofilm biomass in CA-MHB and RPMI + 10% LB (Figure 2A). The optical density (OD_{490}) of solubilized untreated AX biofilm was 1.94 ± 0.36 in CA-MHB and 1.18 ± 0.19 in RPMI + 10% LB, respectively suggesting thicker biofilm formation in CA-MHB. However, AZM antibiofilm activity was more prominent in RPMI+10%LB. Figure 2A notably demonstrates a 26% and 74% reduction in AX Florida biofilm biomass comprised of both non-viable and viable bacterial cells following exposure to 1 mg/L (4× MIC) and 4 mg/L (16× MIC) of AZM, respectively in RPMI + 10% LB for 48 hours. Both AZM concentrations (4× MIC and 16× MIC for AX Florida) are extremely low, readily pharmacologically achievable in humans, and furthermore can concentrate intracellularly within phagocytic cells present both within human circulation and tissues.

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Remaining viable CFU/mL following AZM exposure was also enumerated. Ultimately, a 10% reduction (8.05 $\log_{10}$ cfu/mL vs 9.04 $\log_{10}$ cfu/mL) was observed for viable bacterial cells at 4 mg/L of AZM in comparison to untreated pre-formed biofilm in RPMI + 10% LB (Figure 2B). The reduction in total biofilm biomass by 74% while viability was only reduced by 10% at 4 mg/L of AZM suggests that the biofilm biomass is primarily comprised of nonviable capsular exopolysaccharide (EPS) which can be penetrated by AZM. AZM likely penetrates the thick capsular EPS down to a thin layer of underlying viable bacterial cells resulting in EPS degradation and/or detachment.

In vitro susceptibilities of AX to the antibiotics AZM, TZP, LL-37, AZM + TZP, AZM + LL-37, and TZP + LL-37

MIC, time-kill and checkerboard assays were performed for AZM, TZP, LL-37 and combinations AZM + TZP, AZM + LL-37, and TZP + LL-37 using the clinical isolate AX Florida in CA-MHB or RPMI + 10% LB (Table 2). AX Florida exhibited susceptibility to AZM (MIC = 0.25 mg/L), TZP (MIC = 8 mg/L), and LL-37 (MIC = 4 mg/L) in supplemented RPMI. Additionally, bactericidal activity defined as a reduction in viable bacteria by 3 log10 CFU/mL by kinetic killing assays was observed for AZM and LL-37 in RPMI + 10% LB, and TZP in CA-MHB using antibiotic concentrations identified as the MIC in RPMI + 10% LB (Figure S1, Supplemental Digital Content, http://links.lww.com/IMD/A2). No synergy was appreciated for AX Florida using the combinations AZM + TZP, AZM + LL-37 or TZP + LL-37. However, additivity [fractional inhibitory concentration index (FICI) >0.5 to 1] was observed for AZM + TZP and AZM + LL-37 in both CA-MHB and RPMI + 10% LB, and for TZP + LL-37 but only in supplemented RPMI (Table 2)

AZM sensitizes AX to clearance by serum complement, neutrophils, and whole blood

To assess the interactions of AZM with components of host innate immunity, we conducted targeted ex vivo studies utilizing human serum complement, neutrophils, and whole blood. Neutrophils, the most abundant circulating leukocyte and widely regarded to be the first responders of the innate immune system, help to combat pathogens *via* degranulation with release of soluble antimicrobials including cationic host defense peptides (eg, cathecidin LL-37, human neutrophil peptides, and human β-defensins), phagocytosis and neutrophil extracellular trap (NET) formation. Serum complement (including C3), also a critical component of the innate immune system, kills a wide range of Gram-negative bacteria by triggering the assembly of the membrane attack complex, a transmembrane channel that disrupts bacterial cell membranes resulting in microbial lysis and death. Our ex vivo studies revealed that direct co-incubation of AX Florida with AZM at 1/2× MIC and 1× MIC sensitized the bacterium to serum killing while overnight pre-treatment of AX Florida with sub-bacteriostatic concentrations of AZM sensitized the bacterium to neutrophil killing (Figure 3A and 3B). Additionally, whole blood killing where serum complement, phagocytic cells, and platelets may all contribute to innate immune clearance of AX Florida was notably enhanced by the presence of AZM at 1/4× MIC, 1/2× MIC, and 1× MIC when compared to AX Florida in the absence of antibiotic (Figure 3C).

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Discussion

Our studies were performed using carbapenem-resistant strains of AX, an exemplar of an emerging public health threat particularly amongst patients with cystic fibrosis, infected prostheses, and compromised host immunity.[1] AZM is seldom contemplated for the treatment of AX as it is deemed "resistant" as per conventional AST. Indeed, we confirmed that when MIC testing is performed in the standard bacteriologic medium CA-MHB (as per CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines), AZM shows negligible activity against 12 MDR clinical isolates of AX. In sharp contrast, there was a striking >200-fold decrease in MICs across these same clinical isolates in RPMI + 10% LB.

RPMI is a versatile medium reflective of in vivo physiological conditions. It was originally formulated for use in a 5% CO_2 environment (ie, possessing a bicarbonate buffer system), contains glucose, amino acids, salts (sodium chloride, magnesium sulfate, calcium nitrate, etc) and vitamins commonly present in host tissues, and can be used to support the growth of a variety of mammalian cell types such as human lymphocytes, bone marrow cells, hybridoma cells as well as fungi and bacteria when partially supplemented with serum or bacteriologic media (eg, Todd Hewitt broth, LB). CA-MHB in comparison and contrast, is comprised primarily of beef extract, casein hydrolysate, starch, cations (magnesium and calcium), and is used in the laboratory setting to solely support bacterial growth.

The decrease in MIC observed only in eukaryotic cell media was associated with increased AZM entry and killing of AX at physiological attainable doses of AZM. We also observed AZM-mediated sensitization of AX to killing by human whole blood, serum, and neutrophils. Collectively, our findings indicate that AZM, despite being deemed ineffective by standard MIC susceptibility testing, has significant pharmacodynamic interactions (i) directly against MDR AX, and (ii) indirectly *via* boosting of endogenous effectors of the innate immune system that may translate to clinical utility.

The relevance of our findings was also extended to a complicated, device-related infection secondary to AX. Significant therapeutic benefit was achieved in this patient with refractory mediastinitis, sternal osteomyelitis, and aortic graft infection after 2 months of combination therapy with TZP and AZM, followed by chronic suppressive therapy with AZM alone. The proclivity of AX to form biofilms, makes this organism particularly difficult to treat, resulting in treatment failure and infection recurrence.[25,26] Biofilms are matrix-enclosed bacterial populations and are formed on inactive or bioactive surfaces.[27] After bacteria attach to a surface, a bacterial monolayer within a matrix is produced and continues to thrive sequestered from host immunity. Ultimately, bacterial detachment occurs with reversion to planktonic growth, starting a new cycle. Generally, biofilms are resistant to antibiotics, and this plays an important role in the failure of chemotherapy to eradicate biofilm infections. However, as previously described for other Gram-negative organisms, we identified that AZM has antibiofilm effects against AX.[28–30] The ability of AZM to penetrate thick capsular EPS, and impair and suppress further growth of pre-formed AX biofilms, may have contributed to the therapeutic efficacy of AZM that we observed in our patient.

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Adjunctive bactericidal effects attributable to AZM has been documented.[31,32] For example, there is good evidence that macrolides modulate inflammatory pathways by suppressing pro-inflammatory cytokines, reducing the representation of adhesion molecules, and altering the expression of nitric oxide synthase.[33–35] Large, randomized controlled trials have established the unequivocal benefits of AZM in patients with cystic fibrosis, including improvements in pulmonary function and reductions in exacerbations.[32,36,37] Previous studies from our lab, similarly document the in vitro and in vivo therapeutic benefit of AZM against other MDR resistant strains, such as *Pa* and *Sm*, commonly isolated from patients with cystic fibrosis.[13,14] The current study provides an additional explanation for the clinical efficacy findings of AZM in cystic fibrosis patients, and underscores the importance of further exploring these indirect antimicrobial effects under physiological conditions.

Our study, and the published and unpublished work of various other investigators illustrate and reiterate the inadequacies inherent to standard AST based on bacteriologic CA-MHB completely devoid of host environmental factors (eg, bicarbonate buffer system, serum complement, phagocytic cells, endogenous AMPs, etc).[13–16,23,38] These investigators have helped to identify in vitro, ex vivo, or in vivo activity of currently FDA-approved antibiotics against highly MDR pathogens that have been disqualified and prematurely deemed ineffective by traditional AST. Examples include activity of AZM versus *Ab*, *Pa*, *Kp* and *Sm*, AZM + components of innate immunity (eg, serum complement, neutrophils or the AMP LL-37) versus *Ab*, *Pa*, *Kp*, and *Sm*, BLIs versus *Kp* and *Ab*, BLIs + components of innate immunity (eg, serum complement, neutrophils, whole blood or the AMP LL-37) versus *Kp* and *Ab,* and nafcillin + components of innate immunity (eg, keratinocytes, neutrophils, whole blood or the AMPs LL-37, HNP-1, and RP-1) versus methicillin-resistant *Staphylococcus aureus* to name a few.[13–16,23]

MICs identified by AST tremendously impact physician decision making, playing a critical role in helping to ascertain pathogen susceptibility or resistance to antibiotics based on clinical breakpoints. Antibiotic “pseudo-resistance” (eg, macrolides vs *Ab*, *Pa, Kp*, and *Sm*, BLIs versus *Kp* and *Ab*) and “pseudo-susceptibility” (eg, meropenem vs *Sm*; ampicillin vs *Kp*; ceftriaxone vs *Klebsiella aerogenes* harboring AMP-C; trimethoprim/sulfamethoxazole vs *Enterococcus species*) has long been associated with traditional AST performed in bacteriologic CA-MHB medium.[13–15,39–42] Essentially the identified MIC for certain pathogen antibiotic combinations in CA-MHB depicts resistance despite being clinically effective in vivo or susceptibility despite lacking activity in vivo and resulting in poor patient outcomes. Indeed a recent study conducted by Ersoy et al. identified at least a four-fold change in MIC assays for 459 of 1311 isolates (35%) tested in standard CA-MHB versus host mimicking media.[43]

Given the emergence of highly MDR bacteria with limited or no therapeutic options associated with high morbidity and mortality, AST must be modernized and incorporate host environmental factors to help more accurately identify susceptibility and resistance of both FDA-approved antibiotics within our existing antimicrobial arsenal and new drugs at the start of the drug discovery pipeline. Elements to be considered in next-generation AST include more closely mimicking the host environment by performing testing in mammalian tissue culture media with a bicarbonate buffer system, incorporating components of host

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defense to help determine if the antibiotic may sensitize the pathogen to innate immune clearance, accounting for the type of infection being treated (eg, skin abscess vs pneumonia vs urinary tract infection, etc), pathogen type (extracellular vs intracellular organism, Gram + vs Gram –, aerobic vs anaerobic, etc), and inherent properties of the antibiotic itself (eg, concentrates intracellularly, tissue penetration, bactericidal vs bacteriostatic).

In conclusion, our study reveals the potent bactericidal activity of AZM, its ability to impair biofilm, and its interactions with the innate immune system against MDR AX. However, this remarkable activity was only uncovered by performing AST in a more physiologic environment with mammalian tissue culture media and by using different components of host defense, and highlights the shortcomings of traditional AST currently employed in clinical microbiology laboratories worldwide. Limitations of our study include the modest number of AX isolates tested, technical constraints associated with our biofilm setup (ie, inability to perform testing directly on prosthetic aortic graft material or assess biofilm pharmacodynamics over time), lack of in vivo testing, and lack of clinical trials in humans. Nonetheless, this investigation highlights the potential utility of AZM, the most commonly used antibiotic in the United States, in AX infection. Future in vivo studies and human clinical trials evaluating the efficacy of AZM against AX infection would be required to definitively determine the true applicability of our findings.

Materials and methods

Bacterial strains, media, and antibiotics

MDR clinical isolate AX Florida was utilized in all experiments performed. Additional MDR AX clinical strains isolated from patients with cystic fibrosis (AU 33282, AU 33765, AU 34276, AU 31610, AU 34000, AU 34819, AU 31828, AU 34343, AU 32231, AU 31074, and AU 33282) were obtained from the *Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan, Ann Arbor. All isolates were stored in Luria Bertani (LB) broth + 40% glycerol at –80°C until use. Antibiotics AZM and TZP were purchased from (St. Louis, MO, USA), and the human defense peptide cathelicidin LL-37 was purchased from BaChem (Bubendorf, Switzerland). The mammalian tissue culture medium RPMI 1640 (ThermoFisher Scientific, Carlsbad, CA, USA) was supplemented with 10% LB broth (Hardy Diagnostics, Santa Maria, CA, USA) (RPMI + 10% LB), and the bacteriologic medium Mueller-Hinton broth (Spectrum Chemicals, Gardena, CA, USA) was supplemented with 20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+} (CA-MHB).

Growth curve

AX Florida inoculated into 5 mL of LB was grown overnight to stationary phase (14–16 hours) at 37°C in a shaking incubator. The following day, bacteria were washed 2× with phosphate buffered saline (PBS) and re-suspended in tubes containing 25 mL of RPMI + 10% LB or CA-MHB to an initial OD_{600} = 0.05. Tubes were subsequently placed in a shaking 37°C incubator with re-growth assessed by measuring OD_{600} at selected time intervals (4, 8, 24 hours).

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MIC, checkerboard, and time kill assays

Broth microdilution MIC, checkerboard and time kill assays were performed in RPMI + 10% LB or CA-MHB and in accordance with CLSI and EUCAST guidelines.[17–19] Checkerboard synergy, additivity, and antagonism were defined using the FICI: FICI 0.5 = synergy, FICI >0.5 to 1 = additivity, FICI >1 to <4 = indifference, and FICI 4 = antagonism. Time kill assay bactericidal activity was defined as a reduction in viable bacteria 3 $\log_{10}$ CFU/mL, with bacteriostatic activity defined as a reduction in viable bacteria <3 $\log_{10}$ CFU/mL at 24 hours compared to the starting inoculum using the MIC antibiotic (AZM, TZP, or LL-37) concentration.[20]

Fluorescence microscopy

Fluorescence microscopy was performed as previously described with the following modifications.[21] AX Florida colonies were picked from LB plates and grown in LB broth overnight. Overnight cultures were diluted 1:50 in RPMI + 10% LB or CA-MHB and grown to an OD_{600} = 0.20 before seeding a 96-well plate with 50 μL culture per well. NBD-labeled AZM at 1.25 mg/L was added to exponentially growing bacteria (OD_{600} = 0.20) and placed in a shaker at 37°C for 2–3 hours and collected at OD_{600} = 0.40.[22] The stains: FM4–64 (bacterial cell membrane) at 4–6 mg/L, DAPI (nucleic acid) at 2 mg/L, and SYTOX-Green (nucleic acid; impermeant to live cells) at 0.5 m M (Molecular Probes/Invitrogen, Carlsbad, CA, USA) were added to cultures with and without antibiotic. In the case of NBD-tagged AZM, SYTOX-Green dye was omitted and cell cultures were washed with fresh media before staining. Stained cultures (6 μL) were subsequently transferred onto a 1.2% agarose pad containing RPMI + 10% LB for microscopy. Image analyses were performed using FIJI (ImageJ 1.51w) and CellProfiler 3.0.

Biofilm assay

AX Florida colonies were picked from LB plates and grown in LB broth overnight. Overnight cultures were diluted 1:100 in RPMI + 10%LB and grown to an OD_{600} = 0.40. Bacteria were centrifuged at 4000 rpm for 10 minutes at room temperature and re-suspended in RPMI + 10% LB to an OD_{600} = 0.40 before seeding a 12-well plate with 2 mL culture per well. Plates were incubated for 48 hours at 37°C with 5% CO_2. Planktonic bacteria were removed and 2 mL RPMI + 10% LB + AZM (0–256 mg/L) added to preformed biofilm before incubating plates for 48 hours at 37°C with 5% CO_2. After incubation, media was removed and plates air-dried, then stained using 0.1% Safranin (Acros Organics, Fair Lawn, NJ, USA) for 3 minutes and washed five times with distilled water. Adhering dye was dissolved in 30% acetic acid, and absorption measured at 490 nm using a spectrophotometer to assess both viable and nonviable bacterial cells comprising the biofilm. Alternatively, in lieu of staining, biofilm was disrupted with vigorous pipetting followed by serial dilutions in sterile PBS, with plating on LB to enumerate viable CFU. All results represent a minimum of three independent assays.

Serum killing assay

Human serum was pooled from three healthy donors under protocols approved by the UC San Diego Human Subjects Institutional Review Board for use in established bacterial

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killing assays. Serum killing assays were performed as previously described[13] with the following modifications. AX Florida (2×10^6 CFU/well) was added to RPMI ± 10% pooled human serum, and with or without varying concentrations of AZM (at 1/4× MIC, 1/2× MIC, and 1× MIC). Assays were performed with a final volume of 200 μL using siliconized tubes rotated in a 37°C incubator for 15 minutes before completing serial dilutions in sterile PBS and plating on LB for CFU enumeration. The percentage of surviving bacteria was calculated in comparison to the initial inoculum.

Neutrophil killing assay

Human neutrophils were isolated from healthy donors using the PolymorphPrep system (Axis-Shield, Oslo, Norway) under protocols approved by the UC San Diego Human Subjects Institutional Review Board for use in established bacterial killing assays. Human neutrophil assays were performed as previously described[23,24] with the following modifications. Neutrophils were re-suspended in RPMI to 2×10^6 cells/mL and used to seed a 96 well plate (2×10^5 cells/well). Cells were infected at a multiplicity of infection equal to 50 with AX Florida that were untreated or exposed overnight to a sub-bacteriostatic concentration of AZM (0.003 mg/L). Plates were centrifuged at $500 \times g$ for 10 minutes then incubated for 15 and 30 minutes at 37°C in 5% CO_2. Serial dilutions performed using sterile PBS and Triton-X 100 (0.02%) were plated on LB plates for bacterial enumeration. The percentage of surviving bacteria was calculated in comparison to the initial inoculum.

Whole blood killing assay

Stationary phase bacteria were washed twice, diluted to an inoculum of 1.5×10^6 CFU in 20 μL PBS and mixed with 160 μL heparinized human blood and 20 μL PBS with or without AZM (at 1/4× MIC, 1/2× MIC, and 1× MIC) in siliconized tubes. Tubes were incubated at 37°C and rotated for 15 minutes. Serial dilutions performed using sterile PBS and Triton-X 100 (0.025%) were plated on LB plates for bacterial enumeration. Percentage bacterial survival was defined as CFU enumerated at the end of the assay divided by CFU at time point 0 hours.

Statistics

Statistical analyses were performed using GraphPad Prism 6.0f (GraphPad Software). One-way analysis of variance, two-way analysis of variance, Mann-Whitney or unpaired Student *t*-test were used where appropriate. *P*-values <0.05 were regarded as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

This work was supported by the National Institute of Health grants K12-HD000850 (to ERU), U54-HD090259 (to GS and VN), 1U01AI124316-01 (to MK, GS, JP, and VN), and 1KL2TR001444 (to MK).

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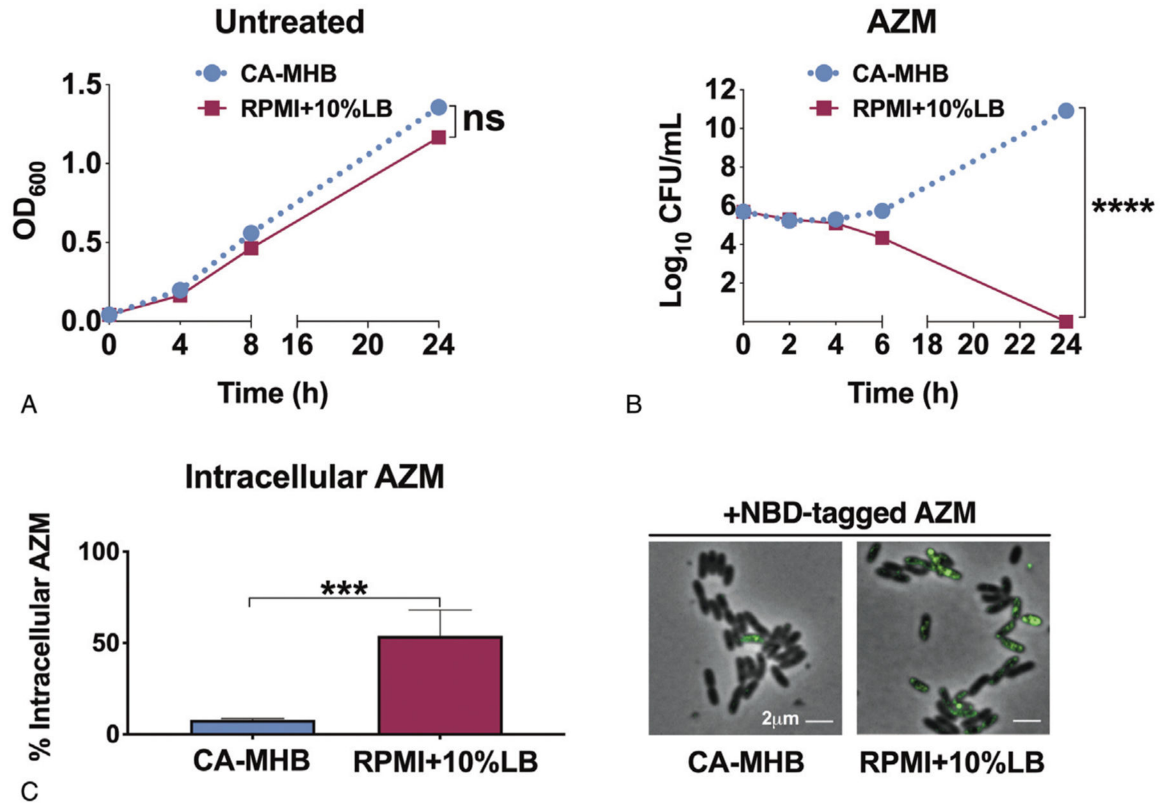

Figure 1. Azithromycin bactericidal activity against AX and intracellular bacterial entry observed under physiologic conditions.

A: Growth of AX Florida in bacteriologic (CA-MHB) versus supplemented tissue culture media (RPMI + 10% LB) at 4, 8, and 24 h was noted to be equivalent. B: Kinetic killing curve demonstrating AZM bactericidal activity against AX Florida but only in RPMI + 10% LB not CA-MHB. C: Fluorescence microscopy based bacterial cytological profiling performed using log-phase AX Florida treated for 3–4 h with 1.25 mg/L of green NBD-tagged AZM. Increased intracellular uptake (with >500 cells counted per condition) was noted in RPMI + 10% LB (53.9% ± 5.4%) not CA-MHB (7.9% ± 0.3%). Data are plotted as mean ± SEM and represent the combination of three experiments performed in triplicate. ***$P < 0.001$ or ****$P < 0.0001$ by two-way ANOVA (A and B) or Mann-Whitney test (C). ANOVA: analysis of variance; AX: *Achromobacter xylosoxidans*; AZM: azithromycin; CA-MHB: cation-adjusted Mueller Hinton Broth; RPMI + 10% LB: Roswell Park Memorial Institute 1640 + 10% Luria Bertani Broth; NS: no statistical significance; SEM: standard error of mean.

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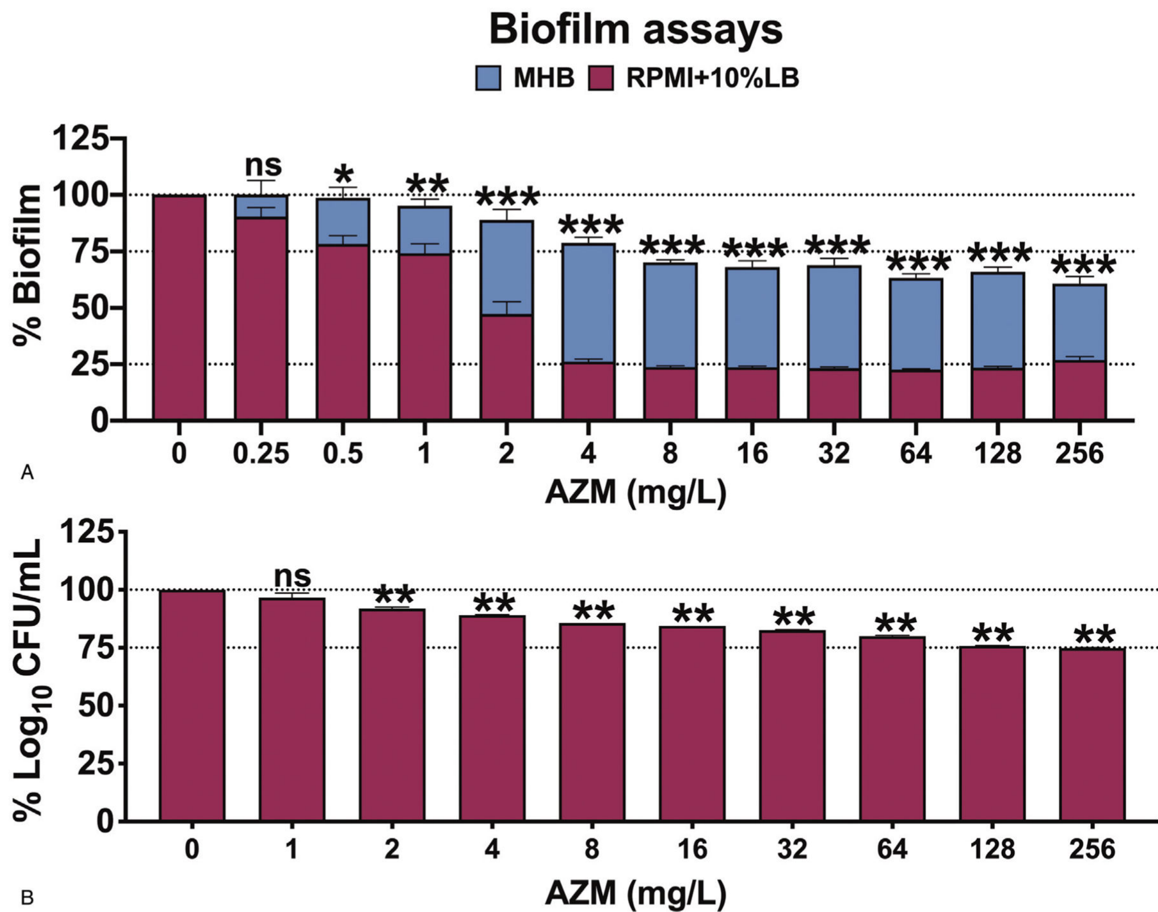

Figure 2. Azithromycin penetrates AX biofilm biomass.

A: AX Florida biofilm was formed following 48 h of growth at 37°C with 5% CO_2, washed to remove planktonic bacteria and subsequently treated with various concentrations of azithromycin (0.25–256 mg/L) for 48 h in bacteriologic (CA-MHB) or supplemented tissue culture (RPMI + 10% LB) media. Biofilm biomass (nonviable and viable bacterial cells) was determined by measuring absorbance after safranin staining and solubilization of the biofilm respectively. At 4 mg/L azithromycin was noted to decrease biofilm biomass by >75% in RPMI + 10% LB and <25% in CA-MHB. B: Remaining viable bacterial cells were enumerated following disruption of biofilm with vigorous pipetting and serial dilutions using sterile PBS, and plating on LB. A 10% reduction in viable bacterial cells was noted for biofilm in RPMI + 10% LB treated with 4 mg/L azithromycin. Data represent the mean ± SEM from a combination of three experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ or NS, no statistical significance by unpaired Student *t*-test comparing azithromycin treated bacteria in RPMI + 10% LB to CA-MHB (A) and by one-way ANOVA comparing azithromycin treated bacteria to untreated bacteria in RPMI+ 10% LB (B). ANOVA: analysis of variance; AX: *Achromobacter xylosoxidans*; AZM: azithromycin; CA-MHB: cation-adjusted Mueller Hinton Broth; RPMI + 10% LB: Roswell Park Memorial Institute 1640 + 10% Luria Bertani Broth; SEM: standard error of mean.

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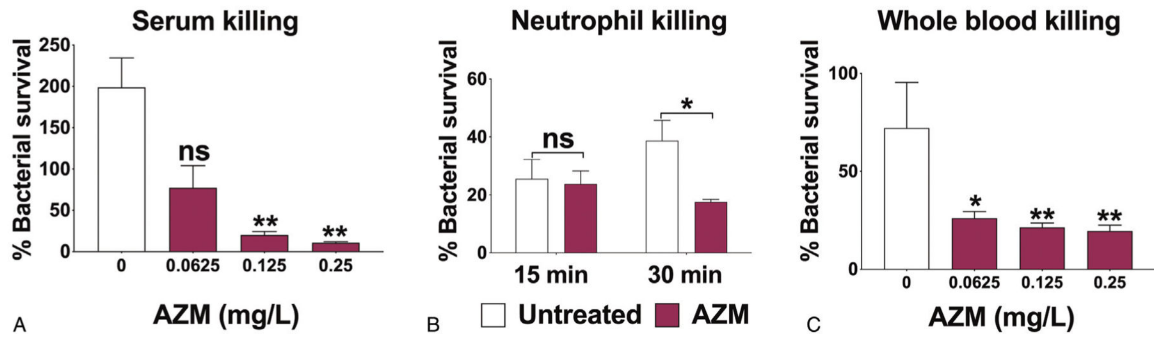

Figure 3. Azithromycin enhances innate immune mediated killing of multi-drug resistant AX.

A: Percentage survival of AX Florida bacteria following co-incubation with 10% human serum and azithromycin (1/4× MIC, 1/2× MIC, 1× MIC) or no antibiotic in a serum killing assay. B: Percentage survival of bacteria pre-treated with a sub-bacteriostatic concentration of azithromycin (1/8× MIC) versus untreated bacteria in a human neutrophil killing assay. C: Percentage survival of bacteria following co-incubation with human whole blood and azithrimycin (1/4× MIC, 1/2× MIC, 1× MIC) or no antibiotic in a whole blood killing assay. Data represent the mean ± SEM from a combination of three experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ or NS, no statistical significance by two-way ANOVA comparing pre-treated or treated bacteria to untreated bacteria (A–C). ANOVA: analysis of variance; AX: *Achromobacter xylosoxidans*; AZM: azithromycin; MIC: minimum inhibitory concentration; SEM: standard error of mean.

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Table 1

The MIC of AZM for 12 clinical AX strains in CA-MHB and RPMI + 10% LB.

	MIC (mg/L)	
AX strain	**CA-MHB**	**RPMI + 10% LB**
AX Florida	128	0.25
AU 33282	256	<0.25
AU 33765	128	0.5
AU 34276	16	0.5
AU 31610	>512	0.5
AU 34000	>512	8
AU 34819	>512	1
AU 31828	32	0.5
AU 34343	>512	2
AU 32231	256	1
AU 31974	512	1
AU 33282	>512	2

AX: *Achromobacter xylosoxidans*; AZM: azithromycin; CA-MHB: cation adjusted Mueller-Hinton Broth; MIC: minimum inhibitory concentration; RPMI + 10% LB: Roswell Park Memorial Institute 1640 + 10% Luria Bertani Broth.

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Table 2

Antimicrobial susceptibility of *Achromobacter xylosoxidans* Florida to AZM and corresponding combinational therapy with TZP and LL-37 assessed by minimum inhibitory concentration (MIC) and checkerboard assays.

	MIC (mg/L)						Checkerboard FICI (interpretation)		
***A. xylosoxidans* Florida**	**AZM**	**TZP**	**LL-37**	**AZM + TZP**	**AZM + LL-37**	**TZP + LL-37**	**$FICI_{AZM + TZP}$**	**$FICI_{AZM + LL\text{-}37}$**	**$FICI_{TZP + LL\text{-}37}$**
CA-MHB	128	4	8	64/1	64/0.125	4/0.25	0.75 (A)	0.515 (A)	1.03125 (I)
RPMI + 10% LB	0.25	8	4	0.125/0.25	0.03125/2	4/0.0625	0.531 (A)	0.625 (A)	0.515 (A)

Fractional inhibitory concentration indices (FICIs) were interpreted as follows: synergy, FICI of 0.50; additivity (A), FICI of >0.50 to 1.0; no interaction or indifference (I), FICI of >1 to 4; antagonism, FICI of >4. Note: LL-37 is expressed in mM.

AZM: Azithromycin; TZP: piperacillin/tazobactam; Cathelicidin LL-37: LL-37; CA-MHB: cation adjusted Mueller-Hinton Broth; RPMI + 10% LB: Roswell Park Memorial Institute 1640 + 10% Luria Bertani Broth.