SUPPLEMENTARY MATERIAL

Supplementary Methods and Results

Bacterial strains. *Acinetobacter baumannii* strains were obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank (Atlanta, GA) and the Military Resistance Surveillance Network (MRSN) at the Walter Reed Army Institute of Research (WRAIR; Silver Spring, MD). MIC data for antimicrobials other than apramycin was determined using a Sensititre GNX35 microdilution panel (Thermo Scientific, Waltham, MA) and interpreted using Clinical and Laboratory Standards Institutes (CLSI) criteria (1). Apramycin MICs were determined using CLSI reference broth microdilution methodology (2).

To identify *A. baumannii* strains suitable for pharmacodynamic studies in neutropenic mice, we selected twelve strains with apramycin MICs ranging from 2 to 64 $\mu g\ ml^{-1}$ (3), injected 10^6 CFU into murine thighs, and harvested tissue 24 hours later for CFU enumeration. In six strains, recovered CFU exceeded inoculum (see Table S1). From this latter set, three virulent strains were chosen for further experiments.

The first (MRSN7465) was multidrug-resistant (MDR), and the latter two (MRSN1450, FDA-CDC 278) were extensively drug-resistant (XDR) based on consensus criteria (4). More specifically, MRSN7465 was resistant to gentamicin and tobramycin, but susceptible to amikacin, doxycycline/minocycline, quinolones, sulbactam, and polymyxins. MRSN1450 was resistant to carbapenems, aminoglycosides and quinolones, but remained susceptible to doxycycline/minocycline, polymyxins, and sulbactam (1). FDA-CDC 278 was susceptible only to polymyxins.

Animals. CD-1 (ICR) female mice weighing 25-30g each were purchased from Charles River Laboratories, Inc. (Kingston, NY). Group size was three for each data point unless otherwise indicated.

Single-dose maximum tolerated dose (MTD) determination. Mice were injected intraperitoneally (IP) with 0, 20, 50, 100, 200, 500, 1000, 1500, or 3000 mg kg^{-1} apramycin (Alfa Aesar, Tewksbury, MA, USA). On day 3 post apramycin dosing, 100 μl of blood was collected in heparinized capillary tubes (Fisher Healthcare, Waltham, MA). Mice were immediately euthanized, and organs harvested and fixed in 10% neutral buffered formalin for histological evaluation.

Multi-dose MTD determination. Mice were injected IP with 500 mg kg^{-1} of apramycin for 14 consecutive days and observed for weight changes, morbidity, and mortality. Kidney and liver tissues were fixed for histological evaluation (see Fig S1).

Serum Creatinine Determination. Plasma creatinine was measured by high performance chromatography coupled to tandem mass spectrometry on a Shimadzu UFLC liquid chromatography system and API 5000 triple quadrupole mass spectrometer (Sciex, Framingham, MA). Calibrators were made from creatinine dissolved in phosphate buffered saline. Plasma samples and calibrators were first mixed with an equal volume of 0.1 mg dL^{-1} ^{13}C isotopically labeled creatinine internal standard. Samples were deproteinized by mixing with an equal volume of trichloroacetic acid, acetonitrile, and water (15:42.5:42.5) and centrifuged for 10 minutes at 14,000 rpm. 10 microliters of extracted supernatant was injected onto a Kinetex 150 x 2.1 mm C18 HPLC column (Phenomenex, Torrance, CA), eluted with 20 mM ammonium formate and 0.1% formic acid in Milli-Q (MilliporeSigma, Burlington, MA) purified water, and

creatinine and its isotopic standard were measured by multiple reaction monitoring using transitions m/z 114.1>44 and 115.1>45 respectively. All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO).

Pharmacokinetic (PK) and pharmacodynamic (PD) studies. Neutropenia was induced through IP injection of 150 mg kg^{-1} or 100 mg kg^{-1} cyclophosphamide (Sigma-Aldrich) in 100 µl sterile saline four days and one day prior to PK and PD experiments, respectively. Uranyl nitrate (Electron Microscopy Sciences, Hatfield, PA) was also injected IP at 5 mg kg^{-1} three days prior to PK and PD experiments, as previously described, to establish renal clearance more closely resembling that in humans (5). For PK analysis, apramycin was injected subcutaneously at 20, 80, or 500 mg kg^{-1}. Blood was collected from the mouse tail vein immediately prior to and at 0.5, 1, 3, 5, 8, 24 hours post injection with heparinized capillary tubes and centrifuged at 1500 relative centrifugal force for 10 minutes. Plasma was frozen at -20˚C for subsequent analysis to determine apramycin levels. Both AUC and C_{max} are expressed as the mean and standard deviations of determinations from three (20 and 80 mg kg^{-1}) or four (500 mg kg^{-1}) mice per dosing group.

Apramycin/Tobramycin LC-MS/MS method. Plasma samples were thawed, and 10 µL of plasma was mixed with 10 µL of tobramycin internal standard dissolved in phosphate buffered saline at concentration of 10 µg mL^{-1} (Sigma-Aldrich). Apramycin and tobramycin internal standard were extracted by protein precipitation by mixing with an equal volume of precipitation solution comprised of trichloroacetic acid, acetonitrile, and water (15:42.5:42.5 v/v). Proteins were pelleted by centrifugation and supernatant was transferred to 96-well plate for analysis. Assays were calibrated using apramycin dissolved in phosphate buffered saline.

Methodology for apramycin quantification was adapted from a previously described tobramycin assay (6). Specifically, 10 microliters of deproteinized plasma and internal standard was injected by autosampler for analysis. Apramycin and internal standard were resolved using ion pairing chromatography and measured using multiple reaction monitoring (MRM) tandem mass spectrometry (intra-assay CV 2.9%). Liquid chromatography was performed using a Kinetex C18 HPLC column, 3.0 x 50 mm, 2.6 μm bead size, (Phenomenex, Torrence, CA) on a Shimadzu Prominence UPLC with autosampler (Shimadzu Scientific Instruments, Columbia, MD). Analytes were resolved using a gradient of 20 mM heptafluorobutryic acid (HFBA) in water (buffer A) and eluted with 20 mM HFBA in acetonitrile (buffer B). For metabolite monitoring, the HPLC was coupled to an API 5000 triple quadrupole mass spectrometer with Turbo V electrospray ion source (Sciex, Framingham, MA). MRM spectra were acquired using Analyst 1.6 software and peaks were quantified by their areas under the curve (AUC). Apramycin AUC values were normalized by dividing by the AUC values for their respective tobramycin internal standard peaks. Apramycin calibrator solutions (0 – 100 μg mL^{-1}) were measured in the same fashion, and the resulting standard curve was used to convert normalized AUC values into apramycin concentrations. Additional liquid chromatography and mass spectrometer settings are detailed in Table S2 and Fig S2.

Table S1. Growth of selected *A. baumannii* strains in the murine neutropenic thigh model.

A. baumannii Strain	Apramycin MIC (μg ml^{-1})	CFU thigh^{-1}
MRSN7465	**2**	**1.2x10^{8}**
MRSN0915	2	No Growth[a]
MRSN7520	4	No Growth
MRSN11712	4	No Growth
MRSN7112	8	5x10^{6}
MRSN7322	8	No Growth
MRSN15067	16	6x10^{7}
MRSN1450	**16**	**6x10^{7}**
FDA-CDC0298	32	No Growth
MRSN2400	32	5x10^{7}
FDA-CDC0278	**64**	**2x10^{6}**
MRSN15081	64	No Growth

Strains selected for *in vitro* and *in vivo* experiments highlighted in bold.

[a]Limit of detection = 1 x 10^{6} CFU per thigh.

Table S2. Metabolite MRM transitions, mass spectrometer and liquid chromatography settings.

Ion source settings		
Curtain gas: 17	GS1: 40	GS2: 40
Ion spray voltage: +2500V	Source temp: 200°C	Positive ion mode
MS settings		
Declustering potential: 100	Entrance potential: 10	Collision energy: 35
Collision gas: 7	CXP: 16	
MRM mass transitions (parent ion m/z -> daughter ion m/z)		
Apramycin (quantifier)	Apramycin (qualifier)	Tobramycin internal stand.
540.6 > 217.2	540.6 > 199.0	468.2 > 163.3
HPLC gradient elution program (flow rate 0.6 mL/min)		
Time (min)	% Buffer B	
0	5%	
0 – 3.5	5% - 35% gradient	
3.6 – 3.9	100% wash	
4 - 6	5% re-equilibration	

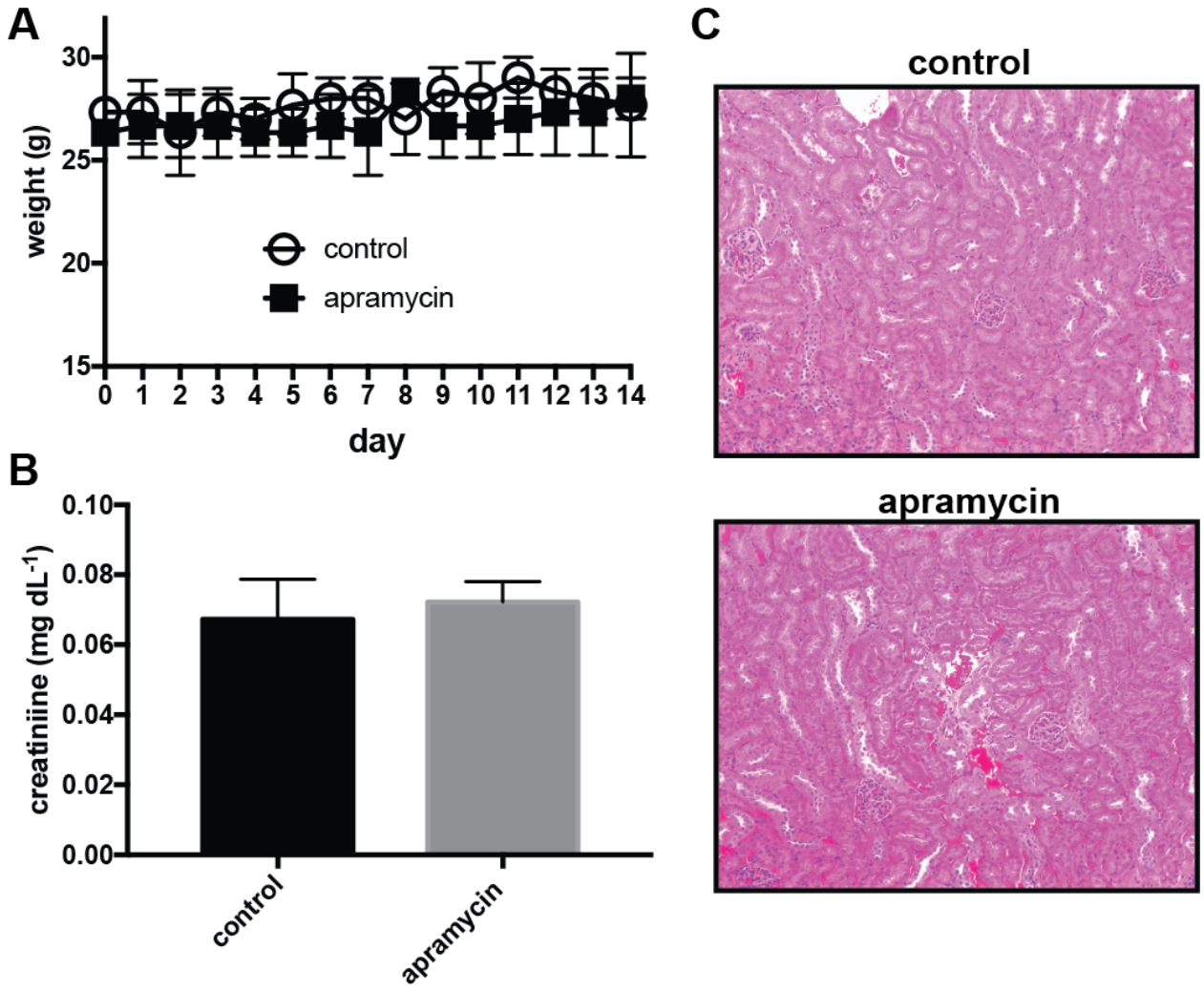

Figure S1. Maximum-tolerated dose. CD-1 mice were dosed with 500 mg kg^{-1} IP each day for 14 days. **(A)** Apramycin treated mice and controls showed no difference in body weight during the course of the experiment. **(B)** Plasma creatinine measurements were also indistinguishable when measured 24 h after the final apramycin dose. **(C)** There was no evidence of pathology in kidney tubules.

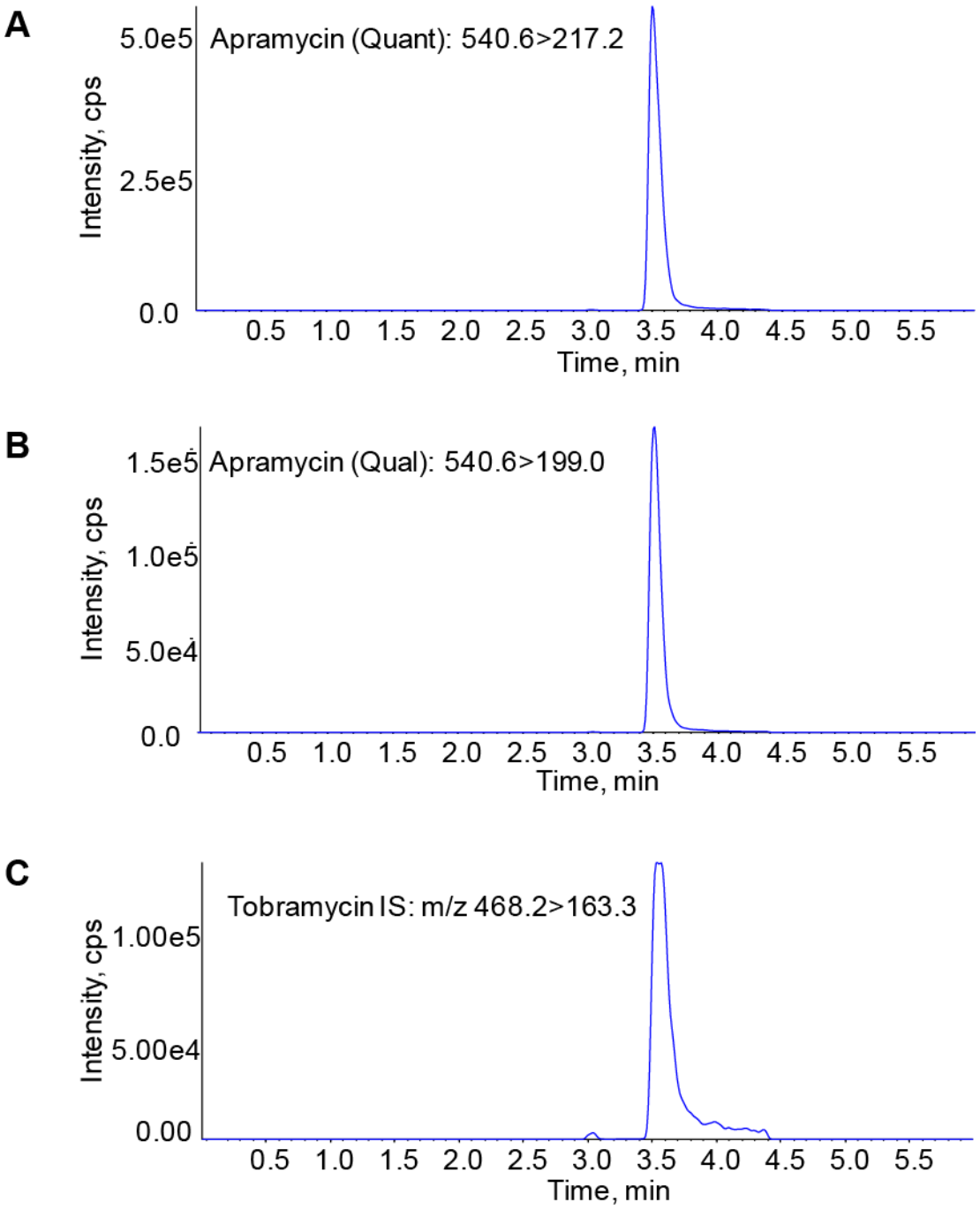

Figure S2. LC-MS/MS chromatograms of apramycin and tobramycin internal standard multiple reaction monitoring peaks. (A) Transition 540.6>217.2 was used to quantify apramycin. **(B)** Transition 540.6>199.0 was included as a confirmatory qualifier peak for apramycin. **(C)** Transition 468.2>163.3 was used to monitor tobramycin internal standard.

Supplementary References

1. **Clinical and Laboratory Standards Institute.** 2017. Performance standards for antimicrobial susceptibility testing; twenty-sixth informational supplement. CLSI document M100-S27. Clinical and Laboratory Standards Institute, Wayne, PA.
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3. **Kang AD, Smith KP, Eliopoulos GM, Berg AH, McCoy C, Kirby JE.** 2017. *In vitro* Apramycin Activity against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Diagn Microbiol Infect Dis **88:**188-191.
4. **Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL.** Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect **18:**268-281.
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6. **Bernardi PM, Barreto F, Dalla Costa T.** 2017. Application of a LC-MS/MS method for evaluating lung penetration of tobramycin in rats by microdialysis. J Pharm Biomed Anal **134:**340-345.