Supplementary Materials and Methods for;

Meropenem-clavulanic acid shows activity against *Mycobacterium tuberculosis in vivo.*

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Bacterial strains and macrophages. *Mycobacterium tuberculosis* strains H37Rv and HN878 (J. Musser) were cultured from frozen aliquots (–80°C) in Middlebrook 7H9 broth with 10% albumin-dextrose and supplemented with 0.05 % Tween 80 (henceforth 7H9 medium) according to standard mycobacterial culture protocols. For growth on solid medium *M. tuberculosis* was grown on 7H11 middlebrook agar supplemented with 10% oleic acid/dextrose/albumin according to standard mycobacterial culture protocols. Bacteria were subcultured and grown to an early exponential phase before experimental use. J774A.1 murine macrophage-like cell line (American Type Culture Collection) was cultured from cryopreserved aliquots in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4mM L-Gln, 4.5g/L glucose, 15mM HEPES, 0.5mM pyruvate, and 1X nonessential amino acids and maintained in a humidified incubator (37°C, 5% CO_2). Macrophages were grown to approximately 70% confluence before harvesting for use in intracellular susceptibility assays.

Mice and Rabbits. New Zealand White (NZW) female rabbits (Charles River Laboratories International, Wilmington, MA) were used in pharmacokinetic studies to obtain PK parameters required for a drug efficacy trial. Preliminary efficacy studies utilized C57Bl/6 mice (Charles

River Laboratories International, Wilmington, MA), which were aerosol infected (100-200 CFU/mouse) with *M. tuberculosis* strain H37Rv as previously described (8). All animal work was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the National Institute of Allergy and Infectious Disease approved the experiments described herein under LCID-3E protocol (NIH Intramural Research Program: A-4149-01), and all efforts were made to minimize suffering. All *M. tuberculosis* infected tissues were maintained and processed in a certified BSL3 facility until the viable organisms had been inactivated.

Antimicrobials. Carbapenems surveyed for intracellular activity in combination with clavulanic acid (Sigma) were meropenem (MERREM, AstraZeneca), imipenem (PRIMAXIN, Merck), ertapenem (INVANZ, Merck), doripenem (DORIBAX, Ortho-McNeil-Janssen), and faropenem (AK Scientific). Intravenous formulations were used for all assays and animal studies. Control drugs used for the assays were rifampicin and isoniazid (Sigma).

Table S1. MIC and MBC evaluations

Beta -Lactam	MIC /MBC(μM)	
	*H37Rv**	*HN878*[†]
meropenem	0.51 / 1.0	0.65 / 3.3
imipenem	0.52 / 2.6	0.84 / 3.3
ertapenem	0.41 / 3.3	nd
doripenem	0.23 / 0.9	nd
faropenem	0.64 / 1.4	0.44 / 0.9

** 100μM clavulanic acid; † 50μM clavulanic acid*

Chromatograpy by LCMS. Plasma extracts for pharmacokinetics and samples from stability testing were analyzed using an Agilent 1100 Series LC/MS (Wilmington, DE, USA) with a diode array UV/Vis detector (DAD, model G1315A) and a single quadrupole mass-selective detector (MSD, model G1946DSL). Chromatographic separations were performed on a Luna NH_2 column, 100 x 2mm i.d., 3mm (Phenomenex) with an NH_2 security guard column, 4 x 2mm i.d., 5 mm (Phenomenex). The mobile phase used was 20mM ammonium acetate and acetonitrile (40:60, v/v). The separation occurred under isocratic conditions with a flow rate of 0.30 mL/min at a column temperature of a 25°C. Meropenem and doripenem (IS) were detected by both UV and MS (m/z) using negative ion mode for the parent ions 383 and 420, respectively.

Determination of the MIC and MBC99. Minimal inhibitory concentrations (MIC) for the various drug compounds were evaluated using the standard 96-well microbroth dilution assay for *M. tuberculosis* and were read by visual inspection. Two-fold serial dilutions of antibiotic in 7H9 medium were first prepared in 96-well round-bottomed microtitre plates (Nunc, USA). An equivalent volume of bacterial broth containing $\sim 10^5$ bacterial CFU/mL (in the presence or absence of 50-100 mM clavulanic acid) was added to each well to give final concentrations of the antimicrobial drugs ranging from 50mg/mL to 0.025mg/mL and the plates were incubated aerobically at 37°C for 7 days. The MIC was recorded as the lowest concentration of drug that prevented visible growth. Minimal bactericidal concentration (MBC) tests were performed directly from the microtitre plates used to determine drug MICs. Wells showing inhibited growth to no growth were resuspended, diluted 1/10 in 7H9 medium, and plated in duplicate onto 7H11 agar plates. Plates were incubated for 4 weeks at 37°C. At 4 weeks, CFU were enumerated. The well containing a minimum of a 3-log reduction in CFU/mL compared to the initial inoculum of $\sim 10^5$ bacterial CFU/mL was defined as the MBC.

Stability study. The stability of meropenem and other carbapenems (faropenem, ertapenem, doripenem, and imipenem) in water, 7H9 medium, and supplemented DMEM was assess by preparing 50 mg/ml stocks in individual HPLC vials (with inserts) at a volume of 50 mL per vial and incubating at 37°C. Individual vials were removed at various time points and analyzed by LCMS as described above.

Intracellular susceptibility in mouse macrophages. J774A.1 cells were harvested at around 70% confluence and cell suspensions were plated in 24-well tissue culture plates at a concentration of 10^5 cells per well in supplemented DMEM. Monolayers were allowed to stabilize 24 hrs prior to infection. Macrophages were infected with 10^5 *M. tuberculosis* bacilli and incubated 4 hours at 37°C. Wells were then thoroughly washed with media to remove extracellular bacilli and media was replaced with DMEM containing different concentrations of carbapenems and 200mM clavulanic acid or control drugs isoniazid and rifampicin as noted in Table 2. All drug combinations and controls were tested in triplicate. Carbapenems in combination with clavulanic acid were spiked every 8 hours due to their instability whereas control drugs were exchanged on day 2 and day 4 over a 6-day assay. In order to maintain the volume in the wells when dosed three times daily, aliquots of media were removed prior to drug addition at equivalent volumes. Media changes were performed as needed and cells incubated at 37°C, 5% CO_2 for the duration of the experiment. Wells were observed daily under the microscope to assess cell viability or detachment. None of the compounds had any discernable cytotoxicity or cytopathic effects over the six day regimen. At each treatment time point, monolayers were gently washed with PBS and lysed using 0.1% SDS/0.05% Tween 80 in PBS. Lysates were serially diluted (1:10) in 7H9 medium and plated on nutrient 7H11 agar (10% oleic acid-albumin-

dextrose complex). Bacterial colony formation was enumerated after 4 weeks incubation at 37°C. Data are represented as $\log_{10}$ mean CFUs for each drug combination and analyzed using a one way ANOVA and Bonferroni's multiple comparison test using Graphpad PRISM 5.0.

Mouse Efficacy study. C57Bl/6 mice were aerosol infected as previously described ((8)) and allowed 3 months to establish a chronic infection. The study consisted of 3 treatment groups with 10 mice per group at dosages of 300mg/kg meropenem, 300 mg/kg meropenem / 50mg/kg clavulanic acid, and a PBS control, which were delivered by twice daily subcutaneous (sq) injections and oral gavage (po), respectively. Merrem I. V. (500mg) was reconstituted with 11.0 mL of PBS (45.5 mg/mL) and the appropriate dilution (15.0 mg/mL) made just prior to the 0.5 mL sq injection per (on average) 0.025kg mouse. Clavulanic acid was dissolved in PBS at 12.5mg/mL and delivered po at 0.1 mL per mouse with each dose prepared immediately prior to gavage. Lungs and spleens were harvested from five mice after either 2 weeks or 4 weeks of treatment for enumeration of CFU by plating on 7H11 supplemented agar. The difference between the three groups was compared using a 1 way ANOVA and the Bonferroni's multiple comparison test.

Rabbit PK Studies. Pharmacokinetic (PK) parameters were assessed in two studies containing four rabbits each. The first PK study involved dosing the animals with 75mg/kg meropenem and 125mg/kg clavulanic acid by I.V. injection through a catheter placed in the left ear vein. Another catheter was place in the right ear vein and blood withdrawn at time points 0, 20, 40, 60, 90, 120, 240 and 300 minutes. Blood samples (approximately 1mL) were collected in Li heparin containing tubes and immediately centrifuged at 4000 rpm for 10 min to isolate plasma. Plasma samples were stored at -80°C until drug extractions could be performed. Extractions utilized

50mL of thawed plasma spiked with 20mL of doripenem (2.1 mg/mL) as an internal standard (IS). A 10mL aliquot of methanol was added to help disperse components upon vortexing and limit clumping of protein precipitates upon addition of 450mL of acetonitrile. This provided a final extraction mixture consisting of 85% acetonitrile. The mixture was vortexed for 2 minutes and then centrifuged at 14000 rpm for 10 min to pellet precipitated proteins and plasma components. Extractions were analyzed by LCMS using a 20ml injection as described above. Clavulanic acid was not detectable by UV or MS. Meropenem and doripenem were detected by both UV and MS (m/z) for the parent ions 383 and 420, respectively. Initial quantification for percent recoveries using naïve plasma and standard calibration curves were also performed. Data from calibration curves provided slope values for the quantification of drug concentrations for each time point. Mean values for triplicate analyses per time point with standard deviations were then graphed using Graphpad PRISM 5. Data analysis using a single decay curve function and AUC data analysis provided values for C_0 (estimated concentration at time zero), $t_{1/2}$ (plasma half-life), and AUC (area under the curve). The second PK study was performed identical to the first with the addition of 75mg/kg cilastatin.