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


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Abbreviations; *Mtb* (*Mycobacterium tuberculosis*); *t*$_{1/2}$ (half-life); AUC (area under the time-concentration curve); DHP-1 (dihydropeptidase I); TB (tuberculosis); MDR (multi drug resistant); XDR (extensively drug resistant); IV (intravenous); MIC (minimum inhibitory concentration); MBC (minimum bactericidal concentration); *C*$_{max}$ (maximum serum concentration); CFU (colony-forming units); IS (internal standard)

Running title: *In vivo* activity of Meropenem-clavulanate against TB
Abstract

The carbapenems imipenem and meropenem in combination with clavulanic acid reduced the bacterial burden in *Mycobacterium tuberculosis* (*Mtb*)-infected macrophages by 2-logs over six days. Despite poor stability in solution and a short half-life in rodents, treatment of chronically infected mice revealed significant reductions of bacterial burden in the lungs and spleens. Our results show that meropenem has activity in two *in vivo* systems but stability and pharmacokinetics of long-term administration will offer significant challenges to clinical evaluation.

The burden of tuberculosis (TB) continues to rise with significant increases in drug resistance in the developing world (19). Rising rates of multidrug resistant (MDR) and extensive drug resistant (XDR)-TB continues primarily due to inadequate treatment programs, poor laboratory capacity and the lack of public health infrastructures needed to manage the disease (19). Demonstration of activity against *M. tuberculosis* (*Mtb*) *in vitro* does not guarantee *in vivo* potency because of differences in the microenvironment within which bacteria reside (16). Recently the combination of meropenem with clavulanic acid (clavulanate) was shown to be active *in vitro* against *Mycobacterium tuberculosis*, including against MDR and XDR isolates (9). This suggested the possibility of repurposing carbapenems (fourth generation β-lactams) in combination with β-lactamase inhibitors to treat MDR and XDR-TB. β-lactams were previously thought to be ineffective against *M. tuberculosis* primarily due to the endogenous mycobacterial BlaC enzyme which effectively hydrolyzes them, however clavulanic acid is effective in inactivating this enzyme (8, 9, 14, 18). The *in vitro* MIC of the meropenem-clavulanate combination was less than 1 μg/mL and resulted in sterilization of aerobically grown cultures within 14 days (9).
Carbapenems are the most potent β-lactams and were developed in the 1980’s to enhance resistance to β-lactamases (4, 11). Meropenem is a broad-spectrum carbapenem active against several clinically relevant Gram-positive and Gram-negative aerobes and anaerobes (4). The bactericidal activity of meropenem results from the inhibition of cell wall synthesis through the inactivation of penicillin binding proteins (4, 20). Carbapenems are not very hydrolytically stable which limits drug administration to a controlled intravenous infusion (2). Meropenem is FDA-approved for the treatment of complicated skin and soft tissue infections, intra-abdominal infections (appendicitis, and peritonitis), and bacterial meningitis (1).

Clavulanic acid is FDA-approved as a β-lactamase inhibitor often administered with amoxicillin (the combination is marketed as Augmentin) to prevent hydrolysis of the active β-lactam (5).

MIC and MBC values for various carbapenems (meropenem, doripenem, faropenem, ertapenem, and imipenem) in combination with clavulanic acid were determined against H37Rv and the virulent Beijing strain used for rabbit infections, HN878 (15). All of these carbapenems when combined with clavulanic acid were highly active, with MICs ranging from 0.23 - 0.84μM and MBC99 ranging from 0.9 - 3.3μM for both strains. It was previously established by Cuffini et al., that meropenem penetrates macrophages and achieves intracellular concentrations high enough for active killing of intraphagocytic pathogens like S. aureus (3). In addition, plasma protein binding is reported to be only 2% (7), therefore binding to albumin in FBS supplemented DMEM would not be expected to be a significant factor. Meropenem has been reported to be thermally unstable in aqueous solutions (10, 12, 17), therefore we determined the stability of meropenem and the other carbapenems at 37°C in water, 7H9 broth medium, and the medium used in the infected macrophage in the susceptibility assay (DMEM) by LC/MS using a Luna NH2 column with a single quadrupole mass-selective detector (Agilent MSD Model G1946DSL). Meropenem was significantly less stable in DMEM than in either water or 7H9 medium with a
half-life of 8hrs compared to 38 hrs in 7H9, and >80 hrs in water. Other carbapenems had similarly short half-lives ranging from 3-10 hrs.

Because of this short half-life we examined intracellular killing by administering carbapenems to infected macrophages 3 times a day (every 8 hrs) with media changes every two days. No evidence of cell toxicity was found under these conditions even at concentrations as high as 400μg/mL of these carbapenems in combination with clavulanic acid up to 200μM (data not shown). Murine J774A.1 macrophages were then infected with H37Rv at a multiplicity of 1-2 bacilli per cell and subsequently treated with various carbapenems at concentrations equivalent to the known C_{max} values in humans receiving standard therapeutic doses (50μg/mL for meropenem, imipenem and ertapenem, 30μg/mL for doripenem and 10 μg/mL for faropenem). Two control drugs rifampicin and isoniazid were also dosed once a day at the human equivalent C_{max} (5μg/mL) and also had their media replaced on days 2 and 4. Treated macrophages were lysed at the indicated times and serial dilutions were plated for enumeration of CFU (Figure 1). These data showed significant growth arrest of intracellular H37Rv by two days (p=0.05) and highly significant killing with all carbapenems by four and six days (p=0.01 and 0.001 for meropenem, for example, at 4 and 6 days, respectively). At six days, the carbapenems demonstrated a 1.5 - 2.0 log reduction in bacterial numbers compared to untreated controls with imipenem and meropenem having the largest effect. For comparison, isoniazid and rifampicin controls demonstrated a 2-log kill over the same time period.

To examine efficacy in an animal model, we infected 30 C57Bl/6 mice with *M. tuberculosis* H37Rv and allowed the infection to progress to a chronic stage. Three months after infection the mice were divided into three groups of 10 and therapy was initiated. One group was treated with meropenem alone at 300mg/kg by subcutaneous injection twice daily, a second group received meropenem at the same dose but in addition was given twice daily...
50mg/kg oral doses of clavulanic acid, the final group received vehicle control treatment (PBS).

Five mice from each treatment group were sacrificed 2 weeks later with the remaining five sacrificed at 4 weeks of treatment and bacterial burden in both lung and spleen were enumerated. The data in Figure 2 illustrates that meropenem did in fact show activity in this very stringent chronic mouse model. Compared to the control group both meropenem alone or in combination with clavulanic acid significantly reduced CFU in both lung and spleen after two weeks of treatment (p=0.008 and p=0.002 ANOVA, respectively) with a further modest reduction in bacterial burden observed after four weeks of therapy. Clavulanic acid did not show a statistically significant enhancement of killing in the lungs or spleen at either time point.

We also planned to conduct an efficacy study in TB-infected NZW rabbits, which develop hypoxic pulmonary lesions (16), therefore we determined pharmacokinetic parameters and tolerability in uninfected rabbits for meropenem-clavulanate at doses of 75mg/kg and 125 mg/kg, respectively. Drugs were delivered sequentially by I.V. bolus in four rabbits. Blood was drawn at scheduled time points and plasma obtained for determination of serum concentrations. Data obtained from the four animals was pooled and evaluated using Graphpad PRISM 5.0 with single decay curve and AUC analyses. PK plots are a representation of pooled data from all four animals (Figure 2). The mean C₀, t½, and AUC were 2.5 mg/mL, 12.1 min, and 15.2 mg-min/mL, respectively, for meropenem-clavulanic acid co-administration. Because of this relatively low exposure we also repeated this PK study with the addition of cilastatin, to prevent hydrolysis of meropenem by DHP-1 (renal dihydropeptidase 1). Treatment of rabbits with cilastatin (75 mg/kg) increased exposure and the corresponding PK parameters were 3.2 mg/mL, 22.0 min, and 51.5 mg-min/mL, respectively. Cilastatin therefore increased the exposure by 4 times even though the half-life was not significantly increased. Unfortunately, the increased exposure caused severe diarrhea that resulted in the death of 2 of the 4 animals 48 hours post
drug exposure. Due to rapid adverse effects in the digestive tract of the rabbit, efficacy in this species was no longer pursued.

Fukasawa et al. reported that humans have a significantly lower activity of DHP-1 compared to other species and that rabbits had a relatively high degree of enzymatic activity (6). Therefore, we hoped that co-administration of cilastatin would substantially reduce renal hydrolysis and help maintain the levels of active compound in circulation. It is noted that meropenem is considered more stable to DHP-1 hydrolysis than imipenem due to its 1-β methyl side chain and the steric hindrance associated with the secondary amine of the C-2 side chain. No information regarding the other carbapenems and DHP-1 hydrolysis in rabbits was identified at this time. Similar complications were reported by Topham et al., for meropenem administered in doses above 125mg/kg (13). The use of a nonhuman primate model was considered as an alternative, however, according to Topham’s study, cynomolgus monkeys that received seven daily intravenous doses of meropenem or imipenem (180 and 500 mg/kg) also had episodes of diarrhea in addition to emesis shortly after dosing. Those monkeys also experienced a reduction in body weight of 12.5 to 25% accompanied by a decreased consumption of food and water, making further therapeutic studies for the treatment of tuberculosis using meropenem or imipenem combinations with clavulanic acid less approachable.

In summary, this report demonstrates that meropenem-clavulanic acid in combination exhibits bactericidal activity in macrophages and modest activity in murine models of disease. Further assessment in animal models is complicated by the comparatively high activity of the renal DHP-1 enzyme in all of the animal models used for experimental chemotherapy of TB. Additional animal studies that utilize controlled infusions of meropenem (as is done clinically for
human use) may provide more efficacy information. The human isoform of DHP-1 has comparably lower activity and these studies suggest that prospective clinical evaluation in humans with chronic, non-responsive disease is warranted.
FIG. 1. Intracellular susceptibility of H37Rv. β-lactams evaluated in combination with 200μM clavulanic acid demonstrated similar killing of intracellular *M. tuberculosis*: untreated controls (•), rifampicin (■), isoniazid (▲), meropenem (x), faropenem (○), doripenem (+), ertapenem (θ), and imipenem (ο). Imipenem was most active at killing with a 2.0 log reduction of bacilli compared to untreated controls. By day 2 all of the drug treated groups are significantly different than the control (p<0.05, repeated measures ANOVA), by day 4 and 6 this difference became even more significant (p<0.01 and p<0.001, respectively for all drug treatments compared with control).
FIG. 2. Mouse efficacy of study. Chronically *M. tuberculosis*-infected mice were treated with 300mg/kg meropenem, 300mg/kg meropenem with 50mg/kg clavulanic acid or PBS (control) for 2 (white bars) or 4 (grey bars) weeks. Treatment with meropenem or meropenem and clavulanic acid for either two or four weeks reduced the bacterial burden in the lungs (P=0.008) and spleen (P=0.002).
FIG. 3. Pharmacokinetic analysis of meropenem-clavulanic acid in NZW rabbits. Plasma concentration as a function of time plotted using nonlinear single decay curve analysis with AUC data analysis; (■) meropenem (75mg/kg)/clavulanic acid (50mg/kg) and (▲) meropenem (75mg/kg)/clavulanic acid (50mg/kg)/cilastatin (75mg/kg). Ranges for C₀ (extrapolated initial concentration after IV bolus), t½ (half-life), and AUC (area under the curve) values for all four rabbits are reported with mean values from pooled data. The addition of cilastatin increased the values to 3.2mg/mL, 22.0min, and 51.5mg-min/mL, respectively.
References


