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In Vitro Susceptibility of *Mycobacterium tuberculosis* Isolates to an Oral Carbapenem Alone or in Combination with β-Lactamase Inhibitors

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We evaluated the antituberculosis (anti-TB) activity of five β-lactams alone or in combination with β-lactamase inhibitors against 41 clinical isolates of *Mycobacterium tuberculosis*, including multidrug-resistant and extensively drug-resistant strains. Of those, tebipenem, an oral carbapenem, showed the most potent anti-TB activity against clinical isolates, with a MIC range of 0.125 to 8 μg/ml, which is achievable in the human blood. More importantly, in the presence of clavulanate, MIC values of tebipenem declined to 2 μg/ml or less.

Tuberculosis (TB) resistant to all the first- and second-line anti-TB drugs, first reported as totally drug-resistant TB (TDR-TB) in 2009, is beginning to jeopardize public health worldwide (1). TDR-TB has been considered to be an incurable disease for which no therapeutic alternatives exist (2). To overcome such drug-resistant TB (DR-TB), 10 anti-TB drug candidates are currently undergoing clinical trials (3). However, some of the trials have been postponed or suspended due to undesirable adverse reactions resulting from long-term and multidrug administration. Therefore, further investigation of novel candidates and regimens is urgently needed to establish and optimize the treatment of refractory DR-TB. According to the World Health Organization and recent reports, the efficacy, safety, and tolerability of amoxicillin plus clavulanate (AMX-CLA) and carbapenems alone or in combination with CLA have been demonstrated in patients suffering from intractable DR-TB (4–8). The clinical usefulness of AMX-CLA, however, has been disputed because of insufficient evidence. Furthermore, although carbapenems such as meropenem (MEM) and imipenem (IPM) are reasonable alternatives to parenteral drugs such as aminoglycosides and cyclic peptides, the route of administration is unfavorable for TB patients and is regarded as unsuitable for prolonged therapy (9). Moreover, MEM needs to be used with AMX-CLA for the time being, because there is no combination drug containing MEM and CLA (10). For these reasons, there is a strong need for a more realistic β-lactam. In the present study, we evaluated the anti-TB activities of five β-lactams alone or in combination with β-lactamase inhibitors against clinical isolates of *Mycobacterium tuberculosis*, including multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) strains.

MICs for each agent against the *M. tuberculosis* H37Rv laboratory strain and 41 clinical isolates that are stored in our laboratory were determined two times by means of a broth microdilution method according to a previous report (11). These strains were precultured until the mid-log phase in Middlebrook 7H9 broth (Difco, United States) supplemented with 10% ADC (5% bovine serum albumin fraction V, 2% dextrose, and 0.005% bovine liver catalase), including 0.05% Tween 80. The bacterial culture was suspended in fresh 7H9-ADC and adjusted to a McFarland tube no. 1 (optical density at 530 nm [OD_{530}] = 0.16 to 0.2) and then diluted 1:100 using the same broth. Two-fold serial dilutions of each agent were prepared in a volume of 100 μl using 96-well microtiter plates, and then 100 μl of bacterial suspension was inoculated into each well. The plates were incubated in an atmosphere of 5% CO_2 with a relative humidity of 95% at 37°C for 7 to 10 days.

Agent suppliers and the sample preparation procedure are shown in Table S1 in the supplemental material. MIC_{50} and MIC_{90} values of each agent against clinical isolates were defined as MICs at which either 50% or 90% of strains were inhibited. The width of MIC distribution was represented as a binary number. The extent of drug resistance was assessed based on the criteria in accordance with previous reports and information published by the European Committee on Antimicrobial Susceptibility Testing (12–14). As for pyrazinamide (PZA) resistance, sequence analysis of the *pncA* gene was implemented using the primers F1 (5′-GTGATCTATC CCGCCGGTTG-3′) and R1 (5′-GAACCCACCGGGTCTTCGA C-3′). An 830-bp amplicon contains the complete *pncA* coding region and a putative promoter region (15). Briefly, PCRs were performed using PCR master mix (Promega, United States) under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of denaturation (94°C for 0.5 min), annealing (63°C for 0.5 min), and extension (72°C for 1.5 min), and a final extension at 72°C for 7 min. PCR products were then purified using PCR cleanup gel extraction (Macherey-Nagel, Germany). DNA sequencing was performed via BigDye Terminator v3.1 cycle sequencing with an Applied Biosystems 3130 genetic analyzer (Life Technologies, United States). As shown in Table S2 in the supplemental material, one frameshift and 11 point mutations, includ-

Received 3 June 2014 Returned for modification 29 June 2014
Accepted 27 August 2014
Published ahead of print 15 September 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.03539-14.

doi:10.1128/AAC.03539-14

TABLE 1 Antimicrobial activity of each drug against *Mycobacterium tuberculosis* H37Rv

Agent (abbreviation)	Inhibitor	Concn of inhibitor (μg/ml)	MIC (μg/ml)
Meropenem (MEM)	None	0	1–2
	CLA	2	0.25–0.5
	CLA	4	0.5
	AVI	2	0.5–1
	AVI	4	0.25–0.5
Biapenem (BPM)	None	0	1–2
	CLA	2	0.5
	CLA	4	0.25–0.5
	AVI	2	0.5
	AVI	4	0.5–1
Tebipenem (TBM)	None	0	0.5–1
	CLA	2	0.125
	CLA	4	0.125
	AVI	2	0.25
	AVI	4	0.125–0.25
Ampicillin (AMP)	None	0	16–32
	CLA	2	0.25–0.5
	CLA	4	0.25
	AVI	2	1–2
	AVI	4	0.5–1
Amoxicillin (AMX)	None	0	16–32
	CLA	2	0.25–0.5
	CLA	4	0.25
	AVI	2	1–2
	AVI	4	0.5–1
Clavulanate (CLA)			64
Avibactam (AVI)			512
Isoniazid (INH)			0.031–0.063
Ethambutol (EMB)			1–2

TABLE 2 Antituberculosis activities of each drug against drug-susceptible clinical isolates of *Mycobacterium tuberculosis* ($n = 20$)

Agent (abbreviation)	Inhibitor	MIC range (μg/ml)	Distribution width	MIC (μg/ml) 50%	MIC (μg/ml) 90%
Meropenem (MEM)	None	1–32	5	16	32
	CLA	≤0.063–8	≥7	2	4
	AVI	0.25–16	6	4	8
Biapenem (BPM)	None	1–32	5	16	32
	CLA	0.25–8	5	2	4
	AVI	0.5–8	4	4	8
Tebipenem (TBM)	None	0.25–8	5	4	8
	CLA	≤0.063–2	≥5	1	1
	AVI	≤0.063–4	≥6	1	2
Ampicillin (AMP)	None	2–>128	>6	64	128
	CLA	≤0.125–32	≥8	2	4
	AVI	≤0.125–32	≥8	8	16
Amoxicillin (AMX)	None	2–>128	>6	128	128
	CLA	≤0.125–32	≥8	2	16
	AVI	≤0.25–32	≥8	16	16
Clavulanate (CLA)		32–256	3	128	256
Avibactam (AVI)		64–>512	>2	NC	NC
Isoniazid (INH)		0.25–0.5	1	0.5	0.5
Rifampin (RIF)		≤0.125	NC[a]	≤0.125	≤0.125

[a] NC, not calculated.

ing two synonymous mutations, were identified. These mutation points, except for the silent mutation, have been reported to confer PZA resistance according to the TB Drug Resistance Mutation Database. Unfortunately, the PCR product of XDR-TB strain no. 7 was not detected even using two sets of primers, implying that the anteroposterior region of the *pncA* gene is completely disrupted (see Table S2). To confirm the existence of genome DNA, PCRs were carried out using the previously reported primer sets for amplifying the *rpoB* gene and *IS6110* insertion sequence (16, 17). All the PCR products of DR-TB strains for *rpoB* and *IS6110* genes were detected (data not shown).

To our knowledge, CLA has been set at 2.5 to 8 μg/ml when assessing *in vitro* whether CLA potentiates the anti-TB activities of β-lactams in *in vitro* studies (11, 18–20). In clinical use, CLA has been principally coadministered with AMX either at 125 mg or 250 mg three times a day, with peak serum levels reported to be 2.55 μg/ml and 5.9 μg/ml, respectively (21, 22). In light of these findings, we determined MICs for β-lactams alone or in the presence of β-lactamase inhibitors to be fixed at either 2 μg/ml or 4 μg/ml. As shown in Table 1, there were striking differences in MIC values between β-lactams alone and β-lactams plus β-lactamase inhibitors, whereas an increased concentration of β-lactamase inhibitors barely affected the MIC values. The best synergistic effect was observed in aminopenicillins plus CLA. For instance, susceptibility of *M. tuberculosis* H37Rv to AMX increased by 32- to 128-fold owing to the presence of CLA (Table 1). Among the five β-lactams exposed solely, tebipenem (TBM) exhibited the most potent anti-TB activity against *M. tuberculosis*, with a MIC value of 0.5 to 1 μg/ml (Table 1). Of note, MIC values for TBM plus β-lactamase inhibitors declined by up to one-eighth compared to that for TBM alone. On the other hand, biapenem (BPM) alone showed potency similar to that of MEM alone, with a MIC value of 1 to 2 μg/ml, and exerted up to a 4-fold increase in susceptibility in the presence of β-lactamase inhibitors (Table 1).

Next, we determined MICs for β-lactams alone or in combination with β-lactamase inhibitors (4 μg/ml) against 20 drug-susceptible (DS) and 21 DR clinical isolates of *M. tuberculosis*. Considering all the evaluation results for MIC range, MIC_{50}, and MIC_{90}, susceptibility of DS-TB strains for β-lactams alone was, in descending order, TBM > MEM = BPM > ampicillin (AMP) > AMX (Table 2). Similar results were obtained when combined with either CLA or avibactam (AVI) (Table 2). In the same manner, susceptibility of MDR-TB and XDR-TB strains for β-lactams alone was, in descending order, TBM = BPM > MEM > AMX > AMP, with MIC_{50} values of 2 μg/ml, 2 μg/ml, 8 μg/ml, 32 μg/ml, and 64 μg/ml, respectively (Table 3). Remarkably, β-lactams with and without β-lactamase inhibitors tended to be more effective against MDR-TB and XDR-TB strains than against DS-TB strains, implying that the cell wall components of DR-TB strains are altered by various mutations. The MIC ranges for MEM-CLA and AMX-CLA against MDR-TB and XDR-TB were 0.25 to 2 μg/ml and <0.25 to 16 μg/ml, respectively; they were in accordance with the previous report (0.23 to 1.25 μg/ml and 0.32 to 10

TABLE 3 Antituberculosis activities of each drug against multidrug- and extensively drug-resistant clinical isolates of *Mycobacterium tuberculosis*[a]

Agent (abbreviation)	Inhibitor	MIC range (μg/ml)	Distribution width	MIC (μg/ml)	
				50%	90%
Meropenem (MEM)	None	0.5–16	5	8	16
	CLA	0.25–2	3	0.5	2
	AVI	0.5–4	3	1	4
Biapenem (BPM)	None	0.5–16	5	2	16
	CLA	0.25–4	4	0.5	4
	AVI	0.25–4	4	1	4
Tebipenem (TBM)	None	0.125–8	6	2	4
	CLA	≤0.063–1	≥4	0.25	1
	AVI	0.125–1	3	0.5	1
Ampicillin (AMP)	None	0.25–>128	>9	64	128
	CLA	≤0.125–8	≥6	0.5	8
	AVI	0.25–32	7	2	8
Amoxicillin (AMX)	None	0.25–>128	>9	32	128
	CLA	≤0.125–16	≥7	0.5	8
	AVI	≤0.125–32	≥8	4	16
Clavulanate (CLA)		32–128	2	64	128
Avibactam (AVI)		64–>512	>3	512	>512
Amikacin (AMK)		≤0.5–>512	>10	1	>512
Isoniazid (INH)		2–>32	>4	16	64
Rifampin (RIF)		1–>128	>7	128	256
Streptomycin (STR)		≤0.25–>256	>10	4	512
Ethambutol (EMB)		0.5–16	5	4	16
Moxifloxacin (MXF)		≤0.063–4	≥6	0.5	2
Levofloxacin (LVX)		0.125–16	7	2	8
Ethionamide (ETH)		1–>64	>6	128	128
p-Aminosalicylic acid (PAS)		≤0.031–>32	>10	0.125	16
Cycloserine (CS)		8–64	3	16	64
Clarithromycin (CLR)		0.125–16	7	4	16

[a] Drug-resistant strains of tuberculosis ($n = 21$) include multidrug-resistant strains ($n = 5$), pre-extensively drug-resistant strains ($n = 13$), and extensively drug-resistant strains ($n = 3$).

μg/ml, respectively) (Table 3) (11). Intriguingly, the MIC range for TBM alone against MDR-TB and XDR-TB strains was comparable with that for AMX-CLA (Table 3). Carbapenems possessed narrow-range MIC spectra and more potent activity against clinical isolates of *M. tuberculosis* than aminopenicillins (Tables 2 and 3) (11). The difference in anti-TB activity between them is ascribable to the mechanisms of action in relation to L,D-transpeptidases (LDTs) and an Ambler class A β-lactamase, BlaC. The drug target LDTs, which are involved in the biosynthesis of peptidoglycan (PG) cross-linking containing 3→3 interpeptide bonds, have been considered to be effectively inactivated by carbapenems but not aminopenicillins (23–25). In addition, carbapenems, including TBM, have been reported to be relatively resistant to decomposition by BlaC that is constitutively produced by *M. tuberculosis* and triggers the hydrolysis of the β-lactam ring (26, 27). Taken together, carbapenems might be ideal compounds to combat MDR-TB and XDR-TB. There was no apparent cross-resistance between existing anti-TB drugs and the β-lactams/β-lactamase inhibitors tested (Table 3; see also Table S2 in the supplemental material).

Overall, TBM plus CLA showed the most potent anti-TB activity, with MIC values of 2 μg/ml or less. Tebipenem pivoxil (TBM-PI) is an oral carbapenem for the treatment of respiratory and otolaryngologic infections. Fortunately, for patients with infectious diseases, TBM is available without cilastatin, which blocks the hydrolysis of carbapenems in kidneys, owing to the stability to dehydropeptidase 1 (drug's interview form, Meiji Seika Pharma Co., Ltd., Japan). Additionally, TBM has been reported to scarcely interact with CYP3A4 and CYP2B6 (28, 29). More importantly, TBM has been proved to exhibit good distribution into the pulmonary epithelial lining fluid in an animal model, which allows TBM to be used for lung disease (28). The dosage form of TBM is fine granules, which is suitable for infants and young children. Also, the tablet form has been developed for the treatment of adult infectious diseases (30). Under fasting conditions, the maximum plasma level of TBM after single dosing of either 200 mg TBM-PI fine granules or tablets has been reported to be 9.4 ± 1.6 μg/ml or 6.6 ± 1.7 μg/ml, respectively, which is the same or more than the MIC range for TBM alone (0.125 to 8 μg/ml) (Table 3) (31, 32). Contrary to the time-dependent β-lactams, the bactericidal activity of tebipenem has been reported to correlate closely with area under the curve (AUC)/MIC and maximum concentration of drug in serum (C_{max})/MIC rather than the percentage of time above MIC (%T_{MIC}) against respiratory pathogens such as *Strep-*

tococcus pneumoniae and *Haemophilus influenzae* (33). In concentration-dependent drugs, such as fluoroquinolones and aminoglycosides, a tolerable and higher dosage is preferable to achieve a sufficient peak serum concentration and AUC. Therefore, in order to set treatment regimens of MDR-TB and XDR-TB, dose optimization using Monte Carlo simulation could be warranted. Intriguingly, tebipenem has a longer postantibiotic effect and postantibiotic sub-MIC effect against other bacteria than oral cephem antibiotics (33). This evidence suggests that tebipenem could be useful for the treatment of TB in spite of its short half-life (32).

In conclusion, TBM with and without CLA would assist the treatment of DR-TB, especially XDR-TB and TDR-TB. Further investigation is needed to evaluate the clinical usefulness of TBM and develop a more effective oral carbapenem.

ACKNOWLEDGMENT

The five β-lactam antibiotics used in this study, potassium clavulanate, and avibactam were kindly provided by Meiji Seika Pharma Co., Ltd.

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