ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2011, p. 2597–2600 Vol. 55, No. 6
0066-4804/11/$12.00 doi:10.1128/AAC.01824-10

Activity of Carbapenems Combined with Clavulanate against Murine Tuberculosis[▽]

Nicolas Veziris,[1,2,3]* Chantal Truffot,[1] Jean-Luc Mainardi,[4,5] and Vincent Jarlier[1,2,3]

UPMC Université Paris 06, ER5, EA 1541, Laboratoire de Bactériologie-Hygiène, F-75005 Paris, France[1]; AP-HP, Hôpital Pitié-Salpêtrière, Laboratoire de Bactériologie-Hygiène, F-75013 Paris, France[2]; Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, F-75013 Paris, France[3]; Laboratoire de Recherche Moléculaire sur les Antibiotiques, Centre de Recherche des Cordeliers, INSERM, UMR S 872, Université Pierre et Marie Curie, Université Paris Descartes, Paris F-75006, France[4]; and Université Paris Descartes, AP-HP, Laboratoire de Microbiologie, Hôpital Européen Georges Pompidou, Paris F-75015, France[5]

Received 28 December 2010/Returned for modification 6 January 2011/Accepted 7 March 2011

Although beta-lactam antibiotics are not considered as antituberculous drugs, it has been recently shown that the combination of carbapenems and clavulanate is bactericidal *in vitro*. We evaluated in a murine model of tuberculosis the activity of carbapenems alone and combined with clavulanate against *Mycobacterium tuberculosis*. Swiss mice infected intravenously with 3×10^5 *M. tuberculosis* H37Rv were treated for 4 weeks with clavulanate alone or imipenem, meropenem, and ertapenem alone or combined with clavulanate, whereas a positive control group was treated with isoniazid, and a negative control group was held without treatment. The combination of imipenem or meropenem plus clavulanate significantly improved survival. Among groups of mice with 100% survival, only isoniazid reduced lung CFU counts; the carbapenem-clavulanate combinations did not prevent bacterial growth. Although less active than isoniazid, the combinations of imipenem or meropenem plus clavulanate improved the survival of mice infected with *M. tuberculosis* and should be further evaluated.

Tuberculosis (TB) is the second most common infectious disease leading to mortality, after AIDS. It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. In 2008, 9.4 million people developed new disease, and 1.8 million people died from TB (32, 33). Inappropriate use of essential anti-TB drugs has led to the emergence of bacilli resistant to one or more of these drugs. Widespread dissemination of these bacilli represents an obstacle to the control of TB. In 2009, the World Health Organization (WHO) estimated that there were more than 400,000 new cases of multidrug-resistant TB (MDR-TB; defined by a resistance to at least isoniazid and rifampin, the two main anti-TB drugs) (35). Unfortunately, the extensive use of second-line drugs led to the emergence of extensively drug-resistant (XDR) bacilli (28). XDR bacilli are MDR-TB bacilli that also resistant to fluoroquinolones and to either amikacin, kanamycin, or capreomycin. XDR-TB cases comprise 4 to 19% of MDR-TB cases (4–6), and these individuals have a very poor prognosis, with mortality rates that can be as high as 65 to 100% (5, 13, 21, 31).

Antituberculous drugs with a novel mechanism of action to provide activity against drug-resistant *M. tuberculosis* are needed. Beta-lactam antibiotics are widely used against most bacterial infections but are not considered as antituberculous drugs. The reasons usually evoked for justifying the lack of activity of beta-lactams against *M. tuberculosis* are impermeability (19) and chromosomic beta-lactamase activity (18). However, the permeability of *M. tuberculosis* has been shown to be equivalent to that of *P. aeruginosa*, a species that is commonly treated with beta-lactam antibiotics (7) and clavulanate, a beta-lactamase inhibitor, irreversibly inactivates the BlaC beta-lactamase of *M. tuberculosis* (16). Moreover, it has been recently reported that the peptidoglycan of *M. tuberculosis* contains an high content of 3→3 cross-links (80%) generated by a novel class of transpeptidase, the L,D-transpeptidases (22). Two candidate *M. tuberculosis* genes were found to encode L,D-transpeptidases (Ldt_{Mt1} and Ldt_{Mt2}) active in the formation of 3→3 cross-links (15, 22). One of these L,D-transpeptidases (Ldt_{Mt2}) is essential for virulence in a mouse model of acute infection (15). Like the other members of the L,D-transpeptidase family (24), Ldt_{Mt1} and Ldt_{Mt2} were inactivated by carbapenems (15, 22), a class of beta-lactam antibiotics that is poorly hydrolyzed by the *M. tuberculosis* beta-lactamase BlaC (16). Thus, L,D-transpeptidases are likely to be the targets of carbapenems in *M. tuberculosis*. Some human and mouse data support the *in vivo* antituberculous activity of imipenem (7). Finally, it has been recently shown *in vitro* that carbapenems were promising agents in the treatment of tuberculosis since these drugs have bactericidal activity against XDR strains of *M. tuberculosis*, if associated with clavulanate (17). Taken together, these data suggest that clavulanate may render *M. tuberculosis* susceptible to carbapenems and that carbapenem-clavulanate combinations may have some activity against tuberculosis.

The aim of the present study was to determine whether, in an *in vivo* murine model, carbapenem-clavulanate combinations are active against *M. tuberculosis*.

MATERIALS AND METHODS

***M. tuberculosis* strain.** *M. tuberculosis* H37Rv strain was grown on Lowenstein-Jensen medium. Colonies were subcultured in Dubos medium for 7 days at 37°C. The turbidity of the obtained suspension was adjusted to match that of a standard

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Faculté de Médecine Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75634 Paris Cedex 13, France. Phone: (33) 1 40 77 97 46. Fax: (33) 1 45 82 75 77. E-mail: nicolas.veziris@upmc.fr.

▽ Published ahead of print on 14 March 2011.

TABLE 1. MICs of imipenem, meropenem, and ertapenem alone and in the presence of 2.5 mg of clavulanate/liter

Treatment	MIC (mg/liter)		
	Without clavulanate	With clavulanate	EUCAST susceptibility breakpoints[a]
Imipenem	16	1	2–8
Meropenem	8	1	2–8
Ertapenem	16	4	0.5–1

[a] EUCAST (9).

1-mg/ml suspension of *M. bovis* variant BCG and further diluted, with saline, to 0.2 mg/ml for mouse inoculation.

Antimicrobial agents. Meropenem (AstraZeneca), imipenem (Merck Sharp Dome), ertapenem (Merck Sharp Dome), and isoniazid (Laphal) were purchased from the manufacturers. Clavulanate was purchased as pure powder from Sigma.

Mice. Swiss mice were purchased from the from the Janvier breeding center (Le Genest Saint-Isle, France).

MIC measurement. MIC was measured for imipenem, meropenem, and ertapenem alone and in the presence of 2.5 mg of clavulanate/liter in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) (17).

Infection of mice. Ninety female 4-week-old Swiss mice were intravenously infected in the tail vein with 0.5 ml of the bacterial suspension prepared as described above and containing approximately 5×10^5 CFU. The animal experiment guidelines of the Faculté de Médecine Pitié-Salpêtrière were followed.

Chemotherapy. On the day after infection, the mice were randomly allocated to seven groups. A negative control group consisted of 20 mice infected that were not treated. The treated groups consisted of 10 mice each. A positive control group was treated with isoniazid alone. The test groups were treated either with imipenem, meropenem, or ertapenem alone or combined with clavulanate. An additional control group was treated with clavulanate alone.

Isoniazid suspension in gelosed water was prepared weekly and kept at 4°C. Beta-lactam solutions were prepared daily in saline according to laboratory advising.

Treatment was started the day after infection (preventive model) and administrated once a day for 5 days a week for 1 month, either by oral gavage (isoniazid) or subcutaneously (beta-lactams).

To match the C_{max} obtained in humans (3, 8, 20, 26) and using the available data for mice (2, 25, 29, 34), the following doses were selected: imipenem, meropenem, ertapenem. and clavulanate at 100 mg/kg and isoniazid at 25 mg/kg.

Assessment of infection and treatment. To provide baseline values before initiation of chemotherapy, 10 control mice were killed on day 1 after inoculation. To assess the activity of treatment, all of the surviving mice were killed on day 28.

The severity of infection and the treatment activity were assessed by determining the survival rate, spleen weight, gross lung lesion score (0, no lesions; +, <10 tubercles; ++, 10 to 50 tubercles; +++, >50 tubercles), and the numbers of CFU in the lungs. Lungs were aseptically removed and homogenized according to a standard procedure (30). Enumeration of CFU was done as previously described (23).

Statistical analysis. Mean CFU counts were compared using a Wilcoxon test. The proportions of surviving mice were compared by using a Fisher exact test since the samples were small. Differences were considered significant at the 95% level of confidence.

RESULTS

MICs. The MICs of carbapenems against *M. tuberculosis* in the presence of clavulanate were reduced 4- to 16-fold (Table 1). The MICs of the combination were below the EUCAST breakpoint of susceptibility for imipenem and meropenem (1 mg/liter; critical concentrations, 2 to 8 mg/liter) (9).

Survival rate. Six of ten untreated mice died between days 18 and 24. All of the isoniazid-treated mice survived. Three clavulanate-treated mice died. Of the carbapenem-treated mice, one died on day 19 in the imipenem group (probably due to a gavage accident), 3 died between days 19 and 23 in meropenem group, and 3 in ertapenem group died between days 19 and 22. The addition of clavulanate improved survival since no mouse died in the imipenem plus clavulanate or the meropenem plus clavulanate groups ($P = 0.01$ versus untreated mice), and only one mouse died in ertapenem plus clavulanate group ($P = 0.06$ versus untreated mice).

TABLE 2. Mean CFU counts in mouse lungs after 28 days of carbapenem treatment (with or without clavulanate)

Treatment	Mean $\log_{10}$ CFU lung count ± SEM	
	CFU on day 1	CFU on day 28[a]
None	5.49 ± 0.53	7.07 ± 0.3*
Isoniazid		4.34 ± 0.82†
Imipenem		6.66 ± 0.18
Imipenem + clavulanate		6.36 ± 0.3†
Meropenem		7.28 ± 0.21
Meropenem + clavulanate		6.88 ± 0.31
Ertapenm		7.23 ± 0.13
Ertapenem + clavulanate		7.08 ± 0.3
Clavulanate		7.30 ± 0.52

[a] Values indicated by "†" were significantly different from the value indicated by "*".

Spleen weights and gross lung lesions. Compared to the untreated control, group only the mice of isoniazid group had reduced spleen weights. All carbapenem-treated mice (alone or in combination with clavulanate) had splenomegaly equivalent to that of untreated mice (data not shown).

All of the mice that died before day 28 and two of the four mice in the negative control group sacrificed on day 28 had developed massive gross lung lesions (++ to +++). Compared to the controls, the isoniazid-treated mice had fewer lung lesions (three mice with lung lesion scores of 0, +, and ++). In contrast, all mice had severe lesions after clavulanate treatment or carbapenem treatment, even when combined with clavulanate.

Enumeration of CFU in the lungs. The mean CFU count in the lungs was 5.5 $\log_{10}$ CFU among mice sacrificed the day after inoculation (Table 2). In the untreated control group, this value increased by 1.6 $\log_{10}$ CFU to reach 7.1 $\log_{10}$ among the four mice surviving on day 28. The lung CFU counts were significantly reduced by 1.2 $\log_{10}$ CFU in the isoniazid group (4.3 $\log_{10}$ CFU, $P = 0.002$) compared to the start of treatment. In all of the carbapenem-treated groups, even with a clavulanate combination, the CFU counts increased by 0.8 to 1.8 $\log_{10}$ CFU. Compared to the CFU counts in the four surviving untreated mice, there was a significant difference in favor of treated mice only in the imipenem-clavulanate group (6.4 versus 7.1 $\log_{10}$ CFU, $P = 0.007$). In all of the other groups, the CFU count at day 28 was not different from that of the surviving untreated mice.

DISCUSSION

We have shown that imipenem, meropenem, and ertapenem alone do not prevent mortality of mice infected with *M. tuberculosis* reference virulent strain H37Rv. The addition of clavulanate significantly improved survival since there was no mor-

tality in the imipenem-clavulanate or meropenem-clavulanate combinations. However, even in the two groups in which all mice survived, there was an increase in the CFU count compared to the start of treatment, which means that these combinations were not bacteriostatic. In contrast, as expected, isoniazid displayed bactericidal activity.

These results can be interpreted as encouraging (improvement of survival) or as discouraging (lack of bacteriostatic activity). When analyzing these results, we must keep in mind that the question is whether these antibiotics can be proposed for TB patients. In order to answer this question, using the results of this experiment, the dosing used for the antibiotics must be compared to the usual human dosing. Regarding isoniazid, we know that a does of 25 mg/kg in mice generates pharmacokinetic parameters equivalent to those obtained in human with the usual 5-mg/kg dosing (14). Regarding imipenem, in human given 1 g (intravenously [i.v.]) the C_{max} is 70 mg/liter, the half-life approximately is 1 h, and the area under curve (AUC) is 100 to 200 mg · h/liter (11, 20). In mice that have received 128 mg/kg subcutaneously the C_{max} is 142 mg/liter, the half-life is 16 min, and the AUC is 61 mg · h/liter (29). For meropenem, in humans given 1 g (i.v.), the C_{max} is 54 mg/liter, the half-life is approximately 1 h, and the AUC is 100 to 200 mg · h/liter (8, 12). In mice given 80 mg/kg subcutaneously, the C_{max} is 55 mg/liter and the half-life is 2 h (25). That this half-life is longer in mice than in humans is surprising and should be confirmed since usually the half-life of antibiotics is shorter in mice than in humans.

Regarding ertapenem, in human given 1 g i.v., the C_{max} is 54 to 150 mg/liter, the half-life is 4 h, and the AUC is 500 to 800 mg · h/liter (10, 34). In mice given 50 mg/kg subcutaneously, the C_{max} is 140 mg/liter, the half-life is approximately 1 h, and the AUC is 586 mg · h/liter (34). Thus, with the exception of meropenem, the half-life of beta-lactams in mice is shorter than it is in humans. Since the antibacterial activity of these antibiotics depends mainly on time over the MIC, the activity observed in this model is probably less than what can be expected in humans. Moreover, in humans imipenem and meropenem are administered three times a day, whereas in this model they were administrated only once a day.

In these conditions, one can ask for which carbapenem should be used? The MIC of ertapenem in the presence of clavulanate does not fall in the range of susceptibility, which is confirmed by the fact that the ertapenem-clavulanate combination does not completely prevent mouse mortality. Regarding imipenem and meropenem, the MIC measured in the presence of clavulanate falls in the range of susceptibility (17). However, in the study by Hugonnet et al. (17), the MIC, measured on 14 strains, displayed more variability with imipenem than with meropenem, which would support the choice of meropenem. Conversely, if we trust the mouse pharmacokinetic data published, then the mouse data presented here are clearly pessimistic for imipenem and optimistic for meropenem, which would favor the use of imipenem.

Regarding the use of clavulanate in humans, it requires the coadministration of amoxicillin since there is no clavulanate-alone formulation. The 2.5 mg/liter that allows synergism *in vitro* can be reached with the usual human dosing of the amoxicillin-clavulanate combination (1, 2). For meropenem the mouse pharmacokinetic parameters should be specified, since the data available in the literature are inconsistent.

In summary, the significant impact of carbapenem-clavulanate combinations on survival that we observed, although encouraging, is not sufficient evidence, and additional murine studies are still needed. Precise carbapenem pharmacokinetic parameters will have to be determined in mice. More frequent dosing of carbapenems (two, three, or four times a day) in order to mimic human pharmacokinetics will probably have to be used in order to know whether these combinations can achieve bactericidal activity *in vivo* against *M. tuberculosis*. If bactericidal activity can be achieved, then the contribution of carbapenem-clavulanate combinations compared to and combined with other second-line drugs will have to be studied in models with a high bacillary load (curative model) in which the selection of resistant mutants can occur.

In humans, the use of meropenem-clavulanate combination has been reported for four XDR-TB patients. Three of them improved, whereas one did not and died. The patient that died was also the only one infected with a strain resistant to linezolid, which makes it difficult to assess the contribution of the meropenem-clavulanate to the antituberculous activity of combinations received by the other patients (27). Finally, it must be emphasized that the parenteral administration and the cost of these antibiotics will probably limit their use to patients in more developed countries where the majority of patients do not harbor XDR strains.

In conclusion, in addition to other data obtained *in vitro* (15, 17, 22), the activity of the carbapenem-clavulanate combinations demonstrated in a murine model here, even if weak, is nevertheless encouraging. Further experiments with larger doses are needed in order to ascertain whether these combinations can be bactericidal.

ACKNOWLEDGMENT

We thank Aurélie Chauffour for technical assistance.

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