Activity of Carbapenems Combined with Clavulanate against Murine Tuberculosis

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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 × 108 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 antibiotic 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 (LdtMt1 and LdtMt2) active in the formation of 3 → 3 cross-links (15, 22). One of these L,D-transpeptidases (LdtMt2) is essential for virulence in a mouse model of acute infection (15). Like the other members of the L,D-transpeptidase family (24), LdtMt1 and LdtMt2 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
TABLE 1. MICs of imipenem, meropenem, and ertapenem alone and in the presence of 2.5 mg of clavulanate/liter

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIC (mg/liter)</th>
<th>Without clavulanate</th>
<th>With clavulanate</th>
<th>EUCAST susceptibility breakpoints*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>16</td>
<td>1</td>
<td>2–8</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>8</td>
<td>1</td>
<td>2–8</td>
<td></td>
</tr>
<tr>
<td>Ertapenem</td>
<td>16</td>
<td>4</td>
<td>0.5–1</td>
<td></td>
</tr>
</tbody>
</table>

* 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 x 10^6 CFU. The animal experiment guidelines of the Faculte de Medicine Pitie-Salpetriere 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. 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.** Treatment was started the day after infection (preventive model) and administered once a day for 5 days a week for 1 month, either by oral gavage (isoniazid) or subcutaneously (beta-lactams).

**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.

**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).

**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 dose of 25 mg/kg in mice generates results of this experiment, the dosing used for the antibiotics for TB patients. In order to answer this question, using the that the question is whether these antibiotics can be proposed (activity). When analyzing these results, we must keep in mind that the employment 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 dose of 25 mg/kg in mice generates results of this experiment, the dosing used for the antibiotics for TB patients. In order to answer this question, using the

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.

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REFERENCES


