Antimicrobial-induced release of endotoxin from *Pseudomonas aeruginosa*: comparison of *in vitro* and animal models

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This study was designed to compare the amount of lipopolysaccharide (LPS) induced following exposure to doripenem, imipenem/cilastatin, meropenem and ceftazidime in an *in vitro* computerized-simulation system (simulating the drug concentration pattern in human plasma after administration of a drug), with that induced by exposure to a drug at a constant concentration. When *Pseudomonas aeruginosa* was exposed to the test drugs at constant concentrations of 0.1 ×, 1 × and 10 × MIC, differential relative induction of LPS was observed as follows: ceftazidime > meropenem, doripenem > imipenem/cilastatin. In the computerized-simulation system, however, the amount of LPS induced by treatment with ceftazidime (1 g) was similar to that by doripenem (250 mg), imipenem/cilastatin (500 mg) and meropenem (500 mg). In a rat model of *P. aeruginosa* bacteraemia, rates of eradication of bacteria from the blood were similar for carbapenems and ceftazidime except for 1 h post-administration of ceftazidime. Serum LPS levels induced by treatment with doripenem (30 mg/kg), imipenem/cilastatin (30 mg/kg), meropenem/cilastatin (30 mg/kg) and ceftazidime (50 mg/kg) were almost the same at 3 h after administration of each drug. Data obtained from computerized-simulation systems might be more applicable than those obtained from organisms exposed to constant drug concentrations for estimating the amount of LPS in the plasma of human patients infected with Gram-negative bacteria.

Keywords: lipopolysaccharide, antibiotics, carbapenems, computerized-simulation system

Introduction

To date, many classes of antibiotics are known to induce release of various amounts of lipopolysaccharide (LPS; endotoxin) *in vitro*.1–5 Among the β-lactams, the carbapenem sub-class has been reported to be the least potent inducer of LPS release.6,7 However, given that there are differences between members of this class in their ability to induce endotoxin release,8 we investigated the endotoxin-releasing properties of doripenem, a new injectable 1-β methyl carbapenem.9 Previous studies have used techniques in which bacteria are exposed to constant concentrations of the compounds under investigation. This does not reflect the situation clinically where concentrations of antimicrobials in tissues vary over time. We speculated that free LPS concentrations induced by antimicrobial agents would be best studied by mimicking the pharmacokinetics in humans, rather than at constant drug concentrations. Accordingly, we developed a computerized-simulation system, in which the drug concentration pattern closely resembles human pharmacokinetics for evaluation of LPS concentrations induced by an individual drug.

We investigated *in vitro* LPS induction following exposure to carbapenems (doripenem, imipenem/cilastatin and meropenem) and ceftazidime in such an *in vitro* system based on predicted human plasma concentrations, and compared the results with LPS induction at constant drug concentrations. We also studied *in vivo* LPS concentrations induced by administration of doripenem, imipenem/cilastatin, meropenem/cilastatin and ceftazidime in a rat model of bacteraemia, and compared it with the *in vitro* results.

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Materials and methods

Bacterial strain and culture conditions

*Pseudomonas aeruginosa* SR24, a blood culture isolate, was used in the *in vitro* and *in vivo* studies and was stored at −80 °C in 10% skimmed milk until use. Bacteria were grown in Mueller–Hinton broth (Difco, Detroit, MI, USA) without shaking at 37 °C for 18 h. For the *in vivo* study, stock bacterial solution was rapidly thawed and immediately diluted in Mueller–Hinton broth.

Antibiotics

Doripenem, meropenem and cilastatin were obtained from Shionogi & Co. (Osaka, Japan), imipenem/cilastatin was obtained from Banyu Pharmaceutical Co. (Tokyo, Japan) and ceftazidime was obtained from GlaxoSmithKline (Tokyo, Japan). Meropenem was combined with cilastatin at a ratio of 1:1 for the *in vivo* study only.

Animals

Six-week-old female Sprague–Dawley (SD) rats (body weight range 140–180 g) were obtained from Clea Japan Inc. (Tokyo). All studies with animals were approved by the Experimental Animal Committee of Shionogi & Co., Ltd.

Determination of MIC

The MIC of each drug was determined by the agar dilution method according to the guidelines of the Japanese Society for Chemotherapy. Mueller–Hinton agar (Difco) plates were inoculated using an automatic inoculator (Sakuma, Tokyo, Japan) to give a final inoculum of ∼1 × 10^5 cfu/mL. Plates were incubated at 37 °C for 18 h, and the MIC was defined as the lowest concentration of the drug that inhibited visible bacterial growth.

Sample preparation to determine antibiotic-induced LPS concentrations

**Constant concentration exposure.** An overnight culture in 0.02 mL of Mueller–Hinton broth was diluted with 200 mL of Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 20% heat-inactivated fetal calf serum (FCS), and then incubated with shaking at 37 °C for 2–3 h. Antibiotic solution was added to the medium to the desired concentration. The drug concentrations were 0.1 ×, 1 × and 10 × MIC, respectively. After the addition of antibiotics, cultures were incubated at 37 °C with shaking, and the number of viable cells and amounts of LPS were assayed at 1 and 6 h.

**Simulation of human plasma concentrations.** An *in vitro* computer-programmed pharmacokinetics simulation system based on human plasma concentration levels was used to compare LPS concentrations induced by carbapenems and ceftazidime. In the present study, this system simulated concentration patterns in human blood induced by doripenem 250 mg/30 min administered by intravenous (iv) infusion, imipenem/cilastatin and meropenem 500 mg/30 min iv and ceftazidime 1 g/60 min iv, respectively (see Figure 1). The *in vitro* model consisted of a tightly sealed 250 mL glass chamber fitted with inflow, outflow and sampling ports. These components were regulated by microcomputer. Eagle’s minimum essential medium supplemented with 20% heat-inactivated FCS was used as the growth medium. The temperature was maintained at 37 °C with a water bath and the medium was continuously mixed with magnetic stirring bars. In this model, the medium containing antibiotic was displaced from the central compartment, and then antibiotic-free medium was pumped into the central compartment with a peristaltic pump. To assay the number of viable cells and amounts of LPS, 2 mL samples were aseptically removed at 0, 1, 2, 4, 6 and 8 h.

In *vivo* drug administration using the systemic infection model. *In vivo* levels of LPS released after treatment with carbapenems and ceftazidime were studied in a rat model of *P. aeruginosa* SR24 bacteraemia. Six-week-old SD rats were immunosuppressed by two intraperitoneal administrations of cyclophosphamide (Shionogi & Co.) 150 mg/kg at 4 days and 75 mg/kg at 1 day before infection. Neutropenic rats were injected intraperitoneally with 5.0 mL of bacterial suspension. The challenge dose was 1.7 × 10^7 cfu/rat. A dose of 50 mg/kg of lead tetraacetate (Sigma, St Louis, MO, USA) was given intravenously to increase the susceptibility of rats to LPS at 1 h after infection. The test drugs were administered subcutaneously 2 h after infection. The dose of each drug was as follows: doripenem 30 mg/kg, imipenem/cilastatin 30 mg/kg, meropenem/cilastatin 30 mg/kg and ceftazidime 50 mg/kg. Animals (n = 9–17 for each group) were killed at 1, 3 and 6 h after administration of each drug, and blood and serum samples were used to assay viable organisms and the amount of LPS, respectively.

Viability counts

Samples were serially diluted 10-fold in Mueller–Hinton broth, and 1.0 mL samples of serial dilutions were incorporated into Mueller–Hinton agar. After overnight incubation at 37 °C, the cfu/mL was determined. Bactericidal activity was taken as a greater than or equal to 99.9% reduction in viable cell count.
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LPS assay

LPS concentrations were measured using an LPS-specific chromogenic Limulus test (Endospecy test; Seikagaku Co., Tokyo, Japan). Sterile, LPS-free specimen containers and pipette tips were used for the LPS assay. In order to remove LPS associated with bacteria, samples were filtered through an LPS-free filter (Steradisc, 0.2 µm; Krabou, Osaka, Japan), which was treated by the perchloric acid method to remove factors interfering with the Limulus test.

In a preliminary experiment, viable cells were not detected in the filtrate passing through a filter.

Microscopy

For the morphological study, *P. aeruginosa* SR24 was incubated on film agar containing 0.1 ×, 1 × or 10 × MIC of the test drugs on glass slides, and the plates were observed by phase-contrast microscopy (Nikon, Tokyo, Japan).

Pharmacokinetic study

Studies of pharmacokinetics in serum after administration of a single dose were carried out with neutropenic, infected rats (*n* = 3) given subcutaneous doses of doripenem (30 mg/kg), imipenem/cilastatin (30 mg/kg), meropenem/cilastatin (30 mg/kg) and ceftazidime (50 mg/kg). An iv dose of 50 mg/kg of lead tetraacetate was given 1 h after inoculum. Each drug was administered 2 h after infection. A sample of blood was obtained 0.25, 0.5, 1, 1.5, 2 and 3 h after drug administration. The concentration of biologically active antibiotic in the sample was determined by the bioassay method using *Escherichia coli* 7437 as the indicator organism. The pharmacokinetic values were calculated by one-compartment analysis using the WinNonlin program (Scientific Consulting, Inc.).

Statistical analysis

The Tukey multiple analysis test was used to compare LPS concentrations in serum samples. *P* values ≤ 0.05 were considered statistically significant.

Results

In vitro killing activity and LPS concentrations induced by constant concentrations of carbapenems and ceftazidime

Bacteria were exposed to drugs at concentrations of 0.1 ×, 1 × and 10 × MIC, and the number of viable organisms and concentrations of LPS derived from *P. aeruginosa* SR24 were 0.2, 1.56, 0.2 and 1.56 mg/L, respectively. At *T*<sub>0</sub>, the number of viable cells in the control group was 4.99 log<sub>10</sub> cfu/mL and the amount of LPS was 7.04 µg/L. Rapid killing was achieved by addition of doripenem and meropenem and the decrease in viable organisms was dependent upon the drug concentration (Table 1). The decrease in viable organisms after treatment with imipenem/cilastatin at a dose of 1 × MIC was roughly the

### Table 1. Bactericidal activities and levels of LPS induced by carbapenems and ceftazidime from *P. aeruginosa* SR24

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>∆Log&lt;sub&gt;10&lt;/sub&gt; cfu/mL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LPS (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fold of MIC</td>
<td>mg/L</td>
<td>1 h&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doripenem</td>
<td>10</td>
<td>2.0</td>
<td>-1.95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.2</td>
<td>-0.51</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.02</td>
<td>+0.04</td>
</tr>
<tr>
<td>Imipenem/cilastatin</td>
<td>10</td>
<td>15.6</td>
<td>-1.84</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.56</td>
<td>-1.84</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.156</td>
<td>+0.10</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10</td>
<td>2.0</td>
<td>-1.95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.2</td>
<td>-0.49</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.02</td>
<td>+0.28</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>10</td>
<td>15.6</td>
<td>-0.71</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.56</td>
<td>-0.42</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.156</td>
<td>+0.02</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>+0.35</td>
</tr>
</tbody>
</table>

<sup>a</sup>Decrease in viable cells between bacterial count at each timed interval and initial inoculum.

<sup>b</sup>Exposure time.
same as that at 10 × MIC. When the bactericidal activity of each drug was compared at a concentration of 10 × MIC, the decrease in viable cells after doripenem treatment was greater than that after imipenem/cilastatin and meropenem. At 1 × MIC, treatment with imipenem/cilastatin resulted in a greater reduction in viable cells compared with the other test drugs. Doripenem and meropenem led to reduced LPS concentrations, correlated with the drug concentration. However, ceftazidime at 10 × MIC reduced viable cells by 2 log10 cfu/mL but failed to decrease the amount of LPS released.

Previous studies reported that the mean peak serum concentration of carbapenems is ∼30 mg/L after iv administration of a single dose of 500 mg.14,15 Accordingly, a constant drug concentration (30 mg/L) was used for assay of viable organisms and LPS concentrations induced by drug treatment. The reduction in viable organisms induced by treatment with the carbapenems was nearly equivalent to, and their bactericidal activity exceeded, that of ceftazidime. When compared at the maximum concentration in serum (30 mg/L), the relative amounts of LPS were as follows: ceftazidime > meropenem = imipenem = doripenem (data not shown).

Phase contrast microscopy

Morphological changes in P. aeruginosa SR24 exposed to 0.1 ×, 1 ×, and 10 × MIC of doripenem, imipenem/cilastatin, meropenem and ceftazidime for 6 h were observed. Cell shape changed and became filamentous during exposure to doripenem at concentrations of 0.1 × MIC. At doripenem concentrations of 1 × and 10 × MIC, cells bulged or became spherical. Morphological changes induced by exposure to meropenem were similar to those induced by doripenem. Morphological changes were not observed after exposure to 0.1 × MIC of imipenem/cilastatin, and spherical forms only were observed in the case of 1 × and 10 × MIC of imipenem/cilastatin. In contrast, during exposure to ceftazidime, only filamentous forms were observed, and their presence did not correlate with drug concentration. Phase-contrast microscopic studies indicated that the concentration of LPS derived from treatment of doripenem and meropenem decreased when cells changed from filaments to spheres. Ceftazidime, which induced filament formation, was associated with higher LPS concentrations than the carbapenems.

Comparative activities of antibiotics on LPS concentrations in an in vitro pharmacokinetic computerized-simulation model

The bactericidal activities of doripenem, imipenem/cilastatin, meropenem and ceftazidime, and LPS concentrations in organisms induced by those drugs, were assayed in the in vitro computerized-simulation system (Figure 1). The bactericidal activity of doripenem was almost the same as that of imipenem/cilastatin and meropenem. These carbapenems reduced viable cells by >2 log10 cfu/mL at 2 h post-incubation, whereas ceftazidime only reduced viable organisms by 1.2 log10 cfu/mL over the same period. After 4 h, the pattern of reduction of viable cells treated with ceftazidime was similar to that induced by the carbapenems.

The amount of LPS in the control group decreased by 30–50% over 2 h incubation, but increased gradually thereafter. At 8 h post-incubation, the concentration of LPS in the medium was 274.3 µg/L, which was seven times greater than that at 1 h. The maximum concentration of free LPS in response to doripenem treatment occurred at 1 h after the addition of the antibiotic. This phenomenon was also observed for imipenem/cilastatin and meropenem. The concentration of free LPS induced by each drug gradually decreased thereafter. The peak concentration of LPS after ceftazidime treatment was observed 4 h after addition of the drug, and gradually
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decreased thereafter. The total amounts of free LPS following treatment with doripenem, imipenem/cilastatin and meropenem from 1 to 8 h ranged from 161.7 to 270.4 µg/L. The concentrations of LPS induced by doripenem, imipenem/cilastatin and meropenem under the computerized-simulation system were almost the same. The total amount of free LPS after ceftazidime treatment (from 1 to 8 h) was 287.9 µg/L. The concentration of free LPS after ceftazidime treatment was similar to that in response to the carbapenems under the computerized-simulation system.

Pharmacokinetic studies using P. aeruginosa-infected rats

The pharmacokinetic parameters of carbapenems and ceftazidime are shown in Table 2. The maximum serum concentration of doripenem was the same as that of imipenem/cilastatin and was lower than that of meropenem/cilastatin and ceftazidime. The area under the plasma concentration–time curve of doripenem was half that of imipenem/cilastatin and meropenem/cilastatin. The elimination half-life value of imipenem/cilastatin was the longest among the three carbapenems and that of ceftazidime was twice or more that of imipenem/cilastatin. The plasma concentration patterns of carbapenems and ceftazidime in neutropenic rats infected with P. aeruginosa were roughly similar to those of human plasma concentrations.14–16

Comparative efficacies of antibiotics in blood clearance and serum LPS levels in rats

The survival of P. aeruginosa in the blood of rats following administration of antimicrobials is shown in Figure 2. Viable cells were reduced by 1.4 log₁₀ cfu/mL at 1 h after administration of doripenem, imipenem/cilastatin and meropenem/cilastatin. With ceftazidime, viable cells were only slightly reduced by 0.2 log₁₀ cfu/mL at 1 h. The viable cell count after ceftazidime treatment, however, was the same as that for the test carbapenems at 3 h after drug administration.

Discussion

In previous studies, the concentration of free LPS from Gram-negative bacilli after drug treatments was assayed using constant drug concentrations.3–6 Recently, Simpson et al.17 reported that ceftazidime-treated patients had significantly higher systemic endotoxin concentrations after the first dose of antibiotics. However, other investigators observed no

Table 2. Pharmacokinetic parameters after administration of doripenem, imipenem/cilastatin, meropenem/cilastatin and ceftazidime to neutropenic infected rats

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dose (mg/kg)</th>
<th>C_{max} (mg/L)</th>
<th>t_{1/2} (h)</th>
<th>AUC (mg·h/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doripenem</td>
<td>30</td>
<td>24.6</td>
<td>0.31</td>
<td>23.1</td>
</tr>
<tr>
<td>Imipenem/cilastatin</td>
<td>30</td>
<td>24.5</td>
<td>1.07</td>
<td>51.3</td>
</tr>
<tr>
<td>Meropenem/cilastatin</td>
<td>30</td>
<td>33.4</td>
<td>0.59</td>
<td>42.2</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>50</td>
<td>94.7</td>
<td>2.87</td>
<td>330.6</td>
</tr>
</tbody>
</table>

The challenge dose was 1.5 x 10^7 cfu/rat. Lead tetraacetate (50 mg/kg) was injected iv at 1 h after infection. Each drug was administered at 2 h after infection; C_{max}, peak level in serum; t_{1/2}, serum elimination half-life; n = 3.
difference in endotoxin release in infections caused by Gram-negative bacilli. Therefore, the phenomenon of antibiotic-induced endotoxin concentration in serum derived from patients with severe Gram-negative infection is still a matter of debate. In the present study, we used both constant drug concentrations and a computerized-simulation system (simulating the concentration pattern in human plasma after administration of a drug) to assay viable organism counts and the concentration of free LPS after drug treatment. The pattern of LPS concentration in the latter model more closely resembled that observed in the serum of infected rats after drug administration than did the former. Therefore, the present study indicated that the reducing ratio of viable organisms to total amount of free LPS in the latter model roughly resembled that observed after administration of a drug to experimentally infected rats until 6 h post-treatment. These results indicate that data obtained from the computerized-simulation system might be more applicable than data from models using constant drug concentrations for prediction of LPS concentration in sera of patients with Gram-negative bacterial infections.

In addition, we observed differences in the amount of LPS released in response to different drugs. When bacteria were exposed to constant concentrations of various drugs at 10×, 1× or 0.1× MIC, the relative amounts of free LPS decreased in the following order: ceftazidime > doripenem, doripenem > imipenem. With carbapenems at 1× MIC, the number of viable organisms was more reduced and the concentration of LPS in the medium was less than that for ceftazidime. Jackson & Kropp reported that the inducibility of LPS from Gram-negative bacilli was dependent upon affinities of the drugs for penicillin-binding proteins (PBPs). In general, carbapenems bind strongly to all seven PBPs in P. aeruginosa organisms, but ceftazidime binds strongly to only PBP1a and PBP3. Among the carbapenems, the affinity of imipenem for PBP2 and the affinity of meropenem and doripenem for PBP3 were higher than for other PBPs, respectively. The present study indicated that bacteria treated with imipenem formed spheroplasts and those treated with meropenem and doripenem formed filaments. Ceftazidime treatment induced formation of longer filaments than did meropenem or doripenem. LPS release is more likely with filamentous than with spheroplast cells. We therefore confirmed that one factor affecting the amount of LPS released was cell morphology following exposure to an individual drug.

In the computerized-simulation system, the peak concentration of free LPS after treatment with carbapenems was observed at 1 h after addition of the drug, whereas it was observed at 4 h after the addition of ceftazidime. This may reflect the rapid bactericidal activity of carbapenems, as the release of LPS occurred following destruction of bacteria. Comparison of the chemical structures of the test drugs reveals that doripenem, imipenem and meropenem all have a penem nucleus, but differ in the methyl group at position 1 and/or 2-side chain of the carbapenem structure, whereas ceftazidime has a cephem nucleus. Thus, a second possible reason for the difference in LPS concentrations between carbapenem and ceftazidime may be related to structural aspects, such as the penem nucleus rather than the 1β-methyl group and/or the side chain at position 2 of carbapenem.

Viable cell counts after drug administration in the computerized-simulation system were similar to those in the rat bacteraemia model. The pattern of LPS release in the computerized-simulation system was different from that in the rat infection model. In the former, the number of organisms was 1×10^5 cfu/mL (total, 2×10^7 cfu) when a drug was added to the culture. In contrast, in the rat infection model, the infective bacterial counts were ∼1×10^7 cfu/animal, but these organisms multiplied in the rats at least until administration of a drug. Craig reported that the time above MIC is the best indicator for predicting the in vivo efficacy of β-lactams. In rats infected with P. aeruginosa, the time above MICs of doripenem, imipenem/cilastatin, meropenem/cilastatin and ceftazidime was 10.4%, 22.1%, 22.5% and 32.5%, respectively. The C_max/MIC ratios of doripenem, imipenem/cilastatin, meropenem/cilastatin and ceftazidime were 123, 15.7, 167

\[ \text{Figure 3. Serum LPS levels in rats. 1, no antibiotic; 2, doripenem; 3, imipenem/cilastatin; 4, meropenem/cilastatin; 5, ceftazidime. Results are expressed as mean ± S.D.} \]
and 60.7, respectively. The AUC/MIC ratios of doripenem, meropenem and ceftazidime were almost the same, and were 4–6 times lower than that of imipenem/cilastatin (data not shown). LPS concentrations induced by carbapenems and ceftazidime in rat serum were roughly the same. Taken together, these data indicate that LPS concentrations may not clearly correlate with PK/PD parameters.

In conclusion, the computerized-simulation system may be a valuable method for assaying free LPS concentration after administration of a drug in order to reflect in vivo conditions. Further experiments in in vivo models are required to analyse in greater detail the influencing factors on the release of LPS.

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References
