Potential Therapeutic Role of Histatin Derivative P-113D in Experimental Rat Models of *Pseudomonas aeruginosa* Sepsis

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**Background.** Morbidity and mortality from *Pseudomonas aeruginosa* sepsis remain high despite the availability of antibiotics to which the microorganism is sensitive.

**Methods.** The in vitro activity of histatin derivative P-113D was investigated against *Pseudomonas aeruginosa*. In addition, its in vivo efficacy was studied in 3 rat models of infection: intraperitoneal injection of 1 mg of *P. aeruginosa* 10 lipopolysaccharide, intraperitoneal injection of 2 × 10⁹ cfu of *P. aeruginosa* ATCC 27853, and intra-abdominal sepsis induced by cecal ligation and puncture. Rats received isotonic sodium chloride solution parenterally (control groups), 1 mg of P-113D/kg of body weight, 1 mg of polymyxin B/kg of body weight, or 20 mg of imipenem/kg of body weight. Main outcomes measured were abdominal exudate and plasma bacterial growth, plasma concentrations of endotoxin and tumor necrosis factor (TNF–α), and lethality.

**Results.** The in vivo studies showed that all compounds reduced lethality, when compared with results for the control group. Overall, P-113D exhibited a slightly lower antimicrobial activity than did imipenem, even though P-113D achieved a substantial decrease in plasma concentrations of endotoxin and TNF–α, compared with the imipenem. No statistically significant differences for antimicrobial and antiendotoxin activities were noted between P-113D and polymyxin B.

**Discussion.** These results provide evidence for double antiendotoxin and antimicrobial activity for P-113D and point to its potential use for the treatment of severe infections.

Gram-negative sepsis has become increasingly significant as a consequence of the growing population of immunocompromised patients [1–3]. In particular, *Pseudomonas aeruginosa* can be a serious pathogen for a variety of patients, including those with cancer who are receiving chemotherapy, patients with AIDS, patients with burns, patients who are undergoing mechanical ventilation, patients in critical care units, and children with cystic fibrosis [2, 4, 5]. Morbidity and mortality from sepsis caused by gram-negative microorganisms—namely, *Pseudomonas* species—remain high despite the availability of antibiotics to which the microorganism is sensitive. In fact, in critically ill patients, bacteremia often is accompanied by symptoms of septic shock and increasing susceptibility to endotoxin, owing to the inflammatory cascade [4–6]. The prominent harmful role of lipopolysaccharides (LPSs) as initiators of septic shock is well known [7–9]. LPSs are composed of an O-polysaccharide chain, a core sugar, and a lipophilic fatty acid (lipid A) and are the major structural and functional components of the outer membrane of gram-negative bacteria [8, 9]. They exhibit a variety of toxic and proinflammatory activities associated with the pathogenesis of gram-negative infection [7–11]. Many of these pathophysiologic phenomena result from the ability of LPS to activate host effector cells through stimulation of receptors on the cell surface. These target cells secrete large quantities of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)–1, IL–6, IL–8, platelet-activating factor, arachidonic acid metabolites, erythropoietin, and endothelin [7, 9, 11–13].

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Current treatments for gram-negative sepsis in critically ill patients are based on prompt administration of adequate antimicrobial agents. An empirical treatment founded on administration of broad-spectrum antimicrobial agents without waiting for microbiological documentation of infection is justified when fever and neutropenia are present, because of the high frequency of severe infection with fulminant course related to a low granulocyte count [2, 5, 14, 15]. *Pseudomonas* species are among the most common pathogens that cause bloodstream infections in clinical settings [16]. Antibiotic resistance among these microorganisms is increasing, and antibiotic options are becoming more limited [17]. Moreover, several studies have shown that exposure of gram-negative organisms to antibacterial agents can result in endotoxin release and have suggested that this phenomenon could have deleterious effects [18, 19].

In recent years, a growing body of research has revealed the potent antimicrobial and antiendotoxin activities of antimicrobial peptides [20–22]. These peptides are positively charged amphipathic molecules that have been isolated from a wide variety of animals and plants in which they act as a natural defense mechanism. The molecules are membrane-active polycationic peptides with an affinity for the negatively charged microbial surface that relies not only on electrostatic interactions but also on biochemical structure [23–26]. Moreover, on the basis of its highly conserved molecular structure among gram-negative bacteria, lipid A may be an appropriate target for polycationic peptides. In fact, the anionic and amphiphilic nature of lipid A enables it to bind either to compounds that are positively charged or to molecules that possess an amphipathic character [24, 27].

Histatins are a group of small, cationic, histidine-rich antimicrobial peptides secreted in human saliva by parotid and submandibular-sublingual glands. Histatin 1 and histatin 3 are encoded by distinct genes, whereas the other 10 histatin peptides that have been isolated from saliva are believed to arise from histatins 1 and 3 by proteolytic processing [28, 29]. Histatins have been found to have antimicrobial activity against a broad spectrum of bacteria and fungi [28–31]. The antimicrobial activity of histatins appears to be a distinctive multistep mechanism involving depletion of the microorganism intracellular ATP content as a result of nonlytic ATP efflux [28–30]. Other activities are ascribed to these peptides, such as a role in formation of the enamel pellicle of teeth, inhibition of hemagglutination, coaggregation, protease activity, and neutralization of LPS by binding to lipid A [31, 32]. In particular, they were found to inhibit LPS-mediated gelation of limulus amebocyte lysate and to reverse the antigencomplement action of LPS and lipid A. The histatin derivative P-113b is composed of a sequence of 12 aa residues contained within the 24 aa residues of histatin 5 and is amidated at its C terminus. Like the other histatins, P-113b is active against clinically important microorganisms, such as streptococci, staphylococci, *Pseudomonas* species, and *Candida albicans* [28–32].

The present experimental study was designed to investigate the in vivo efficacy of P-113b in 2 rat models of *P. aeruginosa* infection and in 1 rat model of cecal ligation and puncture.

**MATERIALS AND METHODS**

**Drugs.** Polymyxin B was obtained from Sigma-Aldrich, and imipenem was obtained from Merck, Sharp & Dohme. Powders were dissolved in accordance with manufacturers’ recommendations. Solutions were made fresh on the day assays were performed.

**Synthetic peptides.** P-113b (d-AKRHHGYKKFH-NH2) was synthesized by use of 9-fluorenlymethoxycarbonyl (Fmoc) solid-phase chemistry, according to the following procedure: (1) 5- and 15-min deprotection steps were done by use of 20% piperidine in the mixture DMF/NMP (dimethylformamide/N-methyl-pyrrolidone) (vol/vol, 1:1) in the presence of 1% Triton, and (2) the coupling reactions were performed with the protected amino acid (AA) diluted in the mixture DMF/NMP (vol/vol, 1:1) in the presence of 1% Triton, by use of N,N′-disopropylcarbodiimide (DIC) as the coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt) (Fmoc-AA:DIC:HOBt:1:1:1) for 1 h. The completeness of each coupling reaction was monitored by means of the chloranil test [33, 34]. The protected peptide resin was treated with a mixture of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane for 1 h. After cleavage, the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether, and lyophilization was done. P-113b was purified by solid-phase extraction on Kromasil sorbent (octylsilone-covered spherical silica, 5 μm, 100 Å) (EKA Chemicals), by use of a protocol described elsewhere [35]. The resulting fractions with purity >97%–98% were tested by use of high-performance liquid chromatography. The peptide was analyzed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.

**Susceptibility testing.** Susceptibility testing was performed by means of the microbroth dilution method, according to procedures outlined by the NCCLS [36]. However, since cationic peptides bind polystyrene, polypropylene 96-well plates (Sigma-Aldrich) were substituted for polystyrene plates. The MIC was the lowest antibiotic concentration at which observable growth was inhibited. Experiments were performed in triplicate.

**Bacterial killing assay.** To study the in vitro killing effect of the compounds, aliquots of exponentially growing bacteria (*P. aeruginosa* ATCC 27853) were resuspended in fresh Mueller-Hinton (MH) broth, at a concentration of ∼107 cells/mL, and were exposed to each peptide at 4× MIC for 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min at 37°C. After these times, samples were serially diluted in 10 mmol/L sodium HEPES buffer (pH
received isotonic sodium chloride solution ip (control group C1), immediately after bacterial challenge, each group of rats received isotonic sodium chloride solution (control group C2), 1 mg of P-113/11002. Immediately after injection, each of the 4 groups of rats received 1 mg of P. aeruginosa ATCC 27853. For model 1, 4 groups of 15 rats each were anesthetized by intramuscular injection of ketamine (30 mg/kg of body weight) and then were injected ip with 1 mg of P. aeruginosa 10 LPS in a total volume of 500 μL of sterile saline. Immediately after injection, each of the 4 groups of rats received isotonic sodium chloride solution ip (control group C0), 1 mg of P-113/11003/kg of body weight, 20 mg of imipenem/kg of body weight, or 1 mg of polymyxin B/kg of body weight.

For model 2, P. aeruginosa ATCC 27853 was grown in brain-heart infusion broth. When bacteria were in the log phase of growth, the suspension was centrifuged at 1000 g for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline. All rats (4 groups of 15 rats each) were anesthetized as described for model 1. The abdomen of each rat was shaved and prepared with iodine. The rats received ip 1 mL of saline containing 2 × 10^10 cfu of P. aeruginosa ATCC 27853. Immediately after bacterial challenge, each group of rats received isotonic sodium chloride solution ip (control group C0), 1 mg of P-113/11003/kg of body weight, 20 mg of imipenem/kg of body weight, or 1 mg of polymyxin B/kg of body weight.

For model 3, all rats (4 groups of 15 rats each) were anesthetized by intramuscular injection of ketamine (30 mg/kg of body weight), or imipenem (20 mg/kg of body weight). Drug levels were measured by use of bioassay: a spore suspension of Bacillus subtilis ATCC 6633 suspended in tryptic soy agar was used. The plates were read after incubation at 30°C for 18 h.

Evaluation of treatment. At the end of the study, the rate of blood-culture positivity, the quantity of bacteria in intra-abdominal fluid, the rate of lethality, and concentrations of plasma endotoxin and TNF-α were evaluated for each type of experimental model. The rats were monitored for the subsequent 72 h.

In all models, the definition of systemic symptoms was analogous to criteria applied to humans. Each rat was considered to be septic if at least 2 of the following criteria were met: increased pulse rate, rectal temperature >38°C or <36°C, increased breathing rate, and >12,000 or <4000 white blood cells/μL. Rats that survived were killed with chloroform, and blood samples for culture were obtained by aseptic percutaneous transthoracic cardiac puncture. In addition, to perform quantitative evaluations of the bacteria in intra-abdominal fluid, 10 mL of sterile saline was injected ip, samples of the peritoneal lavage fluid were serially diluted, and a 0.1-mL volume of each dilution was spread onto blood agar plates. The limit of detection was ≤1 log_{10} cfu/mL. The plates were incubated both in air and under anaerobic conditions, at 35°C for 48 h. The bacterial isolates were identified by biochemical assay.

For blood cultures (models 2 and 3) and for determination of plasma concentrations of endotoxin and TNF-α (all models), 0.2-mL blood samples were obtained, from a tail vein 0, 2, 6, and 12 h after injection (of LPS or bacteria) or after surgical
procedure, into a sterile syringe and transferred to tubes containing EDTA-tripotassium salt.

Biochemical assays. Endotoxin concentrations were measured by the commercially available limulus amebocyte lysate test (E-TOXATE; Sigma-Aldrich). Plasma samples underwent serial 2-fold dilution with sterile endotoxin-free water and were heat treated for 5 min in a water bath at 75°C, to destroy inhibitors that can interfere with the activation. The endotoxin content was determined as described by the manufacturer of the test. Endotoxin standards (0, 0.015, 0.03, 0.06, 0.125, 0.25, and 0.5 EU/mL) were tested in each run, and the concentration of endotoxin in the test samples was calculated by comparison with the standard curve. TNF-α levels were measured by use of a solid-phase sandwich ELISA. The intensity of the color was measured in an MR 700 microplate reader (Dynatech Laboratories), by reading the absorbance at 450 nm. The results for the samples were compared with the standard curve, to determine the amount of TNF-α present. All samples were tested in duplicate. In this assay, the lower limit of sensitivity for TNF-α was 0.05 ng/mL.

Statistical analysis. Mortality rates between groups were compared by use of Fisher’s exact test. Qualitative results for blood cultures were analyzed by use of the χ² test, Yates correction, or Fisher’s exact test, depending on the sample size. Quantitative evaluations of the bacteria in intra-abdominal fluid cultures were presented as mean ± SD; statistical comparisons between groups were made by analysis of variance. Post hoc comparisons were performed by use of the Bonferroni test. Mean values for plasma concentrations of endotoxin and TNF-α were compared between groups by analysis of variance. Each comparison group contained 15 rats. Significance was accepted at P < .05.

RESULTS

In Vitro Studies

According to the broth-microdilution method, P. aeruginosa ATCC 27853 was similarly susceptible to the peptides tested: the MIC for polymyxin B and P-113d was 2.00 mg/L, and the MIC for the control agent imipenem was 0.12 mg/L. Evaluations of in vitro time to killing of P. aeruginosa showed a potent killing activity for P-113d. In fact, killing by P-113d was shown to be the most rapid: activity was complete after a 10–15-min exposure period. Killing by polymyxin B was complete after a 15–20-min exposure period, and killing by imipenem was complete after a 20–25-min exposure period (data not shown).

In Vivo Studies

Model 1: ip administration of LPS. Peak plasma concentrations of endotoxin and TNF-α were observed 6 h after ip administration of 1.0 mg of P. aeruginosa 10 LPS. Nevertheless, ip P-113d and polymyxin B treatments resulted in a marked decrease of TNF-α levels (P < .05) and virtually undetectable

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endotoxin level, EU/mL</th>
<th>TNF-α level, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control group C₀)</td>
<td>25.90 ± 4.14</td>
<td>129.45 ± 15.15</td>
</tr>
<tr>
<td>P-113d, 1 mg/kg of body weight</td>
<td>&lt;0.015 ± 0.0a</td>
<td>0.26 ± 0.01a</td>
</tr>
<tr>
<td>Polymyxin B, 1 mg/kg of body weight</td>
<td>&lt;0.015 ± 0.0a</td>
<td>0.16 ± 0.01a</td>
</tr>
<tr>
<td>Imipenem, 20 mg/kg of body weight</td>
<td>21.67 ± 3.86</td>
<td>100.12 ± 13.6</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD. a P < .05, vs. control group C₀ and the imipenem-treated group.

Table 2. Efficacy of intravenous administration of P-113d, polymyxin B, and imipenem in rat models after intraperitoneal injection of 2 × 10⁸ cfu of Pseudomonas aeruginosa ATCC 27853.

<table>
<thead>
<tr>
<th>Time after bacterial challenge, treatment</th>
<th>Lethality, no. (%) of rats that dieda</th>
<th>Qualitative blood culture positive for bacteria, no. of rats</th>
<th>Bacterial count in peritoneal fluid, mean ± SD, cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control group C₀)</td>
<td>15 (100)</td>
<td>15</td>
<td>8.4 × 10⁸ ± 2.9 × 10⁸</td>
</tr>
<tr>
<td>P-113d, 1 mg/kg of body weightb</td>
<td>4 (26.6)</td>
<td>4</td>
<td>3.3 × 10⁹ ± 8.8 × 10⁸</td>
</tr>
<tr>
<td>Polymyxin B, 1 mg/kg of body weightb</td>
<td>4 (26.6)</td>
<td>4</td>
<td>4.2 × 10⁶ ± 2.0 × 10⁴</td>
</tr>
<tr>
<td>Imipenem, 20 mg/kg of body weightb</td>
<td>3 (20.0)</td>
<td>3</td>
<td>1.8 × 10⁴ ± 0.3 × 10³</td>
</tr>
<tr>
<td>360 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control group C₀)</td>
<td>15 (100)</td>
<td>15</td>
<td>9.0 × 10⁹ ± 3.1 × 10⁹</td>
</tr>
<tr>
<td>P-113d, 1 mg/kg of body weightc</td>
<td>4 (26.6)</td>
<td>4</td>
<td>4.0 × 10⁹ ± 7.8 × 10⁹</td>
</tr>
<tr>
<td>Polymyxin B, 1 mg/kg of body weightc</td>
<td>5 (33.3)</td>
<td>5</td>
<td>6.7 × 10⁶ ± 2.0 × 10⁵</td>
</tr>
<tr>
<td>Imipenem, 20 mg/kg of body weightc</td>
<td>4 (26.6)</td>
<td>5</td>
<td>3.8 × 10⁴ ± 1.1 × 10⁴</td>
</tr>
</tbody>
</table>

NOTE. Each treatment group included a total of 15 rats. a Lethality was monitored for 72 h after bacterial challenge. b P < .05, vs. control group C₀, for all values. c P < .05, vs. control group C₀, for all values.

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Levels of endotoxin in the plasma of peptide-treated groups, compared with those in control group C0, finally, no substantial differences in the plasma levels of both LPS and TNF-α were observed between the imipenem-treated and untreated groups (table 1).

**Model 2: ip injection of** $2 \times 10^{10}$ **cfu of P. aeruginosa ATCC 27853.** All rats were monitored for 72 h. The rate of lethality in control groups C1 and C2 (when antibiotics were administered immediately or 360 min after bacterial challenge, respectively) was 100% within 48 h. All antibiotic treatments led to decreased mortality ($P < .05$). In particular, a survival rate of 80.0% was observed for groups treated with imipenem immediately after intervention, and a survival rate of 73.4% was observed for groups treated with P-1134 or polymyxin B (table 2). Bacteriological evaluation of control group C1 showed that 100% of blood cultures were positive for bacteria and $8.4 \times 10^{4} \pm 2.9 \times 10^{6}$ cfu/mL of bacteria was counted in the intra-abdominal fluid. Imipenem showed the highest antimicrobial activity. P-1134 exhibited antimicrobial activity comparable to that of polymyxin B and only slightly lower than that of imipenem. Similar effects on lethality and bacterial counts were observed when the drugs were administered 360 min after intervention (table 2). As in the previous experiment, bacteriological evaluation showed that 100% of blood cultures were positive for bacteria and cultures of intra-abdominal fluid resulted in $9.0 \times 10^{4} \pm 3.1 \times 10^{3}$ cfu/mL of bacteria. In contrast, the administration of drugs at different times after bacterial challenge had a different impact on plasma concentrations of endotoxin and TNF-α. In fact, significant increases in plasma concentrations of endotoxin and TNF-α, with mean peak levels achieved at 6 h after injection of antibiotics, were observed in control and imipenem-treated rats when the antibiotics were injected immediately after intervention (figures 1A and 2A), whereas a constant decrease in plasma concentrations of endotoxin and TNF-α was produced by administration of P-1134 and polymyxin B 360 min after bacterial challenge (figures 1B and 2B). Overall, P-1134 and polymyxin B produced significant reductions in plasma concentrations of endotoxin and TNF-α, when compared with results for the control and imipenem-treated groups. No significant differences were observed between P-1134 and polymyxin B.

**Figure 1.** Effect on concentrations of endotoxin after administration of antimicrobial agents, in rat models of bacterial challenge. Rats received 1 mg of P-1134/kg of body weight, 1 mg of polymyxin B (POL-B)/kg of body weight, and 20 mg of imipenem (IMP)/kg of body weight, administered intraperitoneally (ip) at 0 min (A) and at 360 min (B) after ip injection of $2 \times 10^{8}$ cfu of *Pseudomonas aeruginosa* ATCC 27853.

**Figure 2.** Effect on concentrations of tumor necrosis factor [TNF-α] after administration of antimicrobial agents, in rat models of bacterial challenge. Rats received 1 mg of P-1134/kg of body weight, 1 mg of polymyxin B (POL-B)/kg of body weight, and 20 mg of imipenem (IMP)/kg of body weight, administered intraperitoneally (ip) at 0 min (A) and at 360 min (B) after ip injection of $2 \times 10^{8}$ cfu of *Pseudomonas aeruginosa* ATCC 27853.
Model 3: cecal ligation and puncture. The rate of lethality in control group C₃ (when antibiotics were administered immediately after surgical procedure) was 100% within 48 h. All antibiotic treatments led to decreased mortality (P < .05). Specifically, at 72 h after surgery, a survival rate of 66.7% was observed for groups treated with P-113d and polymyxin B and 73.4% for the imipenem-treated group (table 3). Bacteremia was detected in all rats in control group C₃, and 96.9% for the imipenem-treated group (table 3). Bacteremia specifically, at 72 h after surgery, a survival rate of 66.7% was observed, with mean peak levels achieved at 6 h after surgical procedure. As shown in models 1 and 2, constant concentrations were observed among the antibiotic-treated groups. As in the other models, P-113d and polymyxin B produced significant reductions in plasma concentrations of endotoxin and TNF-α comparable to that observed in model 2 (figures 3B and 4B).

Thirty minutes after a single intravenous injection of drugs, P-113d, polymyxin B, and imipenem reached peak levels of 2.1 mg/L, 2.5 mg/L, and 79.4 mg/L, respectively. Finally, none of the rats treated with P-113d, polymyxin B, or imipenem showed clinical evidence of drug-related adverse effects, and no changes in physiological parameters were observed in the supplementary, uninfected group treated with 1 mg of P-113d/kg of body weight.

DISCUSSION

Despite the existence of appropriate antibiotics, P. aeruginosa continues to be an aggressive and potentially lethal microorganism in many groups of patients [4, 5]. Patients with neutropenia, for example, are susceptible to gram-negative and gram-positive infections, and sepsis due to P. aeruginosa infection may have a fulminant course in this setting [16]. For this reason, present guidelines for the treatment of suspected P. aeruginosa sepsis recommend the rapid introduction of empirical antimicrobial therapy that includes at least 1 antipseudomonal agent [5, 14, 16]. Nevertheless, the emergence of resistance during this therapy is difficult to detect and may lead to inappropriate definitive therapy, with increased rates of mortality and prolonged hospital stays. The main reason for this bacterial resistance is thought to be the organism’s low outer membrane permeability to antimicrobial agents. Moreover, because of its potent inflammatory activity and association with sepsis, LPS can be another important virulence factor in Pseudomonas infections.

The recent discovery of cationic peptides isolated from various biological sources may offer a good opportunity for treating this lethal condition [25]. In fact, their expression in highly

Table 3. Efficacy of intravenous administration of P-113d, polymyxin B, and imipenem in rat models after peritonitis was induced by cecal ligation and puncture.

<table>
<thead>
<tr>
<th>Time after bacterial challenge, treatment</th>
<th>Lethality, no. (%) of rats that dieda</th>
<th>Qualitative blood culture positive for bacteria, no. of rats</th>
<th>Bacterial count in peritoneal fluid, mean ± SD, cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control group C₃)</td>
<td>15 (100)</td>
<td>15</td>
<td>6.9 × 10⁹ ± 1.6 × 10⁹</td>
</tr>
<tr>
<td>P-113d, 1 mg/kg of body weightb</td>
<td>5 (33.3)</td>
<td>5</td>
<td>3.9 × 10⁹ ± 1.3 × 10⁹</td>
</tr>
<tr>
<td>Polymyxin B, 1 mg/kg of body weightb</td>
<td>5 (33.3)</td>
<td>6</td>
<td>4.4 × 10⁸ ± 1.7 × 10⁸</td>
</tr>
<tr>
<td>Imipenem, 20 mg/kg of body weightb</td>
<td>4 (26.6)</td>
<td>4</td>
<td>2.0 × 10⁴ ± 0.6 × 10⁴</td>
</tr>
<tr>
<td>360 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control group C₃)</td>
<td>15 (100)</td>
<td>15</td>
<td>7.7 × 10⁹ ± 1.3 × 10⁹</td>
</tr>
<tr>
<td>P-113d, 1 mg/kg of body weightc</td>
<td>5 (33.3)</td>
<td>6</td>
<td>3.1 × 10⁸ ± 6.9 × 10⁸</td>
</tr>
<tr>
<td>Polymyxin B, 1 mg/kg of body weightc</td>
<td>6 (40.0)</td>
<td>6</td>
<td>7.9 × 10⁷ ± 2.1 × 10⁷</td>
</tr>
<tr>
<td>Imipenem, 20 mg/kg of body weightc</td>
<td>5 (33.3)</td>
<td>5</td>
<td>4.8 × 10⁴ ± 1.5 × 10⁴</td>
</tr>
</tbody>
</table>

NOTE. Each treatment group included a total of 15 rats.

a Lethality was monitored for 72 h after bacterial challenge.
b P < .05, vs. control group C₃, for all values.
c P < .05, vs. control group C₃, for all values.
divergent species reflects the common need of multicellular organisms to defend against microbial invasion. Cationic peptides are thought to be major factors in antibacterial defense on mucosal surfaces, and they have been considered potent in reducing the level of bacterial load, LPS concentration, and TNF-α biosynthesis in animal models challenged with bacterial endotoxin [21, 22, 37]. In the majority of studies of initial screening of protective agents in rats, sepsis was induced by injection of a bolus of endotoxin [38]. The present study was designed to investigate the effect of treatment with P-113d on the development of P. aeruginosa sepsis in rats after ip administration of LPS and after bacterial challenge with P. aeruginosa ATCC 27853. In addition, in the present study we used not only the above-mentioned models but also a model of cecal ligation and puncture that resembled the clinical condition of a bowel perforation and mixed bacterial infection, to better investigate infections that arise from a nidus. Finally, to better describe the efficacy of the compounds in a more common clinical situation, characterized by the presence of an interval between the onset of sepsis and the initiation of therapy, the same experiments were performed after administration of the drugs 360 min after intervention. Our in vitro studies showed that P-113d is a potent anti-Pseudomonas compound; in addition, preliminary studies performed by use of a sensitive stimulus chromogenic in vitro assay showed that P-113d completely inhibits LPS procoagulant activity at a concentration of ~10-μmol/L and that, compared with the effect of polymyxin B on molar concentration, it exhibited an ~10-fold lower inhibition activity.

In the present study, the antimicrobial and endotoxin-neutralizing effects of P-113d were compared with those of polymyxin B and imipenem, both in vitro and in vivo. The polymyxins are a group of cyclic cationic polypeptides originally derived from Bacillus polymyxa; they share remarkable structural similarity with cationic peptides. Polymyxins direct antimicrobial activity and bind stoichiometrically (1:1) to the lipid A moiety of bacterial LPS, with the complete neutrali-
zation of endotoxin activity [39]. Imipenem was used as a control agent, since it is one of the most used antimicrobial agents in empirical antibiotic therapy for septicemia and shock. It kills all pathogens that can cause intra-abdominal infections, including anaerobes, gram-positive cocci, Enterobacteriaceae, and *Pseudomonas* species [40]. On the other hand, the clinical use of many antibiotics, such as imipenem, can promote endotoxin release when administered for the treatment of severe gram-negative bacterial infections. In fact, in the present study, the administration of imipenem significantly increased LPS and TNF-α levels, compared with levels in the peptide-treated rat groups. These data are in agreement with the assertion that the clinical use of antibiotics can be harmful when administered to treat severe gram-negative infections, because they can stimulate the release of endotoxin and thus increase the rates of occurrence of symptoms and of life-threatening complications.

For this reason, alternative compounds, such as cationic peptides, that are able to kill bacteria without production of cytokines by LPS-stimulated macrophages could be very useful in the treatment of gram-negative sepsis. Furthermore, the need for new antimicrobial therapies is evident because of the recent increase in the prevalence of multiple-antibiotic resistance among bacteria and the increasing size of populations of immunocompromised and elderly patients. In our study, P-113δ was effective, compared with all other parameters considered, regardless of the animal model used. Importantly, single ip doses of P-113δ produced a significant reduction in plasma concentrations of TNF-α, compared with concentrations in both the control and imipenem-treated groups. In contrast, compared with polymyxin B, P-113δ produced a similar decrease in the activity of plasma endotoxin.

Models 2 and 3, which also evaluated the antimicrobial effects of the 3 compounds at different times, showed that P-113δ and imipenem exhibited comparable antibacterial activity and that both are more effective than polymyxin B. However, in these models, no significant differences were observed in the rate of lethality among the P-113δ-, imipenem-, and polymyxin B–treated groups. Overall, because of its potent antimicrobial activity and ability to neutralize the biological effects of endotoxin, P-113δ shows promise as a new approach in strategies for the treatment of sepsis in immunocompromised patients.

**References**


