Analysis of Paradoxical Efficacy of Carbapenems against Carbapenemase-Producing *Escherichia coli* in a Murine Model of Lethal Peritonitis

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ABSTRACT The clinical benefit of carbapenems against carbapenemase-producing *Enterobacteriaceae* (CPE) remains in question. MICs of imipenem (IMP) and ertapenem (ERT) against isogenic derivatives of the wild-type strain *Escherichia coli* CFT073 producing KPC-3, OXA-48, or NDM-1 were 0.25, 2, 16, and 64 mg/liter for IMP and 0.008, 0.5, 8, and 64 mg/liter for ERT, respectively. Swiss ICR-strain mice with peritonitis were treated for 24 h with IMP or ERT. Despite a limited duration of time during which free antibiotic concentrations were above the MIC (down to 0% for the NDM-1-producing strain), IMP and ERT significantly reduced bacterial counts in spleen and peritoneal fluid at 24 h ($P < 0.005$) and prevented mortality. Several possible explanations were investigated. Addition of 4% albumin or 50% normal human serum did not modify IMP activity. Bacterial fitness of resistant strains was not altered and virulence did not decrease with resistance. In the presence of subinhibitory concentrations of ERT, growth rates of OXA-48, KPC-3, and NDM-1 strains were significantly decreased and filamentation of the NDM-1 strain was observed. The expression of *bla*_{NDM-1} was not decreased *in vivo* compared to *in vitro*. No zinc depletion was observed in infected mice compared with Mueller-Hinton broth. In conclusion, a paradoxical *in vivo* efficacy of IMP and ERT against highly resistant carbapenemase-producing *E. coli* was confirmed. Alternative mechanisms of antibacterial effects of subinhibitory concentrations of carbapenems may be involved to explain *in vivo* activity. These results are in agreement with a potential clinical benefit of carbapenems to treat CPE infections, despite high carbapenem MICs.

KEYWORDS *Escherichia coli*, antibiotic resistance, carbapenemase, carbapenems, peritonitis

Antibiotic resistance, in particular in Gram-negative bacilli, is a major threat facing modern medicine (1). The emergence and subsequent global spread of extended-spectrum β -lactamase-producing *Enterobacteriaceae* (ESBL-E) has led to a dramatic increase in carbapenem consumption. In this context, carbapenemase-producing *Enterobacteriaceae* (CPE) have appeared and spread worldwide. The emergence of CPE is of particular concern because such strains, as well as jeopardizing the use of carbapenems, are resistant to most β -lactams as well as other classes of antibiotics such as fluoroquinolones, aminoglycosides, and co-trimoxazole. Then, very few therapeutic

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TABLE 1 MICs and pharmacokinetics/pharmacodynamics in mice treated with ERT and IMP for each study strain, compared with human^a

Antibiotic	Species	Regimen	Strain	MIC (mg/liter)	MIC + 4% albumin (mg/liter)	T>MIC (%)	fT>MIC (%)
ERT	Human	1 g q 24 h	TOPO	0.008	0.008	100	100
	Mouse	100 mg/kg q 6 h	TOPO	0.008	0.008	100	100
			OXA-48	0.5	0.5	100	14
			KPC-3	8	8	44	0
			NDM-1	64	64	11	0
IMP	Human	1 g q 8 h	TOPO	0.25	0.25	100	100
	Mouse	100 mg/kg q 2 h	TOPO	0.25	0.25	100	100
			OXA-48	2	2	75	65
			KPC-3	16	16	45	40
			NDM-1	64	64	17	0

^aFor ERT: S ≤ 0.5; R > 1; for IMP: S ≤ 2; R > 8.

options remain (2). The best known antibiotics available today are either toxic or poorly effective, explaining high mortality rates in CPE bacteremia, reaching up to 40% (3). Alternative therapeutic regimens are urgently needed; however, few novel antimicrobials are being developed, especially against metallo- β -lactamase (MBL) producing CPE. Optimizing currently available antibiotics is therefore essential.

Interestingly, although CPE have different levels of resistance to carbapenems, antibiotic therapies, including at least one carbapenem, have been associated with a better outcome in severe infections caused by CPE *Klebsiella pneumoniae*, especially in critically ill patients (4–7). Concerning MBL specifically, although little clinical data are available, several reports of satisfactory outcomes with carbapenems, despite highly resistant strains *in vitro*, have been published (8, 9). This unexpected *in vivo* activity of carbapenems alone or in combination against MBL-producing strains has also been reported by others in mice models of either pneumonia or thigh infections (9, 10). Fitness cost associated with the CPE plasmids, poor *in vivo* expression of resistance or zinc depletion have been, among other explanations, suggested to support this phenomenon (11).

To gain insight into the discrepancy between *in vitro* resistance and the reports of *in vivo* efficacy of carbapenems in patients and animal models, we used a severe *Escherichia coli* peritonitis murine model complicated by severe sepsis and bacteremia to evaluate the activity of carbapenems against CPE with different levels of carbapenem resistance, and to explore several biological hypotheses which could explain how carbapenems maintain some efficacy *in vivo* against *in vitro*-resistant isolates.

RESULTS

Antibiotic activities *in vitro*. MICs of imipenem (IMP) and ertapenem (ERT) for each strain are shown in Table 1. *E. coli* strains CFT073 and OXA-48 remained in the susceptible range while KPC-3 and NDM-1 strains were highly resistant according to EUCAST breakpoints.

Antibiotic concentrations and PK/PD parameters. Antibiotic concentrations over time and pharmacokinetic/pharmacodynamic (PK/PD) indexes for each antibiotic and strain are shown in Table 1. Regimens of one injection of IMP every 2 h and one injection every 6 h of ERT reproduced the fT>MIC (time during which free-drug concentrations of antibiotic exceeded the MIC) obtained in humans for the susceptible strain (12–14). Values of fT>MIC were 0% for ERT against KPC-3 and for ERT and IMP against NDM1-producing strains.

Antibiotic activities in murine peritonitis. Mean bacterial load 2 h after inoculation was 6.61 log₁₀ CFU/ml (range 2.53 to 8.17) in peritoneal fluid, with no differences between the four study strains except NDM-1, which tended to have higher bacterial titers (7.17 log₁₀ CFU/ml, *P* < 0.005). All infected and untreated mice were bacteremic and spontaneous mortality at 24 h was 100%. When treated, survival rates at 24 h were 100% in all treatment groups for all strains except for ERT-treated mice infected with NDM-1, who had a 90% (9/10) survival rate. Figure 1 represents bacterial counts in peritoneal fluid before and after treatment, for each strain. The two therapeutic

FIG 1 Bacterial counts in peritoneal fluid before treatment and after 24 h treatment with IMP or ERT for each strain. Results are expressed in log₁₀ CFU/ml.

regimens (IMP and ERT) produced a significant decrease in viable bacterial counts compared to baseline, against TOPO, OXA-48, and NDM-1 strains ($P < 0.05$). Against KPC-3 strains, however, the decrease was significant only with ERT. For all strains, there was no significant difference in bacterial counts after 24 h of treatment between the therapeutic groups. For the OXA-48 and KPC-3 strains, as well as for the susceptible control, all mice survived when treated by ERT or IMP. No IMP- or ERT-resistant mutant was detected at the end of treatment for any of the tested strains.

Analysis of discrepancy between *in vitro* and *in vivo* efficacy. As PK/PD parameters could not explain the activity observed *in vivo* with both carbapenems, particularly against the carbapenem-resistant NDM-1 strain (Table 1), analysis of the possible causes of discrepancy between *in vitro* and *in vivo* efficacy was performed.

Technical issues. The first explanations to be ruled out were technical issues. No plasmid loss was evidenced in bacterial strains at the end of treatment, indicating that the resistant gene was still present at the end of the experiment. Antibiotic concentrations in peritoneal fluid were below the detection limit for IMP and ERT at the time of sacrifice (0.5 mg/liter). Therefore, there was no potential carry-over effect.

Resistance gene expression *in vivo*. In order to verify that the resistant gene was not only present but fully expressed *in vivo*, expression of *bla*_{NDM-1} (compared with *rrsA*) was measured in *E. coli* CFT073-NDM-1, in cells grown *in vitro* or *in vivo*, but no differences were found (Fig. 2).

Influence of albumin and human serum. The presence of 4% albumin did not modify MICs of IMP and ERT (Table 1). The activity of IMP against CFT073 (Fig. 3A) and NDM-1 (Fig. 3B) in time-kill studies was not modified by the presence of 50% normal human serum. Zinc depletion did not modify IMP MICs against CFT073, OXA-48, or KPC-3 strains, but decreased IMP MICs from 64 to < 0.06 mg/liter against NDM-1, as previously described (11).

Bacterial growth and morphology. No differences were found in terms of maximal growth rate (MGR) or time necessary to achieve maximal growth rate (T_{max}) between the four study strains growing without antibiotics (data not shown), suggesting there was no fitness cost of resistance. Similarly, no differences were found in terms of MGR or T_{max} for the strains growing with subinhibitory concentrations of IMP against pTOPO, OXA-48, KPC-3, or NDM-1 (data not shown). However, in the presence of subinhibitory concentrations of ERT, the T_{max} of OXA-48, KPC-3, and NDM-1 was significantly decreased (Fig. 4). MGR was also significantly decreased against NDM-1 only (data not shown). Microscopic examination of NDM-1 strains growing under subinhibitory concentrations ($1/8 \times$ MIC) of ERT found filamentation of *E. coli*, which was not the case for the isolates exposed to IMP or to no antibiotics (Fig. 5).

FIG 2 Normalized expression of bla_{NDM-1} (compared to $rrsA$) in *E. coli* CFT073-NDM1 cells grown *in vitro* or *in vivo* conditions. ns, not significant.

Zinc concentrations. The median zinc concentration in MH broth was 1.1 mg/liter (range: 1.086 to 1.177 mg/liter). The median concentration of zinc in the sera from healthy mice was 2.02 mg/liter (1.59 to 3.36), while the median was 2.72 mg/liter (1.94 to 3.87) in the sera from infected mice treated for 24 h with IMP ($P = 0.41$).

DISCUSSION

By means of isogenic *E. coli* strains carrying different types of carbapenemases with various IMP and ERT MICs *in vitro* and in a murine peritonitis model, we here attempted to decipher the observation that IMP and ERT alone were unexpectedly effective *in vivo* against isogenic CPE according to the usual PK/PD indexes associated with *in vivo* β -lactam activity, despite *in vitro* resistance (Table 2). Indeed, both IMP and ERT were effective in decreasing bacterial counts in spleen and peritoneal fluid and allowing all mice to survive in this otherwise lethal model. However, considering $ft > MIC$ as the relevant PK/PD index associated with carbapenem activity, values of 0% were obtained for free ERT against KPC-3-producing strains, as well as free ERT and IMP against NDM1-producing strains (Table 1). The results for NDM-1 are especially striking, given that the levels of resistance were the highest (MIC = 64 mg/liter) and the $ft > MIC$ for both free ERT and IMP was 0%. For OXA-48 and KPC-3 isolates, because the levels of resistance were lower, some $ft > MIC$ was achieved, which may explain at least partially the efficacy of the drugs.

This unexpected *in vivo* activity of carbapenems against CPE, which cannot be explained by current PK/PD indexes (15), has been reported by others mainly for NDM-1 producers and in one report for KPC (10, 11, 16, 17). Here, we purposefully tested isogenic strains to ensure that all things were otherwise equal, apart from resistance mechanisms and levels. This allowed us to investigate several hypotheses that may be involved in this phenomenon (Table 2). First, technical issues were eliminated, such as loss of the resistance plasmid or a carry-over effect. A fitness cost of resistance (as measured by *in vitro* growth) was not involved either. Decreased virulence of carbapenemase-producing strains was not an issue here either, as mortality rates of untreated mice reached 100% for all strains.

A lower than expected impact of protein binding may explain part of what we found here. Indeed, we found no differences in the experiments evaluating *in vitro* bacteriostatic or bactericidal activity of ERT and IMP whether they were performed with or without 4% albumin, even though ERT protein binding is theoretically 95%. Taking into account total concentrations of the antibiotics, time above the MIC would be in a range of values associated with *in vivo* antibacterial effect against the KPC-3-producing strain

A. Strain *E. coli* CFT073 **B. Strain *E. coli* CFT073-NDM-1**

FIG 3 Time-kill curves of IMP at the MIC alone or in combination with or without 50% pooled human serum against susceptible *E. coli* CFT073 (A) or resistant *E. coli* CFT073-NDM-1 (B).

(44 to 45% for ERT and IMP, respectively) and 11 and 17% for ERT and IMP against the NDM-1 strain (Table 1). Although the percentage of protein binding is considered “gold standard” for the evaluation of protein binding, the impact of protein binding on the activity of antibiotics is multifaceted and more complex than indicated by the numerical value of protein binding alone (18). In particular, intrinsic properties of serum proteins may mediate serum-antibiotic synergism. However, standardization of the experiments to substantiate that phenomenon remains to be done (18).

Because NDM-1 has the highest resistance levels, we focused further investigations on this strain. One hypothesis was that a decrease in the expression of the NDM-1 could occur *in vivo*, explaining the discrepancy between *in vitro* and *in vivo* resistance. However, normalized expression of *bla*_{NDM-1} did not differ between cells grown *in vitro* and *in vivo* (Fig. 2). The hypothesis that zinc concentrations in the infectious focus may play a role in the expression of the MBL enzymes has recently been suggested by Asempa et al. (11). Indeed, authors found concentrations of zinc in the epithelial lining fluid of mice with lung infections to be much lower than zinc concentrations in the broth used to test the strains' susceptibility, suggesting they may not have been resistant to carbapenems in the focus of infection, despite *in vitro* resistance. However, we assayed zinc in MH broth and in the plasma of control and infected mice and found concentrations in the same range (1 to 2 mg/liter), comparable to those found by Asempa et al. (11) in MHB and in mice plasma.

Another explanation could be related to an antibacterial effect resulting from a direct antibiotic/bacteria interaction but at subinhibitory concentrations. Our results suggest an impact of ERT on bacterial growth associated with filamentation at subin-

FIG 4 Time to achieve maximal growth rate ($T_{max} \times 10^4$ s) of the study strains in the presence of subinhibitory concentrations of ERT (left) and IMP (right).

inhibitory concentrations of $1/8 \times \text{MIC}$ against the NDM-1-producing strain; however, this was not observed with IMP. ERT predominantly inhibits penicillin-binding protein (PBP)-3, although it also has an impact on PBP-2. The former is responsible for cell septation during division. Sub-MICs of other antibiotics that target PBP-3, such as piperacillin-tazobactam, alter septation of *E. coli* due to PBP-3 inhibition without inhibiting division, leading to the generation of filamentous cells (19, 20). Several reports have found that these filamentous *E. coli* are accompanied by the inhibition of production of virulence factors, biofilm formation, and motility, leading to a reduced pathogenicity of these strains in mice (20). IMP, on the other hand, targets only PBP-2 and at sub-MICs does not induce filaments but round forms (21, 22).

Other mechanisms may involve the interaction between antibiotic and host immune responses leading to an enhanced antimicrobial effect targeted by the antibiotic. Bacteria exposed even to subinhibitory concentrations of antibiotics may change their adherence properties, cell surface antigens, excretion of enzymes and toxins, and cell wall thickness (23). Reports have found that *E. coli* grown in the presence of sub-MICs of IMP were phagocytized and killed in numbers significantly higher than untreated bacteria. Prior treatment with a sub-MIC of IMP also resulted in an increased susceptibility of *E. coli* to the bactericidal activity of immune serum (21). IMP demonstrated a stimulating effect of innate immunity in mice that was greater with the lowest IMP doses, and also modified some acquired immune functions that would be useful to investigate in humans (20, 24). More recently, Ulloa et al. (25) found that avibactam had immune sensitizing activities against NDM-producing *K. pneumoniae* that was not appreciated by standard antibiotic testing and led to a reduction in bacterial counts in lung from infected mice (25). To begin to explore the innate immune hypothesis, we tested the bactericidal activity of IMP in the presence and absence of normal human serum, but observed no differences, suggesting the intrinsic bactericidal activity of serum may not play a pivotal role here. A combination of these different phenomena, and possibly others, cannot be excluded.

In conclusion, we found an unexpected *in vivo* efficacy of both ERT and IMP against carbapenemase-producing *E. coli* with high levels of resistance to both drugs. This was especially surprising for NDM-1 producers, where therapeutic options are particularly limited, as carbapenems, despite at no time reaching above the MIC levels, allowed for mice survival and significant decreases in bacterial counts.

This may be the result of the impact of subinhibitory concentrations of antibiotics both on bacterial fitness and the host's immune response and/or other unknown factors. These data may explain clinical reports indicating therapeutic benefit from combinations, including at least one carbapenem against CPE, even against highly resistant strains (4, 7).

FIG 5 Photographs of rod-shaped NDM-1-producing *E. coli* after 24 h growth in MH medium with no antibiotic (A), ERT at 8 mg/liter ($0.125 \times \text{MIC}$) (B), or IMP at 8 mg/liter ($0.125 \times \text{MIC}$) (C). Photographs were taken using a light microscope at magnification of $\times 1,000$ before (upper) and after (lower) Gram staining.

MATERIALS AND METHODS

Bacterial strains, conjugation assays. Three clinical isolates were used as sources of carbapenemase genes: KPC-3-producing *E. coli* 13832, OXA-48-producing *E. coli* 13828, and NDM-1-producing *E. coli* UR20. *E. coli* DH5a was used for cloning experiments, and uropathogenic *E. coli* CFT073 (O6:K2:H1), previously used to produce pyelonephritis and peritonitis in mice, was used as the final recipient strain (26, 27). The plasmid pCR-Blunt II-TOPO (Life Technologies, Saint-Aubin, France), which carried a kanamycin resistance gene, was used for cloning experiments. In order to avoid any problem of expression of resistance *in vitro*, carbapenemase genes were cloned into pTOPO but with their own promoter. In order to avoid plasmid loss, all subcultures were done in the presence of kanamycin (400 mg/liter). PCR, cloning, and DNA sequencing have been described previously (27). For NDM-1, cloning into pTOPO was performed using the NDM-1-F (5'-AGAGAAATTTGCTCAGCTTGTGA-3') and NDM1-R (5'-GATGGCAGATTGGGGTGAC-3') primers. The four isogenic strains carrying the empty vector (pTOPO) and recombinant plasmids with the *bla*_{KPC-3}, *bla*_{OXA-48}, and *bla*_{NDM-1} genes are referred to as TOPO, KPC-3, OXA-48 and NDM-1, respectively.

In vitro antibiotic activities. MIC values of imipenem (IMP) and ertapenem (ERT) were determined by the broth microdilution method in accordance with EUCAST guidelines (www.eucast.org). Time-kill curves were performed to test for the bactericidal effect of each drug. To assess the impact of protein binding on antibiotic activity, tests were also performed in the presence of 4% albumin (to approach plasma albumin concentrations in humans of around 40 g/liter). To assess the impact of human serum on IMP activity, time-kill curves were also performed in the presence of 50% normal human serum (S4190-100 EURO BIO). To assess the influence of zinc depletion, MICs were also assayed in zinc-depleted Mueller-Hinton broth (MHB) by addition of 300 mg/liter of EDTA, as described (11).

For time-kill curves, exponentially growing *E. coli* cells were diluted in glass tubes containing 10 ml MHB to obtain an inoculum of ca. 10^6 CFU/ml and incubated with IMP or ERT in concentrations equal to 0.25 to 2 times the MIC value for the tested strain, or with no antibiotic. Viable counts were enumerated by plating 100- μ L serial dilutions of cultures onto MH agar plates after 0, 1, 3, 6, and 24 h of incubation at 37°C, and expressed in $\log_{10}$ CFU per milliliter. The lower limit of detection was 1 $\log_{10}$ CFU/ml. A bactericidal effect was defined as a decrease of at least 3 $\log_{10}$ in CFU counts compared to the initial inoculum.

Growth experiments. To measure growth rates of the different isolates with or without subinhibitory concentrations of ERT and IMP, bacterial cells were grown overnight in Luria-Bertani medium at 37°C with constant agitation at 250 rpm, and were transferred into fresh medium at a dilution of 1:100,00. On the same plate, wells contained no antibiotic or ERT or IMP at concentrations of 1/4, 1/8, or 1/16 of the MIC for each strain (from 0.0625 to 0.0156 mg/liter of IMP for TOPO, to 4 to 16 mg/liter IMP for the NDM-1 strain). Growth was recorded by an Infinite 200 Tecan spectrophotometer, which measured the optical density at 600 nm (OD_{600}) in each well every 5 min at 37°C for 24 h. OD_{600} values were collected and log-transformed. The time in minutes to reach maximum growth rate was measured and the maximum growth rate was calculated and expressed in h^{-1} (27). All *in vitro* experiments were repeated at least three times. Gram staining was performed on the bacteria growing at 24 h.

Experimental murine model. Swiss ICR-strain immunocompetent female mice aged 5 to 6 weeks and weighing 25 to 30 g were used in the experimental model of intraabdominal infection (27).

TABLE 2 List of possible mechanisms to explain the paradoxical activity of carbapenems *in vivo* against carbapenemase-producing strains

Hypothesis	Test /results	Comments
Loss of plasmid	All subcultures were performed in the presence of kanamycin (400 mg/liter) (a kanamycin resistance gene was present within the pTOPO plasmid)	No loss of plasmid
Carry-over effect	Antibiotic concentrations in peritoneal fluid were below the detection limit (0.5 mg/liter) for IMP and ERT at the time of sacrifice	No carry-over effect
<i>In vitro</i> fitness cost of resistance	Comparison of <i>in vitro</i> growth between susceptible and resistant strains: no difference found	No <i>in vitro</i> fitness cost of resistance
Decreased <i>in vivo</i> virulence	Mortality of untreated mice was 100% for all strains	No decrease in <i>in vivo</i> virulence
Impact of antibiotic protein binding	<i>In vitro</i> bacteriostatic and bactericidal activity of ERT and IMP tested with or without 4% albumin: no difference found	If total antibiotic concentrations taken into account (instead of free concentrations only), PK/PD parameters allow for some $T > \text{MIC}$ of ERT and IMP against all strains (see Table 1). The impact of protein binding on the activity of antibiotics may be more complex than indicated by the numerical value of protein binding
Decrease in expression of carbapenemase <i>in vivo</i> versus <i>in vitro</i>	No difference was found in terms of expression of <i>bla</i> _{NDM-1} (compared to <i>rrsA</i>) in <i>E. coli</i> CFT073-NDM-1 between cells grown <i>in vitro</i> or <i>in vivo</i>	No decrease in <i>bla</i> _{NDM-1} expression <i>in vivo</i>
Zinc concentrations <i>in vivo</i> may differ and explain discrepancy between resistance levels measure <i>in vitro</i> and <i>in vivo</i> for NDM-1 strains	Measured zinc concentrations in MH broth, serum of control and infected mice. Median zinc concentrations in MH broth, healthy mice, and infected mice were 1.1 mg/liter (1.086–1.177), 2.02 mg/liter (1.59–3.36), and 2.72 mg/liter (1.94–3.87), respectively ($P = 0.41$)	No differences were found in zinc concentrations between Mueller-Hinton broth and mice serum
Antibacterial effect of sub-inhibitory concentrations of antibiotics	<i>In vitro</i> growth curves in the presence of sub-inhibitory concentrations of IMP and ERT. -For IMP: no effect -For ERT: prolonged T_{max} for study strains and decreased maximum growth rate for NDM-1. Filamentation of <i>E. coli</i> at $1/8 \times \text{MIC}$ was evidenced.	Sub-MIC concentrations of ERT (which targets PBP-3) may inhibit septation without inhibiting division (as other beta-lactams), leading to the generation of filamentous cells and growth impairment as seen here. IMP targets only PBP-2 and does not induce filamentation.
Interaction between antibiotic and host immune response leading to an enhanced antimicrobial effect	Bactericidal activity of IMP in the presence and absence of pooled human serum was not different	No intrinsic bactericidal activity of serum was evidenced. The interaction between antibiotic and host immune response should be further explored.

Animal experiments were performed in accordance with prevailing regulations regarding the care and use of laboratory animals and approved by the Departmental Direction of Veterinary Services (Paris, France, agreement no. 75-861). The peritonitis protocol (APAFIS number 4949-2016021215347422 v5) was approved by the French Ministry of Research and the ethics committee for animal experiments.

Antibiotic concentrations in mice. To determine the therapeutic regimen in mice that best reproduces the $fT > \text{MIC}$ obtained in humans with standard regimens (1 g every 8 h for IMP and 1 g every 24 h for ERT), pharmacokinetic studies were performed on mice. A dose of 100 mg/kg was chosen for both IMP and ERT as they had previously been shown to reproduce the plasma peak concentrations that corresponded to human concentrations (28, 29). Free-drug concentrations of IMP and ERT had previously been determined in mice in our laboratory, and were found to be 37% for IMP and 1% for ERT (28).

Blood samples of a least 500 μl were obtained by intracardiac puncture from 4 anesthetized mice 30, 60, 90, and 120 min after a single subcutaneous injection of IMP (100 mg/kg) or 30, 60, 120, 240, 360, and 480 min after a single subcutaneous injection of ERT (100 mg/kg) (15). Concentrations of IMP and ERT were determined by high-performance liquid chromatography (HPLC) with UV detection at 237 nm, and the limit of detection was 0.5 mg/liter for both compounds (29).

Treatment experiments. Pellets of overnight *E. coli* cultures were mixed with porcine mucin 10% (Sigma-Aldrich, Saint-Quentin Fallavier, France). Mice, kept in single units during the experimental time, were inoculated with a 250- μl intraperitoneal injection of bacteria/mucin mix, corresponding to a final inoculum of approximately 10^8 CFU/ml. Two hours after inoculation, 15 mice for each strain were sacrificed to determine start-of-treatment bacterial load. We had previously shown that the death rate of

TABLE 3 Deoxynucleotide primers used in this study

Primer	Nucleotide sequence (5' to 3')	Gene purpose
RT-Q-PCR-NDM1_F	TGGCCCGCTCAAGGTATTTT	Quantification of <i>bla</i> _{NDM-1}
RT-Q-PCR-NDM1_R	TGCTGTCCTTGATCAGGCAG	
RT-qPCR_rrsA_F	CTCTTGCCATCGGATGTGCCCA	
RT-qPCR_rrsA_R	CCAGTGTGGCTGGTCATCTCTCA	

this model with the same bacterial strains was almost 100% in the absence of treatment and all mice were bacteremic; thus, for ethical reasons no untreated control group was used (27).

In the therapeutic groups, treatment was started 2 h postinfection and lasted 24 h. Mice received either IMP 100 mg/kg subcutaneously every 2 h or ERT 100 mg/kg subcutaneously every 6 h. Mice were sacrificed by intraperitoneal injection of 200 μ l of sodium thiopental 4 h after the last antibiotic injection. Immediately after sacrifice, a peritoneal wash was performed by intraperitoneal injection of 2 ml of sterile saline solution followed by gentle massage of the abdomen and opening of the peritoneum to collect 1 ml of fluid. Spleen was extracted and homogenized in 1 ml of sterile saline solution. In mice that did not survive the infection, only spleen was extracted within 2 h after death to avoid sample contamination. Samples diluted by a factor of 10 were plated onto MH agar for quantitative culture, containing or not IMP and ERT at a concentration of 4 $\times$ MIC to detect the selection of resistant mutants *in vivo*. Results were expressed as log₁₀ CFU/g for spleen and log₁₀ CFU/ml for peritoneal fluid. The lower limit of detection was 1.3 log₁₀ CFU/g or ml. Lack of plasmid loss was verified on surviving microorganisms by determining ERT and IMP MICs and by enumerating CFU on kanamycin-containing plates (400 mg/liter).

RNA extraction and qRT-PCR. To measure the expression of the *bla*_{NDM-1} gene, total RNA was extracted from bacterial cells grown to the late exponential phase *in vitro* and from bacterial cells extracted from the peritoneal cavity 2 h after inoculation *in vivo*. Total RNA was extracted using the RNeasy PowerMicrobiome kit (Qiagen Hilden, Germany) and residual chromosomal DNA was removed by treating samples with the TURBO DNA-free kit (Life Technologies, Saint-Aubin, France). Samples were quantified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France). cDNA was synthesized from total RNA (approximately 1 μ g) using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions.

Since it is impossible to know if cells are in the same growth phase *in vitro* and *in vivo*, we performed an absolute quantification of the expression of *bla*_{NDM-1} gene. The amounts of transcript levels were normalized using the *rrsA* gene as a housekeeping control gene. The number of transcript copies was determined by extrapolation from the linear regression of the standard curve obtained for each set of primers amplifying *bla*_{NDM-1} and *rrsA* genes (Table 3). The ratio *bla*_{NDM-1} copy number/*rrsA* copy number was calculated to compare the *bla*_{NDM-1} expression in *in vitro* and *in vivo* samples.

Zinc concentrations. Levels of Zn²⁺ were measured in MH broth and in mouse serum by inductively coupled serum mass spectrometry (ICP-MS) on an X-Series II from Thermo Scientific (Platform AEM2, University of Rennes 1/Biochemistry Laboratory, University Rennes Hospital) (30).

Statistical analysis. Continuous variables are expressed as the median followed by the range (minimum to maximum) in brackets, and were compared using a nonparametric test (Kruskal-Wallis). Maximal growth rates and time to achieve maximal growth rate values were compared using the Kruskal-Wallis test. A *P* value of less than 0.05 was considered significant. All statistical analyses were conducted using the Prism software (version 7.0a).

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