Evaluation of Ceftazidime and NXL104 in Two Murine Models of Infection Due to KPC-Producing *Klebsiella pneumoniae*[∇]

Andrea Endimiani,^{1,2} Kristine M. Hujer,^{1,2} Andrea M. Hujer,^{1,2} Mark E. Pulse,³
William J. Weiss,³ and Robert A. Bonomo^{1,2,4*}

Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio¹; Research Service, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, Ohio²; University of North Texas Health Science Center, Fort Worth, Texas³; and Department of Pharmacology, Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio⁴

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We evaluated the efficacy of NXL104, a novel β -lactamase inhibitor, in combination with ceftazidime (CAZ) in two murine infection models (septicemia and thigh infection). We chose two KPC-producing *Klebsiella pneumoniae* strains (VA-361 and VA-406) showing MICs of CAZ of ≥ 256 $\mu\text{g/ml}$. Septicemia was induced by the intraperitoneal injection of KPC-producing *K. pneumoniae* followed 30 min later by a single subcutaneous treatment with CAZ alone or CAZ-NXL104 in ratios of 2:1, 4:1, 8:1, and 16:1. In this model, the median effective doses for 50% (ED_{50}) of the animals for CAZ alone versus VA-361 and VA-406 were 1,578 and 709 mg/kg of body weight, respectively. When combined with NXL104 at 2:1, 4:1, 8:1, and 16:1 ratios, the CAZ ED_{50} s for VA-361 and VA-406 were reduced to 8.1 and 3.5 mg/kg, 15.1 and 3.8 mg/kg, 16.9 and 7.2 mg/kg, and 29.5 and 12.1 mg/kg, respectively. For thigh infection, neutropenia was induced by the intraperitoneal injection of cyclophosphamide at days -4 and -1 preinfection. Infection was established by the intramuscular injection of KPC-producing *K. pneumoniae* into the right thigh. Mice were treated 1.5 h postinfection with either CAZ alone or CAZ-NXL104 at constant ratios of 4:1. When thighs were removed at 24 h postinfection, a >2 -log CFU reduction was observed for mice treated with CAZ-NXL104 at doses of $\geq 128:32$ mg/kg. In contrast, CAZ doses of $\geq 1,024$ mg/kg were unable to reduce the numbers of CFU. Despite resistance to CAZ and possessing a complex β -lactamase background, NXL104 combined with CAZ proved to be very effective in murine models of infection due to contemporary highly resistant KPC-producing *K. pneumoniae* isolates.

The spread of class A KPC carbapenemases among the family *Enterobacteriaceae* (particularly *Klebsiella pneumoniae*), *Pseudomonas* spp., and *Acinetobacter baumannii* is drawing significant attention due to the important clinical implications that this resistance determinant bears for infected patients (11, 21, 25). KPC-producing *K. pneumoniae* isolates are endemic in certain hospitals and are responsible for increasing numbers of outbreaks in many health care facilities located in the United States, Puerto Rico, Israel, Greece, and Germany (10, 17, 27). Sporadic detection in Central and South America, the Far East, and other European countries has also been reported (1, 9, 17, 18, 20, 23).

KPC-producing *K. pneumoniae* isolates demonstrate resistance or reduced susceptibility to most commercially available β -lactams and β -lactam/ β -lactamase inhibitor combinations as well as many other alternative classes of antimicrobials (e.g., fluoroquinolones and aminoglycosides) (5, 17). Thus, colistin, tigecycline, and fosfomycin are becoming the “last-line” therapeutic options for infections due to KPC-producing *K. pneumoniae* isolates. Unfortunately, the clinical use (e.g., dosage

and time of treatment) of these antimicrobials is not yet fully defined, and their resistance rates for KPC-producing *K. pneumoniae* strains are also quickly increasing (7, 11, 15, 23, 28).

Relatively few novel compounds are in development that promise to be active against Gram-negative multidrug-resistant (MDR) pathogens, such as those producing KPC enzymes (3, 6). NXL104 (Novoxel SA, Romainville, France, and Astra-Zeneca Pharmaceuticals, Boston, MA) is a new β -lactamase inhibitor that is active against class A (e.g., TEM, SHV, and CTX-M types) and class C β -lactamases and is currently in clinical trials (<http://clinicaltrials.gov>) (2, 14, 22). Recently, Endimiani et al. demonstrated its *in vitro* activity in combination with standard β -lactams against a large collection of KPC-producing *K. pneumoniae* isolates collected in the Eastern United States (4). In particular, the MICs of the combination of NXL104 at a constant concentration of 4 $\mu\text{g/ml}$ with piperacillin, extended-spectrum cephalosporins (e.g., ceftazidime and cefotaxime), and aztreonam were in the susceptible range for all tested strains (overall, MIC_{90} values of ≤ 2 $\mu\text{g/ml}$) (4).

In the present work, we evaluated the *in vivo* antibacterial efficacy of ceftazidime (CAZ) in combination with NXL104 in a mouse septicemia model and a mouse thigh infection model using two well-characterized KPC-producing *K. pneumoniae* strains. Our data show that the addition of NXL104 results in a significant restoration of ceftazidime efficacy and the ability to eradicate infections due to KPC-producing *K. pneumoniae* isolates.

* Corresponding author. Mailing address: Infectious Diseases Section, VISN 10 GRECC, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, 10701 East Blvd., Cleveland, OH 44106. Phone: (216) 791-3800, ext. 4399. Fax: (216) 231-3482. E-mail: robert.bonomo@med.va.gov.

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TABLE 1. Characteristics of the two KPC-producing *K. pneumoniae* isolates used for the *in vivo* experiments^a

Molecular characteristic	KPC-producing <i>K. pneumoniae</i> isolate	
	VA-361	VA-406
<i>bla</i> genes	<i>bla</i> _{KPC-2} , <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-11}	<i>bla</i> _{KPC-2} , <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-11} , <i>bla</i> _{SHV-12}
aIEF	5.4, 6.7, 7.6, 8.2	5.4, 5.8, 6.7, 7.6
<i>ompK</i> genes	<i>ompK35</i> (FS, Ins of G at bp 123) <i>ompK36</i> (multiple M, del, and Ins) <i>ompK37</i> (multiple M, Ins)	<i>ompK35</i> (FS, Ins of G at bp 123) <i>ompK36</i> (Ins of ~2.5 kb) <i>ompK37</i> (multiple M, Ins)

^a aIEF, analytical isoelectric focusing; FS, frameshift; M, mutations; Ins, insertions; Del, deletions.

MATERIALS AND METHODS

Clinical isolates. *In vivo* experiments were conducted by using two KPC-producing *K. pneumoniae* isolates (i.e., VA-361 and VA-406) characterized previously (4, 5). Both strains were collected in the Eastern United States and are clonally related by pulsed-field gel electrophoresis (PFGE) and repetitive extragenic palindromic PCR (rep-PCR) analyses (5). The molecular and phenotypic profiles of the two KPC-producing *K. pneumoniae* isolates are shown in Table 1. *K. pneumoniae* isolate ATCC 13883 was also used as a control. This ATCC strain has an MIC value of CAZ of 2 µg/ml.

Mice. Female 5- to 6-week-old CD-1 mice (Harlan Laboratories, Houston, TX) were used for both acute lethal septicemia and thigh infection models (see below). All studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee (University of North Texas Health Science Center at Fort Worth). During the study, mice were housed in groups of 3 to 5 with free access to food and water in accordance with National Institutes of Health guidelines.

Murine acute lethal septicemia. Mice were infected by the intraperitoneal (i.p.) injection of the KPC-producing *K. pneumoniae* strain resulting in the death of untreated controls within 24 to 48 h. In particular, a fresh predetermined bacterial inoculum of approximately 3.3×10^5 to 3.6×10^5 CFU in 5% hog gastric mucin grown overnight was used for both KPC-producing *K. pneumoniae* isolates. Thirty minutes postinfection, a single subcutaneous dose of ceftazidime (Sigma-Aldrich, St. Louis, MO) with and without NXL104 (see below for dosages) was initiated, and the survival ratio was monitored for 5 days twice a day.

For *K. pneumoniae* VA-361, 5 mice/dose level for each of the following treatment regimens were tested: CAZ alone (doses of 512, 1,024, and 2,048 mg/kg of body weight) and CAZ plus NXL104 at ratios of 2:1, 4:1, 8:1, and 16:1 (CAZ doses were 4, 8, 16, 32, and 64 mg/kg for each ratio). Tests were performed three times for each dose on separate days. Similarly, for *K. pneumoniae* VA-406, the following treatment regimens were evaluated: CAZ alone (doses of 256, 512, 1,024, and 2,048 mg/kg) and CAZ plus NXL104 at ratios of 2:1 and 4:1 (CAZ doses of 1, 2, 4, 8, and 16 mg/kg for each ratio) and 8:1 and 16:1 (CAZ doses of 2, 4, 8, 16, and 32 mg/kg for each ratio). Tests were performed three times for each dose on separate days. The median effective dose for 50% (ED₅₀) of animals was determined by a computerized program of Probit analysis for both KPC-producing *K. pneumoniae* strains and regarding the different dose regimens tested (26). Survival rates, stratified for different dose regimens, were also obtained.

Neutropenic murine thigh infection. Mice were rendered neutropenic by the i.p. injection of cyclophosphamide (Cytosan; Baxter Healthcare, Deerfield, IL) with 150 mg/kg at day -4 and 100 mg/kg at day -1 preinfection. Infection was induced by the intramuscular (i.m.) injection of 0.1 ml of sterile saline containing 10⁶ CFU of the KPC-producing *K. pneumoniae* isolate into the right thigh of each animal. Mice were treated subcutaneously 1.5 h postinfection with either CAZ alone or CAZ combined with NXL104 at a constant ratio of 4:1. For each dose group, 3 mice were tested, including the untreated control group. In particular, for VA-361, the ranges of concentrations of CAZ-NXL104 tested were 1,024:256 mg/kg to 32:8 mg/kg, whereas for VA-406, the ranges were 1,024:256 mg/kg to 8:2 mg/kg.

At 24 h postinfection, mice were euthanized by CO₂ inhalation, and thighs were aseptically removed for CFU enumerations. In particular, thighs were homogenized (Polytron tissue homogenizer; Kinematica, Luzern, Switzerland) on ice in sterile saline, diluted, and plated onto Trypticase soy agar with charcoal (to prevent the effects of any carryover of compound). Plates were incubated overnight at 37°C prior to counting of CFU (24). The relationship of dose versus change in CFU/thigh was determined for all doses administered.

TABLE 2. MICs for the two KPC-producing *K. pneumoniae* isolates used for *in vivo* experiments

Antimicrobial	MIC (µg/ml)	
	VA-361	VA-406
Piperacillin	≥512	≥512
Piperacillin + NXL104 (1 µg/ml)	32	16
Piperacillin + NXL104 (2 µg/ml)	8	8
Piperacillin + NXL104 (4 µg/ml)	≤0.06	0.5
Piperacillin + tazobactam (4 µg/ml)	≥512	≥512
Cefotaxime	64	≥512
Cefotaxime + NXL104 (1 µg/ml)	0.5	0.5
Cefotaxime + NXL104 (2 µg/ml)	0.5	0.5
Cefotaxime + NXL104 (4 µg/ml)	0.125	0.125
Cefotaxime + clavulanate (4 µg/ml)	16	256
Cefotaxime + tazobactam (4 µg/ml)	16	256
Ceftazidime	256	≥512
Ceftazidime + NXL104 (1 µg/ml)	8	8
Ceftazidime + NXL104 (2 µg/ml)	4	2
Ceftazidime + NXL104 (4 µg/ml)	0.25	≤0.06
Ceftazidime + clavulanate (4 µg/ml)	128	256
Ceftazidime + tazobactam (4 µg/ml)	128	256
Cefepime	32	256
Cefepime + NXL104 (1 µg/ml)	0.5	0.5
Cefepime + NXL104 (2 µg/ml)	0.25	0.5
Cefepime + NXL104 (4 µg/ml)	≤0.06	0.125
Cefepime + clavulanate (4 µg/ml)	8	128
Cefepime + tazobactam (4 µg/ml)	16	128
Imipenem	4	256
Imipenem + clavulanate (4 µg/ml)	2	128
Imipenem + tazobactam (4 µg/ml)	4	256
Meropenem	4	256
Meropenem + clavulanate (4 µg/ml)	2	128
Meropenem + tazobactam (4 µg/ml)	4	128

RESULTS AND DISCUSSION

Finding new antimicrobial agents or combinations is becoming an important priority to combat the spread of MDR Gram-negative organisms, such as those producing the KPC carbapenemases (19). Due to their low toxicity and high bactericidal activity, the use of β-lactam antibiotics in combination with new and more-active β-lactamase inhibitors seems to be an attractive strategy (2). Several new β-lactam/β-lactamase inhibitor combinations have recently demonstrated *in vitro* activity against KPC-producing *Enterobacteriaceae* (3, 4, 16). Although very promising, the *in vivo* activity of those combinations has not yet been fully evaluated.

In the present work, the *in vivo* antimicrobial activity of an experimental combination of CAZ plus the new β-lactamase inhibitor NXL104 was tested at different ratios in two animal models of infection due to KPC-producing *K. pneumoniae*. In particular, the two isolates tested (i.e., VA-361 and VA-406) were clinical strains representative of the main clone that is spreading in the United States and widely (5).

Both KPC-producing *K. pneumoniae* strains were nonsusceptible *in vitro* to all β-lactams and standard β-lactam/β-lactamase inhibitor combinations. However, VA-406 possessed higher MICs (with particular emphasis on carbapenems) than VA-361 (Table 2). This phenomenon is frequently observed in the clinic and is due to a combination of a different *OmpK* background and/or variable expression levels of KPC enzymes (8, 12, 13).

TABLE 3. ED₅₀ for CAZ with and without NXL104 at selected ratios against a septicemia infection model due to *K. pneumoniae* isolates

Strain	ED ₅₀ (mg/kg) (95% confidence limit) ^a				
	CAZ	CAZ-NXL104 at ratio of:			
		2:1	4:1	8:1	16:1
ATCC 13883	1.9 (1.4–2.3)	1.0 (0.75–1.6)	1.1 (0.4–1.4)	0.62 (0.41–0.91)	NT
KPC-producing <i>K. pneumoniae</i> VA-361	1578 (1244–2011)	8.1 (6.2–10.3)	15.1 (12.1–18.7)	16.9 (13.6–20.8)	29.5 (21.9–39.8)
KPC-producing <i>K. pneumoniae</i> VA-406	709 (517–961)	3.5 (2.7–4.7)	3.8 (2.9–4.9)	7.2 (5.4–9.4)	12.1 (9.3–15.9)

^a NT, not tested.

Murine acute lethal septicemia. In Table 3 we show the results of the acute lethal septicemia analysis. For VA-361 and VA-406, CAZ alone exhibited ED₅₀ values of 1,578 and 709 mg/kg, respectively. These elevated ED₅₀s were reduced with the coadministration of NXL104 at the ratios tested. The administration of CAZ-NXL104 at a ratio of 4:1 effectively reduced the ED₅₀s for VA-361 and VA-406 to 15.1 and 3.8 mg/kg, respectively (Table 3). As shown in Fig. 1A and B, animal survival increased in relation to the ratio of CAZ to NXL104 for both KPC-producing *K. pneumoniae* isolates tested. In particular, the addition of NXL104 to CAZ remarkably reduced the amount of CAZ required to treat favorably the systemic infection induced in mice.

Neutropenic murine thigh infection. As shown in Table 4, following the administration of CAZ-NXL104 at a constant ratio of 4:1, VA-361 exhibited CFU reductions with doses of ≥256:64 mg/kg, whereas for VA-406, CFU reductions were achieved at doses of ≥128:32 mg/kg. The static doses were determined to be 216:54 mg/kg for VA-361 and 116:29 mg/kg for VA-406. Dosages of ≤64:16 mg/kg were unable to reduce the CFU count for both KPC-producing *K. pneumoniae* isolates (Table 4).

In both murine models, the more resistant KPC-producing *K. pneumoniae* isolate VA-406 was more susceptible *in vivo* (i.e., lower ED₅₀ and higher CFU reduction) to the CAZ-NXL104 treatment than was VA-361. We do not have a clear

explanation for this phenomenon. However, we speculate that the two KPC-producing *K. pneumoniae* strains possess different virulence factors. Based upon these observations, we maintain that the treatment outcome of infections due to KPC-producing *K. pneumoniae* isolates is influenced by multiple factors and not only the antimicrobial phenotype obtained in the clinical laboratory.

Conclusion. These data show that the addition of NXL104, a potent β-lactamase inhibitor, to CAZ results in the restoration of CAZ efficacy in these two mouse models of infection. Our analysis with *K. pneumoniae* isolates expressing the clinically relevant KPC β-lactamase demonstrates that the usefulness of the CAZ-NXL104 combination was maintained even when there was a disruption of the OmpK channels, as VA-406 possessed MICs against the carbapenems that were well above any reasonably achievable serum levels. The latter feature has significant importance, as current microbiology laboratories will identify KPC-producing *K. pneumoniae* but will be able to report only a limited amount of information about the strains to clinicians (e.g., susceptible or resistant, modified Hodge test positive or negative, and MIC values), as the MIC endpoints are not routinely measured (8). Lastly, our observations also raise interesting questions regarding the growth characteristics and antibiotic permeability attributes of strains of *K. pneumoniae* with OmpK changes, as the efficacy of this CAZ-NXL104 com-

FIG. 1. Survival curves for mice treated with ceftazidime (CAZ) with and without NXL104 in the murine septicemia model due to KPC-producing *K. pneumoniae*. (A) Data regarding KPC-producing *K. pneumoniae* strain VA-361. (B) Data regarding KPC-producing *K. pneumoniae* strain VA-406.

TABLE 4. Log₁₀ CFU/thigh values for CAZ plus NXL104 (constant ratio of 4:1) in the neutropenic thigh infection model versus the two KPC-producing *K. pneumoniae* isolates

Dose of CAZ-NXL104 (mg/kg) at ratio of 4:1 ^c	Log ₁₀ CFU/thigh at 24 h vs CFU at 1.5 h postinfection	
	VA-361 ^a	VA-406 ^b
1,024:256 ^c	-1.76	-1.59
512:128	-1.47	-1.03
256:64	-0.85	-1.02
128:32	+1.88	-0.46
64:16	+2.19	+2.06
32:8	+2.32	+2.33
16:4	NT	+2.69
8:2	NT	+2.93

^a The 1.5-h-postinfection count was 6.16 log₁₀ CFU; without treatment, this value increased to 8.93 log₁₀ CFU by 24 h. NT, not tested.

^b The 1.5-h-postinfection count was 6.07 log₁₀ CFU; without treatment, this value increased to 8.67 log₁₀ CFU by 24 h.

^c For CAZ alone at 2,048 mg/kg, the value for VA-361 was -0.34 log₁₀ CFU/thigh (i.e., 8.59 CFU/thigh), and that for VA-406 was +0.03 log₁₀ CFU/thigh (i.e., 8.70 CFU/thigh). For CAZ alone at 1,024 mg/kg, the value for VA-361 was -0.32 log₁₀ CFU/thigh (i.e., 8.61 CFU/thigh), and that for VA-406 was +0.25 log₁₀ CFU/thigh (i.e., 8.92 CFU/thigh).

bination was better against VA-406. We plan further experiments to explore these significant observations.

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