

Activity of the New β -Lactamase Inhibitor NXL104 against KPC β -Lactamases

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ABSTRACT

Background: NXL104 is a novel non β -lactam β -lactamase inhibitor that has been shown *in vitro* and *in vivo* to inhibit both class A and class C β -lactamases. Within class A enzymes, KPCs represent a new family having potential for wide dissemination; KPC production confers resistance to β -lactams including carbapenems. In this study, we evaluated the *in vitro* activity of NXL104 against KPC-2, and of various β -lactam/NXL104 combinations against bacteria that express this and other β -lactamases.

Methods: Enzymatic activity of recombinant purified KPC-2 was measured with nitrocefin as reporter substrate. Enzyme kinetic parameters and NXL104 inhibition parameters (IC_{50} and turnover number) were determined. Clavulanic acid and tazobactam were included as comparators. MIC values of various β -lactams combined with NXL104 at 4 mg/L were determined by the CLSI method against KPC-producing strains (three KPC-2 and one KPC-3 *Enterobacteriaceae*).

Results: NXL104 showed an extremely potent inhibitory activity against KPC-2 enzyme, with a low IC_{50} (38 nM) and a low turnover number ($T_n=1$). In contrast, clavulanic acid and tazobactam were very much less active with IC_{50} values in the μ M range.

The four KPC producers were highly resistant to β -lactams: MIC values ≥ 128 mg/L for ceftazidime and ceftioxone and 16 - 128 mg/L for imipenem. The addition of NXL104 protected the antibiotics from β -lactamase hydrolysis: MIC values were ≤ 0.125 - ≤ 2 mg/L for ceftioxone/NXL104 and imipenem/NXL104, and 0.5 - 4 mg/L for ceftazidime/NXL104.

Conclusion: NXL104 was remarkably active against KPC-2 enzyme; it efficiently restored the antimicrobial activity of ceftazidime, ceftioxone and imipenem against KPC-2 and KPC-3 producing strains of *Enterobacteriaceae* species.

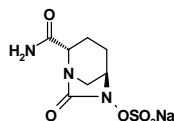
INTRODUCTION

Carbapenems play an important role in the treatment of severe and difficult to treat infections caused by multiresistant gram-negative bacteria. The recent emergence and spread of carbapenem resistance is a major concern in the clinic. The most prevalent resistance mechanism is the production of β -lactamases that are capable of hydrolyzing carbapenems. *Klebsiella pneumoniae* carbapenemases (KPCs) are currently emerging in clinical settings in the eastern United States (1). KPC enzymes are unique in the class A β -lactamases since they efficiently hydrolyse β -lactams, including carbapenems. In addition, the transfer of KPCs has led to the appearance of resistant *Enterobacteriaceae* strains (2).

NXL104 (Figure 1) is a new non- β -lactam inhibitor of β -lactamases in clinical development. It displays a broad spectrum inhibition profile against both class A and class C enzymes; both types of enzyme are inactivated very efficiently with low IC_{50} values, low turnover numbers and a highly stable covalent complex (3). NXL104 has virtually no intrinsic antibacterial activity, but efficiently protects β -lactams from hydrolysis in a variety of class A and class C producing strains, including ESBL producers (4). Protection against acute lethal infections has been demonstrated in murine models (5, 6).

The objective of this study was a kinetic characterization of purified KPC-2 and its inhibition by NXL104, and an evaluation of the *in vitro* antibacterial activity of various β -lactam/NXL104 combinations against highly resistant strains.

Figure 1: Chemical structure of NXL104



METHODS

KPC-2 purification:

*bla*_{KPC-2} was cloned into pET29 to allow the induction of the expression of the protein in *E. coli* BL21. KPC-2 was then purified by serial column chromatography (data not shown). The enzyme was concentrated to 2.5 mg/mL and protein purity assessed by SDS-PAGE (Fig. 2).

β -lactamase assay:

Enzyme activity and inhibition was quantitated by spectrophotometric measurement of nitrocefin hydrolysis at 485 nm and at 37°C after 30 min inhibitor/enzyme pre-incubation. Nitrocefin was present at 100 μ M and KPC-2 at 3 nM in 50 mM phosphate pH 7.0 buffer, 2% glycerol, and 0.1 mg/mL bovine serum albumin. Data were processed using Grafit (Erihtacus Software).

Turnover number:

The turnover number (T_n) is the number of inhibitor molecules required to inactivate one enzyme molecule. Kinetics of inactivation were determined at 37°C with 120 nM KPC-2 and different molar inhibitor/enzyme ratios. Residual β -lactamase activity was measured in the standard conditions after appropriate enzyme dilution. One hour of inhibitor / KPC-2 incubation was chosen for the T_n determination, corresponding to the minimal time period required for maximum inhibition (Fig. 5a). The T_n values were deduced from the extrapolated value for 100% inactivation from the plot of the residual activity versus inhibitor/enzyme ratios (7) - see Fig. 5b, 5c and 5d.

MIC determination:

MICs were determined using CLSI methods for antimicrobial susceptibility testing with Mueller-Hinton broth. MIC was defined as the lowest concentration which inhibited all visual growth.

Bacterial strains:

KPC-producing bacterial strains were isolated in French hospitals in the Paris area: Pitié-Salpêtrière hospital for *E. coli* 2138 and *E. cloacae* 7506 (8), Tenon hospital for *E. cloacae* MAC (kind gift of Dr. G. Arlet) (9) and Kremlin-Bicêtre hospital for *K. pneumoniae* YC (kind gift of Dr L. Poirel) (10).

Tested compounds:

The following antimicrobials were used: ceftazidime pentahydrate (CAZ), piperacillin (PIP), ceftioxone (CRO), amoxicillin (AMX) and imipenem (IMP). The following β -lactam inhibitors were used: clavulanate lithium (CLA), NXL104 and tazobactam (TAZ).

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RESULTS

Figure 2: SDS-PAGE of the purified KPC-2

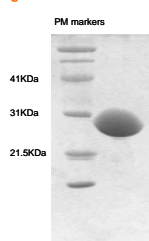
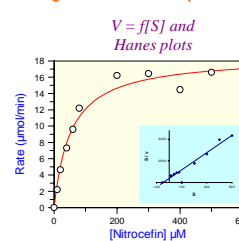


Figure 3: KPC-2 kinetic parameters

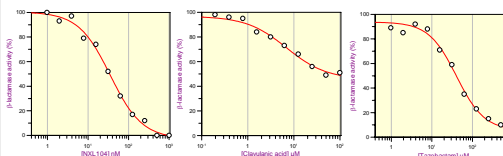


Kinetic parameters	Mean values
K_m	52 μ M
K_{cat}	54 s^{-1}

Figure 4: Inactivation of KPC-2 by NXL104 and comparators

NXL104 showed a stronger inhibition of KPC-2 (IC_{50} value in the nanomolar range), than the comparators clavulanic acid and tazobactam (IC_{50} values in the micromolar range).

In the experimental conditions described in the Methods section, complete inhibition of KPC-2 could not be obtained with clavulanic acid, even at the highest concentrations tested: ~ 50% KPC-2 activity remained with clavulanic acid at 100 μ M. In contrast, KPC-2 activity was totally inhibited by NXL104 at 1 μ M.



β -lactamase	IC_{50} (μ M)		
	NXL104	CLA	TAZ
KPC-2	0.038	6.5	80.0

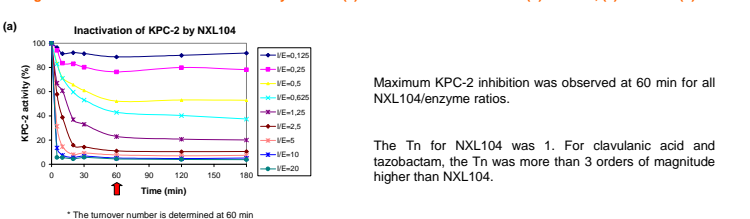
* 30 min preincubation inhibitor/enzyme

CONCLUSION

NXL104 was shown to be a remarkably potent inhibitor of KPC-2 carbapenemase.

- NXL104 inhibited purified KPC-2 with an IC_{50} of 38 nM. Clavulanic acid and tazobactam were respectively more than 100- and 1000-fold less active.
- Efficiency of NXL104 was confirmed by a very low turnover number as compared with β -lactams.
- NXL104 restored the antimicrobial activity of ceftazidime, ceftioxone, imipenem and piperacillin against KPC-2 and KPC-3 producing strains of *Enterobacteriaceae* species.

Figure 5: Kinetics of KPC-2 inactivation by NXL104 (a) and turnover numbers for (b) NXL104, (c) CLA and (d) TAZ



Maximum KPC-2 inhibition was observed at 60 min for all NXL104/enzyme ratios.

The T_n for NXL104 was 1. For clavulanic acid and tazobactam, the T_n was more than 3 orders of magnitude higher than NXL104.

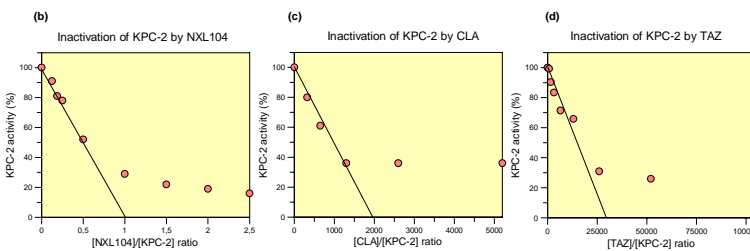


Table 1: Comparative *in vitro* antibacterial activity of different β -lactam / β -lactamase inhibitor combinations against strains of *Enterobacteriaceae* species (MIC – mg/L)

	<i>K. pneumoniae</i> YC	<i>E. coli</i> 2138	<i>E. cloacae</i> 7506	<i>E. cloacae</i> MAC
	KPC-2	KPC-2, TEM-1	KPC-2, TEM-1, KLUC-2	KPC-3, TEM-1, OXA-9
CAZ	>128	128	>128	>128
CAZ / CLA	64	32	128	>128
CAZ / TAZ	>128	16	>128	>128
CAZ / NXL104	1	0.5	4	4
PIP	>128	>128	>128	>128
PIP / TAZ	>128	>128	>128	>128
PIP / NXL104	16	4	32	16
CRO	>128	>128	>128	>128
CRO / NXL104	0.25	≤ 0.125	0.5	1
AMX	>128	>128	>128	>128
AMX / NXL104	>128	64	>128	128
AMX / CLA	>128	>128	>128	>128
IMP	32	16	128	64
IMP / NXL104	≤ 0.125	0.25	0.25	2

* MICs for β -lactamase inhibitors alone were ≥ 128 mg/L.

* β -lactamase inhibitors were at the fixed concentration of 4 mg/L when used in combination with a β -lactam antibiotic.