

The β -lactamase Inhibitor NXL104 Does Not Induce *ampC* β -lactamase Expression in *Enterobacter cloacae*: Evaluation of *ampC* Expression by Quantitative Polymerase Chain Reaction (Q-PCR).

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ABSTRACT

Background: Induction of AmpC β -lactamase can be triggered by several β -lactams, such as cefoxitin (FOX), imipenem and clavulanic acid (CLA), possibly compromising antibiotic treatment. NXL104 is the first of a new chemical series, and is a potent inhibitor of both class A and class C β -lactamases; its potential for induction of *ampC* expression in *Enterobacter cloacae* was investigated. Due to high stability of the NXL104-AmpC covalent complex, *ampC* expression was necessarily evaluated by mRNA quantitation rather than by measurement of enzymatic activity.

Methods: 3 *E. cloacae* strains were cultivated in the presence of FOX, CLA or NXL104 at various concentrations. Total RNA was extracted and used as a template for reverse transcription and PCR amplification of *rplS* and *ampC* cDNA. *ampC* mRNA was quantified relative to *rplS* mRNA. β -lactamase activity was measured using nitrocephin.

Results: Basal *ampC* mRNA expression was very low in all 3 *E. cloacae* strains. A major increase of *ampC* mRNA was observed in cells treated with 1 - 32 μ g/mL FOX, with a maximum reached after 1-2 hours of culture (x 10-250); at higher concentrations, one strain exhibited a peak of *ampC* mRNA at 0.5-1 hour (up to x750). Increases of β -lactamase activity remained detectable 2 hours after treatment. In cells exposed to CLA, a similar response was observed, although the level of *ampC* mRNA induction was less significant (maximum was x40 at 64 μ g/mL). Finally, no significant induction of *ampC* mRNA was observed in any of the 3 *E. cloacae* strains when treated with NXL104 at the maximum concentration tested (64 μ g/mL) during the 6-hour period of incubation.

Conclusion: FOX and CLA were confirmed as *ampC* inducers in *E. cloacae*. In contrast, NXL104 was found to exert no effect on *ampC* expression, implying that NXL104 does not further compromise the activity of co-administered β -lactam antibiotics.

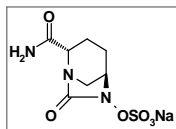
BACKGROUND

Several species of *Enterobacteriaceae* and *Pseudomonas aeruginosa* have a chromosomally encoded AmpC-type cephalosporinase, whose expression can be induced transiently by certain β -lactams; carbapenems and cephamycins generally have the highest induction potential (1). Induction does not necessarily correlate with a risk of clinical failure, particularly when bactericidal activity can be rapidly reached. However, potential for AmpC induction has to be carefully examined when considering a β -lactamase inhibitor since it can antagonize the antibacterial activity of its partner β -lactam (2). Indeed, the antibacterial activity of a given β -lactam with a limited stability to AmpC is preserved provided that its potential for AmpC induction is low; in contrast, its activity would be compromised if associated with a β -lactamase inhibitor that induces significant AmpC production.

NXL104 is a new non- β -lactam inhibitor of β -lactamases that displays a broad spectrum inhibition profile for both class A and class C enzymes; both types of enzymes are inactivated very efficiently at low IC_{50} values, with low turn-over numbers and short covalent intermediate half-lives (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).

The aim of this study was to investigate the *in vitro* ability of NXL104 to induce AmpC expression in *E. cloacae* strains. Because β -lactamase / NXL104 complexes are known to have a long half-life (around 7 days for P99 AmpC), it was not possible to evaluate AmpC induction by measuring directly the β -lactamase activity in NXL104 treated cells. Thus it was measured by quantification of cellular *ampC* mRNA. Cefoxitin and clavulanate were used as reference AmpC inducers.

Figure 1: chemical structure of NXL104



METHODS

Bacterial strains, susceptibility testing, and induction experiments

In addition to *E. cloacae* P99, 3 *E. cloacae* strains were selected in Novoxel bacterial collection on the basis of resistance to FOX and susceptibility to ceftazidime (CAZ). Minimal Inhibitory Concentration (MIC) determinations were performed according to CLSI methods.

For induction studies, mid-log phase cells were incubated up to 6 hours in the presence of FOX at 1-32 μ g/mL, or CLA or NXL104 both at 16-64 μ g/mL. Control cultures were performed in Luria-Bertani (LB) broth. 5 x 10⁸ cells were harvested at each time point for RNA extraction and for β -lactamase assay.

RT-PCR

Total cellular RNA was extracted with a RNeasy RNA Protect Mini Kit (Qiagen) according to manufacturer's instructions. After treatment with RNase-free DNase Set (Qiagen) RNA was quantified using an Agilent system.

PCR primers were designed with Primer Express software (Table 1). Real time PCR reactions were carried out in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using a Quantitect Probe RT-PCR Kit (Qiagen). Individual reactions were set-up in triplicates for either *ampC* or *rplS* gene, according to manufacturer's instructions. Reverse transcription was carried out at 50°C for 30 min; PCR reaction conditions were as follows: initial activation of DNA polymerase at 95°C for 15 min, and PCR for 40 cycles at 95°C for 15 s, 60°C for 60 s. Absence of DNA contamination was verified for each RNA preparation by running the RT-PCR in absence of reverse transcriptase. Data were analysed using Sequence Detection software. Relative quantification was carried out by using the 2^{- $\Delta\Delta$ CT} method. *E. cloacae rplS* (coding for ribosomal protein 19) was the calibrator gene for *ampC*; values obtained were then normalized to that of P99 strain *ampC* for measurement of basal expression, or to that of *ampC* in the same strain before induction. Each induction was performed at least twice for all three *E. cloacae* strains, and the results of a representative experiment are shown here (Figures 3, 4, 5).

β -lactamase assay

Crude bacterial extracts were prepared by vortexing 2x10¹⁰ bacterial cells with glass beads in 20 μ L of 100 mM phosphate buffer. After addition of 100 μ M nitrocephin, β -lactamase activity was measured spectrophotometrically at 485 nm for 15 min using crude cell lysates with appropriate dilution (1/20 in induction experiments). Results are expressed as initial reaction rates (Vi, $\Delta A_{485nm}/min$) per 10⁸ cells or per mg of protein (Figure 2).

Table 1: Primers for real time PCR

Primer	Sequence
<i>ampC</i> forward	5'-TGGCCTATCGGGTCAATGT-3'
<i>ampC</i> probe *	5'-TCAGGCTGTGGCTGGGAGATGC-3'
<i>ampC</i> reverse	5'-CTCCACCGGCCAGTTG-3'
<i>rplS</i> forward	5'-CAGGTGACACCGTGAAGTG-3'
<i>rplS</i> probe *	5'-AAGTATGGGTTGTGAAGTTCCAA-3'
<i>rplS</i> reverse	5'-CGAATGCCTGCAGACGTTT-3'

*: 6-FAM (6-carboxy-fluorescein) modification at 5' end and TAMRA (6-carboxy-tetramethyl-rhodamine) at 3' end

Table 2: MIC values for *E. cloacae* strains (μ g/mL)

Both cefoxitin and ceftazidime are good AmpC substrates; cefoxitin is also a good AmpC inducer while ceftazidime has a limited potential for induction. In order to select potentially inducible strains in this study, 3 *E. cloacae* strains were chosen on the basis of resistance to cefoxitin and susceptibility to ceftazidime.

<i>E. cloacae</i> strain	FOX	CAZ	CAZ + CLA 4 μ g/mL	CAZ + NXL104 4 μ g/mL
P99	>128	>128	>128	1
293LA2	>128	2	4	1
293HT107	64	0.5	2	<0.125
293UC1	128	8	16	0.25

RESULTS

Table 3: Basal expression of *ampC* mRNA in *E. cloacae* strains

Basal expression of *ampC* mRNA in 3 *E. cloacae* strains was compared to that of P99 strain (stably depressed AmpC mutant). *ampC* mRNA was ~150-300 fold higher in P99 than in the 3 other *E. cloacae* strains.

β -lactamase activity against nitrocephin was higher (1000-1800 fold) in P99 than in the other strains.

<i>E. cloacae</i> strain	P99	293LA2	293HT107	293UC1
Normalized <i>ampC</i> mRNA expression	1.0000	0.0035	0.0040	0.0066
Activity against nitrocephin (VI/10 ⁸ cells)	229	0.13	0.22	0.18

Figure 2: Kinetics of *ampC* mRNA expression and β -lactamase activity in *E. cloacae* 293HT107 treated with cefoxitin (1 and 2 μ g/mL)

At each time point, the amount of *ampC* mRNA is expressed as a ratio to the value at T0. mRNA increase was detectable early after incubation start (10 min in other kinetic experiments; data not shown) and peaked after 1-2 hours of culture.

β -lactamase activity appeared slightly delayed compared to *ampC* mRNA, and continuously increased throughout the 4 hours of the incubation with cefoxitin.

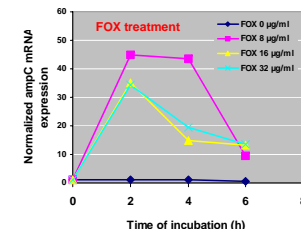
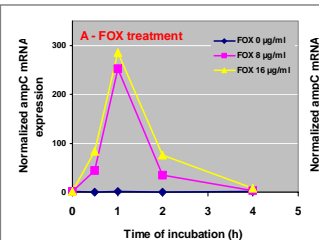
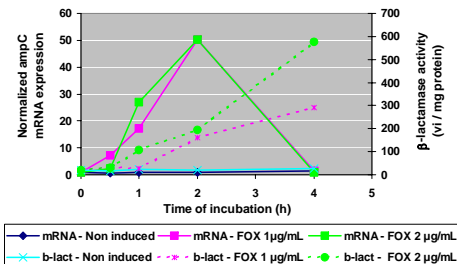


Figure 3: *ampC* mRNA expression in *E. cloacae* 293UC1 treated with cefoxitin

Cefoxitin induced a major increase of *ampC* mRNA in 293UC1 cells; after 2 hours, about 40-fold increase at 8-64 μ g/mL.

Both clavulanate and NXL104 had no effect on *ampC* mRNA levels in 293UC1 cells during the 6 hour culture duration.

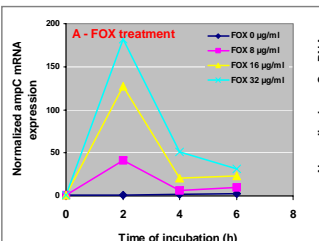


Figure 4: *ampC* mRNA expression in *E. cloacae* 293LA2 treated with cefoxitin, clavulanate or NXL104

Cefoxitin induced a major increase of *ampC* mRNA in 293LA2 cells; after 2 hours, x250 at 8-16 μ g/mL.

Clavulanate had a moderate effect with about a 8-fold increase of *ampC* mRNA at 64 μ g/mL after 2 hours.

NXL104 had no effect on *ampC* mRNA levels in 293LA2 cells during the 6 hour culture duration.

CONCLUSIONS

1 - As compared with P99 strain, the 3 *E. cloacae* strains studied expressed low basal levels of AmpC, when evaluated both by *ampC* mRNA quantification and by measurement of β -lactamase activity against nitrocephin.

2 - At sub-MIC concentrations, cefoxitin induced a dose-dependent synthesis of AmpC in all three *E. cloacae* strains (*ampC* mRNA increased x10-x250, depending on strain and on cefoxitin concentration); mRNA peaked at about 2 hours of incubation.

3 - At the highest concentration tested (64 μ g/mL), clavulanate triggered synthesis of *ampC* mRNA in two of the 3 *E. cloacae* strains (after 2 hours, x8 in 293LA2 and x40 in 293HT107).

4 - In the range of concentrations tested (16-64 μ g/mL), NXL104 had no effect on cellular *ampC* mRNA concentration in all 3 *E. cloacae* strains during the 6 hour culture duration.

NXL104 demonstrated no potential for *ampC* induction in *E. cloacae* species, implying that it does not further compromise the activity of co-administered β -lactam antibiotics.

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