F1-1493

Bactericidal Activity of Ceftaroline Combined with NXL104 Against Critical Targeted Organisms Possessing Various Resistance Mechanisms

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ICAAC 2009
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Abstract

Background: Ceftaroline (CPT), a broad-spectrum cephalosporin with gram-positive activity (including anti-MRSA), was tested in combination with NXL104 (NXL), a potent inhibitor of AmpC, ESBL and KPC β-lactamases (βL) against a selected group of characterized *Enterobacteriaceae* (ENT).

Methods: 6 βL-producing ENT (CMY-2, derepressed AmpC, CTX-M-15, KPC-2 and -3, and a KPC-cured with SHV-27) and 1 wildtype (WT) strain were tested. MIC and MBC were assessed according CLSI guidelines in Mueller-Hinton broth ±10% human serum (HS). Time kill analysis (TK) used CPT, CPT/NXL combinations (fixed 4 µg/mL [CPT/NXL4] and 2:1 ratio) and NXL alone at 1X, 2X, 4X and 8X the MIC. Expression of βL genes was determined by quantitative real-time PCR. Plasmid curing was performed by culturing isolate with DNA intercalating compounds.

Results: CPT and NXL MBC results were generally elevated with MBC/MIC ratios of 2 to 32 among βL-producing strains; while CPT/NXL4 had low MIC values and MBC/MIC ratios at 1 or 2 (Table). 10% HS did not adversely influence CPT or CPT/NXL MIC or MBC values. βL-producing ENT had CPT/NXL4 MIC values at ≤1/4 µg/mL and MBC/MIC of 1 or 2 (1 strain). NXL showed direct antimicrobial activity (MIC, 8-16 µg/mL) against WT and 4 of 6 βL-producing strains. TK detected rapid bactericidal action of CPT/NXL combinations at ≥2X MIC, with some strains having highest enzyme expression showing regrowth at 4-12 hours at 1X MIC. KPC-cured strain was killed rapidly by CPT at ≥2X MIC.

Species	Resistance Mechanism	CPT		CPT/NXL (fixed 4)		CPT/NXL (2:1)		NXL104	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	Wild type	0.12	0.12	0.06/4	0.06/4	0.12/0.06	0.12/0.06	8	32
E. coli	CMY-2	256	>2048	0.25/4	0.5/4	2/1	2/1	8	16
E. cloacae	AmpC	1024	>2048	1/4	1/4	4/2	4/2	16	128
K. pneumoniae	CTX-M-15	2048	>2048	0.12/4	0.12/4	1/0.5	1/0.5	16	>128
K. pneumoniae	KPC-3	128	>2048	0.25/4	0.25/4	2/1	2/1	16	32
K. pneumoniae	KPC-2	1024	>2048	1/4	1/4	4/2	4/2	128	>128
K. pneumoniae	KPC-cured	4	4	0.25/4	0.5/4	1/0.5	1/0.5	>128	>128

Conclusions: NXL demonstrated a remarkably wide and potent βL inhibitory potency against contemporary isolates producing clinically important βL. These results should be used to optimize CPT/NXL dosing regimens.

Introduction

The goal of this study was to evaluate the activity of ceftaroline alone and in combination with NXL104, a new developmental β-lactamase inhibitor (see Poster F1-1492; Figure 1), against resistant gram-negative isolates from Group A and C β-lactamases.

β-lactamases, due to their potential to disseminate and their ability to expand their hydrolytic profile as a result of gene mutations, are particularly threatening. Genes encoding β-lactamases are often located in readily acquired mobile elements that encode resistance to other antimicrobial classes, thus narrowing the therapeutic options to treat infections caused by these organisms.

Ceftaroline is a novel broad-spectrum cephalosporin currently under development for the treatment of skin and skin structure infection and community acquired pneumonia. Ceftaroline possesses both gram-positive and gram-negative activity with extended action against methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* (PRSP).

As with other cephalosporins, ceftaroline is less active against extended-spectrum β-lactamase (ESBL)-producing organisms. To expand the spectrum of activity of ceftaroline against ESBLs, its utility when combined with NXL104 was investigated. NXL104 has demonstrated activity against class A β-lactamases, such as TEM, SHV, CTX-M, and KPC enzymes, and class C cephalosporinases. The bactericidal activity (by MIC/MBC comparisons and killing curves) of ceftaroline alone, NXL104 alone, and ceftaroline combined with NXL104 was tested against critical target organisms.

Strains possessing various β-lactamases were selected for elevated expression of the genes encoding these enzymes as measured by quantitative real-time PCR (qRT-PCR) and compared with an internal control gene. Additionally, 1 strain harboring bla_{KPC-2} was cured of the plasmid carrying this gene, and parental and cured strains were compared.

Materials and Methods

Bacterial Isolates

Expression of the genes encoding the β-lactamase was measured in 16 clinical strains (Table 1). 5 strains with the highest gene expression and a cured strain (4207J) were selected for the evaluation of bactericidal activity. These were 3 *Klebsiella pneumoniae* isolates that carried genes encoding KPC-3, KPC-2, and CTX-M-15; 1 *Escherichia coli* carrying bla_{CMY-2}; 1 *Enterobacter cloacae* with stably derepressed chromosomal AmpC; and a *K. pneumoniae* cured of the plasmid carrying KPC-2. *E. coli* ATCC 25922 was tested for quality control purposes.

The presence of acquired β-lactamase-encoding genes was evaluated by PCR using primers targeting genes encoding several variants of these genes. All amplicons were sequenced and analyzed.

Determination of β-Lactamase Genes Expression

Quantification of β-lactamase genes transcriptional levels was evaluated by qRT-PCR on iCycler iQ™5 System (Bio-Rad; Hercules, CA, USA). Total RNA was extracted from mid-log-phase bacterial cultures using RNA Protect Reagent and RNeasy Mini Kit (Qiagen; Hilden, Germany). Residual DNA was eliminated with Promega RNase-free DNase (Madison, WI, USA). Reverse-transcription PCR was performed using One Step RT-PCR Kit (Qiagen) with SYT09 (Invitrogen; Carlsbad, CA, USA). Relative quantification of target genes expression was performed in duplicate by normalization to an endogenous reference gene (*gyrA*). The critical threshold cycle (C_T) numbers were determined by the detection system software and the amount of target was given as $2^{-\Delta\Delta T}$, where $\Delta\Delta T$ is the difference between the target and reference gene C_T values.

Plasmid Curing

Plasmid curing was performed by culturing a high inoculum of isolate 27-908M in the presence of ethidium bromide (DNA intercalating). Colonies were screened in plates with and without imipenem at 4 µg/mL. Colonies that were able to grow only in plates without imipenem were confirmed by PCR to have lost the gene encoding KPC. Isolates were also evaluated by pulsed-field gel electrophoresis to verify that they were derived from 27-908M.

MIC, MBC, and Time-kill Curve Studies

MIC and MBC were determined with and without 10% human serum using Clinical and Laboratory Standards Institute (CLSI) procedures. Ceftaroline was tested alone and in combinations with NXL104 at a fixed concentration of 4 µg/mL and at a 2:1 ratio. The lowest concentration of the tested agent that killed ≥99.9% of the initial inoculum was defined as the MBC end point.

Time-kill bactericidal activity was performed for ceftaroline/NXL104 on 5 selected strains according to methods described by Moody and Knapp, NCCLS M21-A3, and M26-A. The compounds were tested at 1X, 2X, 4X, 8X MIC and colony counts were performed at T_0, T_2, T_4, T_8, and T_{24}.

Results

- Five clinical isolates were selected based on their highest expression levels of β-lactamase genes, as shown by qRT-PCR (Table 1). Included was a KPC-2 producing strain (27-908M) and its plasmid-cured derivative (4207J) lacking the KPC-2 β-lactamase gene.
- MIC and MBC results were generally high for ceftaroline and NXL104 when tested alone (Table 2).
- Ceftaroline MIC results were greatly decreased in the presence of NXL104 at fixed 4 µg/mL and 2:1 ratio (data not shown): >512-fold for β-lactamase-producing strains and 8- to 16-fold for the plasmid-cured strain (4207J).
- The addition of 10% pooled human serum in the media did not significantly alter MIC or MBC results (Table 2).
- Ceftaroline/NXL104 demonstrated a sustained, rapid killing effect on *E. cloacae* (2-77C) expressing high levels of AmpC β-lactamase for 2X, 4X, and 8X MIC experiments (≥4 $\log_{10}$ CFU/mL killing). At 1X MIC, the killing was slightly slower and regrowth was detected at 8 to 12 hours (Figure 2a).
- *K. pneumoniae* (24-1318A) producing CTX-M-15 when treated with ceftaroline/NXL104 at 2X to 8X MIC demonstrated rapid killing with ≥5 $\log_{10}$ reduction in CFU/mL (Figure 2b). At 1X MIC, a 2 $\log_{10}$ CFU/mL reduction in the initial inoculum for 12 hours was followed by regrowth at 1X MIC.
- NXL104 at fixed 4 µg/mL combined with ceftaroline produced bactericidal effects (>4 $\log_{10}$ CFU/mL killing) at concentrations of 2X to 8X MIC (0.25/4 to 2/4 µg/mL) for KPC-3-producing *K. pneumoniae* 129-482A. No regrowth was observed (Figure 2c).
- *K. pneumoniae* 27-908M producing high levels of KPC-2 showed complete killing (to limit of test sensitivity) at 2X to 8X MIC concentrations and regrowth at 1X MIC (1/4 µg/mL; 4-8 hours) (Figure 2d).
- *K. pneumoniae* 4207J (isolate 27-908M cured of plasmid carrying bla_{KPC-2}) harbored bla_{TEM-1} and bla_{SHV-27}. Rapid and continued bactericidal effects (≥4 $\log_{10}$ CFU/mL reductions) were evident for 2X to 8X MIC. Ceftaroline/NXL104 killing was not achieved for 1X MIC until 24 hours, although ceftaroline alone at 2X MIC was effective against this strain (Figure 2e).
- Ceftaroline/NXL104 at 2:1 ratio showed rapid killing and bactericidal effect for all strains tested, similar to the results observed with ceftaroline/NXL104 at a fixed concentration of 4 µg/mL (data not shown).

Table 1. Results for Gene Expression Experiments Performed on 16 *Enterobacteriaceae* Clinical Isolates to Determine Relative Expression of β-lactamase-encoding Genes.

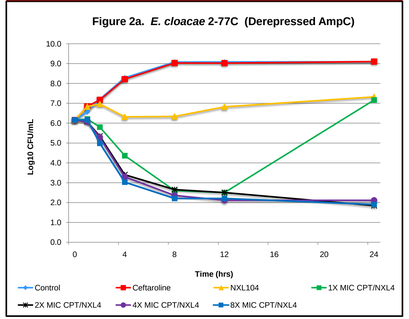
Isolate[a]	Year isolated	Organism	Resistance mechanism	Mean of normalized expression of β-lactamase gene[b]
4-211M	2005	*Klebsiella pneumoniae*	KPC-2	1.69E-01
4-221M	2005	*Klebsiella pneumoniae*	KPC-2	1.71E-01
27-908M	2007	*Klebsiella pneumoniae*	KPC-2	6.43E-01
02-484M	2005	*Klebsiella pneumoniae*	KPC-2	2.51E-01
82-3665A	2007	*Klebsiella pneumoniae*	KPC-3	1.79E-01
82-7515A	2007	*Klebsiella pneumoniae*	KPC-2	2.47E-01
129-482A	2007	*Klebsiella pneumoniae*	KPC-3	7.55E-01
24-1318A	2007	*Klebsiella pneumoniae*	CTX-M-15	4.01E+02
3-13091A	2007	*Klebsiella pneumoniae*	CTX-M-15	3.85E+02
30-996M	2007	*Klebsiella pneumoniae*	CTX-M-15	5.34E+01
29-1940M	2007	*Escherichia coli*	CMY-2	1.33E+01
29-2783M	2006	*Escherichia coli*	CMY-2	1.15E+01
29-2802M	2006	*Klebsiella pneumoniae*	CMY-2	5.99E+06
2-77C	2007	*Enterobacter cloacae*	De-repressed AmpC	2.04E+02
82-1065C	2007	*Enterobacter cloacae*	De-repressed AmpC	2.07E+01
30-12871A	2007	*Enterobacter cloacae*	De-repressed AmpC	1.01E+02

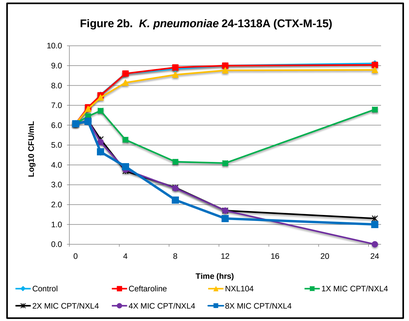
a. Isolates selected for kill-curve analysis are highlighted.
b. Gene expression was performed quantified by normalization of the target gene in relation to an endogenous reference (*gyrA*).

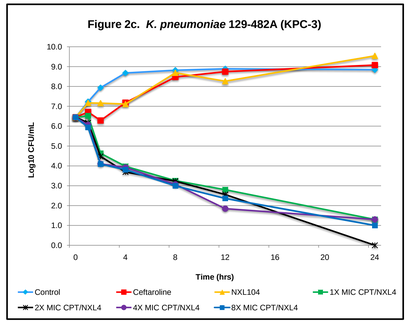
Table 2. Ceftaroline with and without NXL104 MIC/MBC Comparisons and Different Testing Conditions Against 7 Strains of *Enterobacteriaceae*, 6 Producing Broad-Spectrum β-Lactamases.

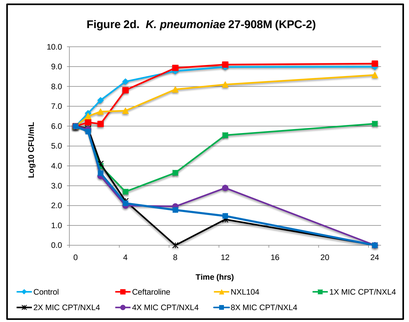
Species (isolate no.)	Resistance mechanism	10% serum	Antimicrobial Agent (µg/mL)							
			Ceftaroline		Ceftaroline/NXL104 (fixed 4 µg/mL)		Ceftaroline/NXL104 (2:1 ratio)		NXL104 alone	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (29-1940M)	CMY-2	-	256	>2048	0.25/4	0.5/4	2/1	2/1	8	16
		+	256	>2048	0.12/4	0.25/4	1/0.5	2/1	16	>128
E. cloacae (2-77C)	AmpC	-	1024	>2048	1/4	1/4	4/2	4/2	16	128
		+	512	>2048	0.5/4	1/4	4/2	4/2	16	128
K. pneumoniae (24-1318A)	CTX-M-15	-	2048	>2048	0.12/4	0.12/4	1/0.5	1/0.5	16	>128
		+	2048	>2048	0.12/4	0.12/4	0.5/0.25	1/0.5	32	>128
K. pneumoniae (129-482A)	KPC-3	-	128	>2048	0.25/4	0.25/4	2/1	2/1	16	32
		+	128	>2048	0.25/4	0.25/4	1/0.5	2/1	16	>128
K. pneumoniae (27-908M)	KPC-2	-	1024	>2048	1/4	1/4	4/2	4/2	128	>128
		+	2048	>2048	0.5/4	0.5/4	2/1	4/2	>128	>128
K. pneumoniae (4207J)	KPC (-)	-	4	4	0.25/4	0.5/4	1/0.5	1/0.5	>128	>128
		+	2	8	0.25/4	0.5/4	1/0.5	1/0.5	>128	>128
E. coli (ATCC 25922)	Wildtype	-	0.12	0.12	0.06/4	0.06/4	0.12/0.06	0.12/0.06	8	32
		+	0.06	0.06	0.06/4	0.06/4	0.12/0.06	0.12/0.06	64	128

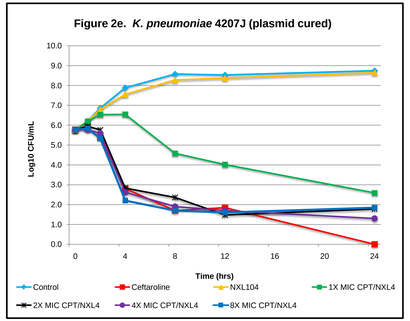
Figure 2. Time-kill Curves of Ceftaroline/NXL104 at Fixed 4 µg/mL (CPTNXL4) Concentration Tested for 5 Selected β-Lactamase-Producing Isolates (2a-2e).











Conclusions

- MBC and time-kill curve results indicated that NXL104 possesses wide and potent β-lactamase-inhibitory activity against strains harboring contemporary challenging β-lactamases, each with high-level expression of β-lactamase-encoding genes.
- All 6 tested strains showed rapid killing with no regrowth when tested by time-kill curve at 2X to 8X MIC, whereas 3 of 6 strains showed regrowth at 1X MIC.
- A secondary finding from this study showed that the addition of human serum (10%) does not compromise β-lactamase-inhibitory activity of NXL104.
- The results of this investigation indicate that ceftaroline, when combined with NXL104, may be a treatment option for infections caused by multidrug-resistant *Enterobacteriaceae* with isolates that carry genes encoding KPC, CTX-M, and AmpC.

References

1. Clinical and Laboratory Standards Institute (2009). *M07-A8. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard - eighth edition.* Wayne, PA: CLSI.
2. Clinical and Laboratory Standards Institute (2009). *M100-S19. Performance standards for antimicrobial susceptibility testing. 19th informational supplement.* Wayne, PA: CLSI.
3. Endimiani A, Choudhary Y, Bonomo RA (2009). In vitro activity of NXL104 in combination with beta-lactams against *Klebsiella pneumoniae* isolates producing KPC carbapenemases. *Antimicrob Agents Chemother* 53: 3599-3601.
4. Lorian V (1996). Antibiotics in laboratory medicine. Baltimore, Williams and Wilkins.
5. Moody J, Knapp C (2004). Time-kill assay for determining synergy. *Clinical Microbiology Procedures Handbook* H. D. Isenberg. Washington, DC, ASM Press: 5.10.31-16
6. National Committee for Clinical Laboratory Standards (1999). *M21-A3. Methodology for the serum bactericidal test; approved guideline.* Wayne, PA: NCCLS.
7. National Committee for Clinical Laboratory Standards (2001). *M23-A2. Development of in vitro susceptibility testing criteria and quality controls parameters, 2nd edition.* Wayne, PA: NCCLS.
8. National Committee for Clinical Laboratory Standards (1999). *M 26-A. Methods for determining bactericidal activity of antibacterial agents; approved guideline.* Wayne, PA: NCCLS.
9. Stachyra T, Levasseur P, Pechereau MC, Girard AM, Claudon M, Miossec C ,Black MT (2009). *In vitro* activity of the β-lactamase inhibitor NXL104 against KPC-2 carbapenemase and Enterobacteriaceae expressing KPC carbapenemases. *J Antimicrob Chemother* 64: 326-329.

Acknowledgement

Supported by Forest Laboratories, Inc.