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High susceptibility of MDR and XDR Gram-negative pathogens to biphenyl-diacetylene-based difluoromethyl-*allo*-threonyl-hydroxamate LpxC inhibitors

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Objectives: Inhibitors of uridine diphosphate-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC, which catalyses the first, irreversible step in lipid A biosynthesis) are a promising new class of antibiotics against Gram-negative bacteria. The objectives of the present study were to: (i) compare the antibiotic activities of three LpxC inhibitors (LPC-058, LPC-011 and LPC-087) and the reference inhibitor CHIR-090 against Gram-negative bacilli (including MDR and XDR isolates); and (ii) investigate the effect of combining these inhibitors with conventional antibiotics.

Methods: MICs were determined for 369 clinical isolates (234 Enterobacteriaceae and 135 non-fermentative Gram-negative bacilli). Time–kill assays with LPC-058 were performed on four MDR/XDR strains, including *Escherichia coli* producing CTX-M-15 ESBL and *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* producing KPC-2, VIM-1 and OXA-23 carbapenemases, respectively.

Results: LPC-058 was the most potent antibiotic and displayed the broadest spectrum of antimicrobial activity, with MIC_{90} values for Enterobacteriaceae, *P. aeruginosa*, *Burkholderia cepacia* and *A. baumannii* of 0.12, 0.5, 1 and 1 mg/L, respectively. LPC-058 was bactericidal at 1× or 2× MIC against CTX-M-15, KPC-2 and VIM-1 carbapenemase-producing strains and bacteriostatic at ≤4× MIC against OXA-23 carbapenemase-producing *A. baumannii*. Combinations of LPC-058 with β-lactams, amikacin and ciprofloxacin were synergistic against these strains, albeit in a species-dependent manner. LPC-058's high efficacy was attributed to the presence of the difluoromethyl-*allo*-threonyl head group and a linear biphenyl-diacetylene tail group.

Conclusions: These *in vitro* data highlight the therapeutic potential of the new LpxC inhibitor LPC-058 against MDR/XDR strains and set the stage for subsequent *in vivo* studies.

Introduction

The emergence of MDR and XDR Gram-negative bacilli is weakening the efficacy of antimicrobial agents currently used to treat infections caused by these microorganisms.[1–3] This alarming situation emphasizes the need to develop novel antibiotics directed at previously unexploited targets in order to overcome resistance to existing antibiotic classes. The development of novel antibiotics targeting uridine diphosphate-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) appears to be a promising strategy, as this essential enzyme catalyses the first, irreversible step in the biosynthesis of lipid A—the membrane anchor of lipopolysaccharide and the predominant lipid species in the outer leaflet of the Gram-negative outer membrane.[4]

In the late 1990s, phenyloxazoline-based LpxC inhibitors were found to be active against *Escherichia coli* both *in vitro* and *in vivo*, thus validating LpxC as a viable antibiotic target.[5] The subsequent discovery of the compound CHIR-090 (characterized by a threonyl-hydroxamate head group coupled to a morpholine-conjugated biphenyl-acetylene tail group) led to the development

of the first, active, LpxC-targeting antibiotic against *Pseudomonas aeruginosa* (Figure 1).[6,7] Replacing the biphenyl-acetylene tail of CHIR-090 with a linear biphenyl-diacetylene group has yielded the compounds LPC-009 and LPC-011, which have enhanced antibacterial activity (Figure 1).[8,9] Recently, another promising class of LpxC inhibitors (pyridine-methylsulfone-hydroxamate-based compounds, e.g. PF-5081090) was also found to show potent activity against Enterobacteriaceae and *P. aeruginosa in vitro* and in murine models of acute septicaemia and pulmonary infection.[10] However, none of these compounds displayed activity against *Acinetobacter baumannii*, a pathogen often associated with nosocomial infections and outbreaks. Recently, the use of NMR spectroscopy to probe the dynamic interactions between LpxC and its bound inhibitors in solution led to the development of the difluoromethyl-*allo*-threonyl-hydroxamate-based compound LPC-058, which contains a *para*-amino substituted biphenyl-diacetylene tail group (Figure 1). LPC-058 was demonstrated to be 5- to 100-fold more active than CHIR-090 against representative strains of Enterobacteriaceae, *P. aeruginosa*, *Vibrio cholerae* and *Chlamydia trachomatis*.[11] Most importantly, LPC-058 was the first LpxC inhibitor to display potent antibiotic activity against the *A. baumannii* ATCC 17978 strain; this finding indicated the feasibility of developing LpxC-targeting antibiotics against this notorious, opportunistic pathogen. Despite this exciting progress, the antibiotic activity of LPC-058 has only been determined against reference strains that lack well-characterized resistance mechanisms and thus are not representative of MDR and XDR strains. In the present study, we evaluated the potency of LPC-058 against a broad panel of Gram-negative clinical isolates and compared it with those of the reference compound CHIR-090 and two other biphenyl-diacetylene-based LpxC inhibitors that differed from LPC-058 in terms of the head group (LPC-011) or the tail group (LPC-087, which has an additional morpholine group). We hypothesized that studying clinical strains would provide information on the effects of antibiotic resistance mechanisms [such as ESBL, carbapenemase or overexpression of efflux resistance–nodulation–division (RND) pumps] on LpxC inhibitor potency. Lastly, we investigated the putative synergy between LpxC inhibitors and the conventional antibiotics commonly used to treat serious infections caused by MDR strains.[12,13]

Materials and methods

Bacterial strains

Reference strains of *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *A. baumannii* (ATCC 17978) were studied, along with 369 Gram-negative clinical isolates from Lille University Hospital (Lille, France). A total of 34 MDR (9%) and 44 XDR (12%) strains were identified, according to the interim standard definitions for acquired resistance.[14] Two MDR strains (CTX-M-15 ESBL-producing *E. coli* and KPC-2 carbapenemase-producing *Klebsiella pneumoniae*) and two XDR strains (VIM-1 carbapenemase-producing *P. aeruginosa* and OXA-23 carbapenemase-producing *A. baumannii*) were used in time–kill assays. Lastly, *Enterobacter aerogenes*, *P. aeruginosa* and *A. baumannii* strains overexpressing the AcrAB-TolC, MexAB-OprM/MexCD-OprJ and AdeABC efflux pumps, respectively, were also examined, along with the corresponding parental strains.[15–18]

Antimicrobial compounds

Cefotaxime, ceftazidime, imipenem, ciprofloxacin and amikacin were purchased from Sigma–Aldrich (Lyon, France). The LpxC inhibitor CHIR-090 and the biphenyl-diacetylene-based LpxC inhibitors LPC-058 and LPC-011 (Figure 1) were prepared at the Duke University Small Molecule Synthesis Facility (Durham 27710, NC, USA), according to published procedures.[7–9] The synthesis of LPC-087 is described in the Supplementary methods (available as Supplementary data at *JAC* Online). Stock solutions of conventional antibiotics and LpxC inhibitors were stored at −80°C until use.

MIC assays

Susceptibility of the bacterial strains to LpxC inhibitors at final concentrations ranging from 0.015 to 64 mg/L was determined using the CLSI standard agar dilution and broth microdilution methods in CAMHB.[19] An inoculum of $\sim 10^4$ cfu/spot or 5×10^5 cfu/mL was used for the agar dilution and microdilution methods, respectively. The inoculated agar and 96-well plates were subsequently incubated for 24 h at 37°C (except for *Yersinia* species, which were incubated for 48 h at 28°C). Each assay was performed at least twice on separate days and three quality control strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 17978) were included on each day of testing (depending on the species investigated). The MIC was defined as the lowest concentration of drug that prevented visible growth after incubation.

Time–kill assays

The bactericidal activity of LpxC inhibitors was investigated with the following strains: CTX-M-15 ESBL-producing *E. coli*, KPC-2 carbapenemase-producing *K. pneumoniae*, VIM-1 carbapenemase-producing *P. aeruginosa* and OXA-23 carbapenemase-producing *A. baumannii*. Assays were performed using LPC-058 alone at concentrations of 1× to 8× MIC or mixed at 0.25× to 1× MIC with the following conventional antibiotics: cefotaxime, ceftazidime, imipenem, ciprofloxacin and amikacin. For time–kill assays, the MIC of each antibiotic was determined by using the CLSI reference microdilution method.[19] The various combinations were added to an inoculum of 5×10^5 cfu/mL from a bacterial culture growing exponentially in CAMHB. After 0, 3, 6, 9 and 24 h of incubation at 37°C with shaking, aliquots were removed and subcultured in triplicate onto bromocresol purple agar plates. Viable cells were then counted. Bactericidal activity was defined as a 3 $\log_{10}$cfu/mL reduction, relative to the initial inoculum. Synergy and indifference were, respectively, defined as a $\geq$2 $\log_{10}$ and $<$2 $\log_{10}$ decrease in the cfu count when the combination was compared with the most active single drug after 24 h incubation. The limit of detection (LOD) was $\leq$1.3 $\log_{10}$ cfu/mL. Each assay was repeated at least twice.

Results

Inhibitory activity of the LpxC inhibitors against clinical isolates of Enterobacteriaceae and non-fermentative Gram-negative bacilli

The antimicrobial activities of LPC-058, LPC-011 and LPC-087 were tested against 369 clinical isolates (234 Enterobacteriaceae and 135 non-fermentative Gram-negative bacilli) and compared with that of the reference compound CHIR-090 (Table 1 and Table S1). All the biphenyl-diacetylene-based compounds showed a high level of activity against common Enterobacteriaceae, with MIC_{50} values of $\leq$0.25 mg/L and MIC_{90} values of $\leq$0.5 mg/L. LPC-058 was the most active compound, with MIC_{50} (0.06 mg/L) and MIC_{90} (0.12 mg/L) values that were 6- to 8-fold lower than those of the reference compound CHIR-090 (with an MIC_{50} of 0.5 mg/L and MIC_{90} of 2 mg/L). The second most active compound was LPC-011, with MIC_{50} (0.12 mg/L) and MIC_{90} (0.25 mg/L) values $\sim$2-fold higher than those of LPC-058 for the various Enterobacteriaceae strains (Table S1); however, its MIC_{50} and MIC_{90} values were 2-fold lower than those of LPC-087 (MIC_{50}=0.25 mg/L and MIC_{90}=0.5 mg/L).

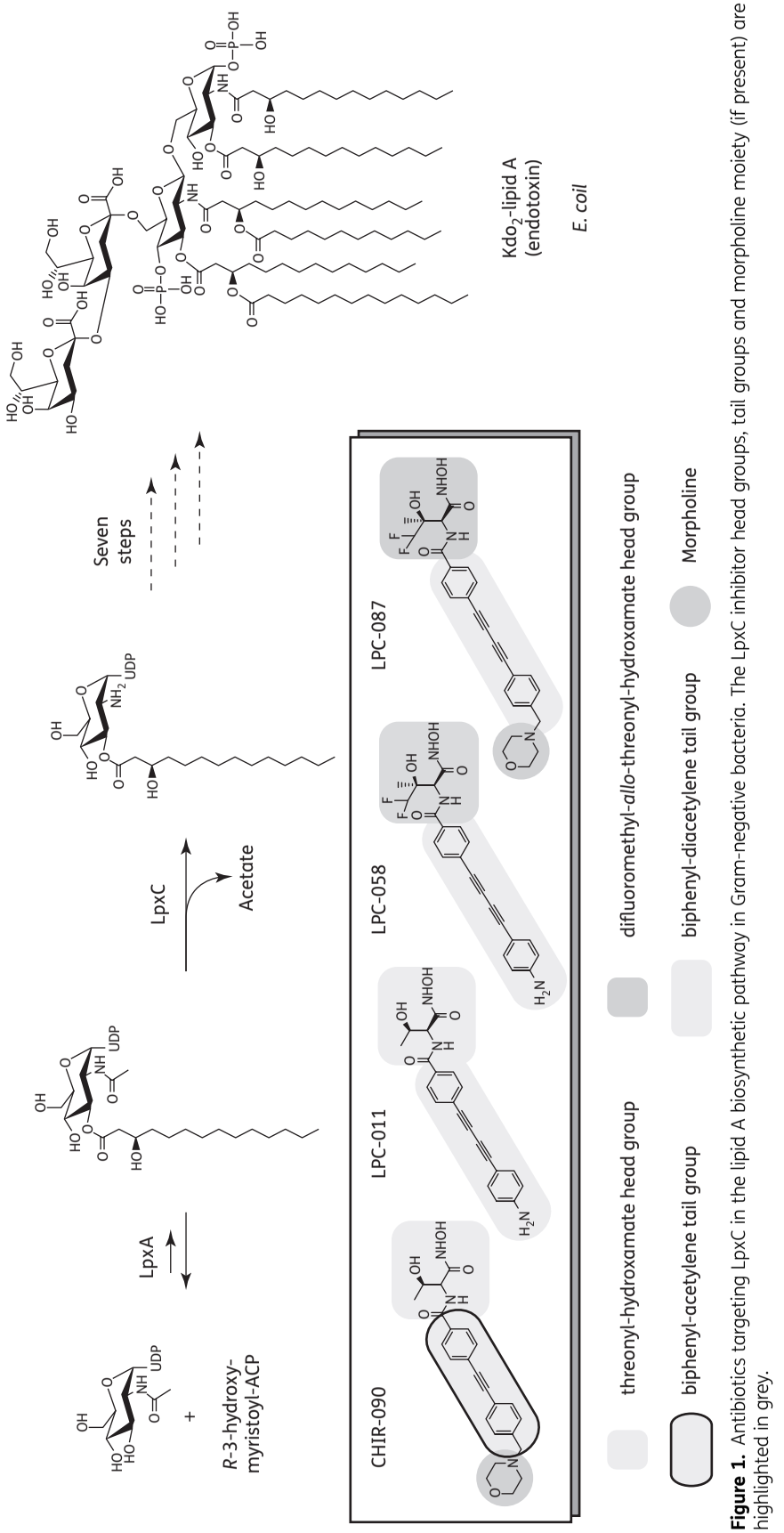

Figure 1. Antibiotics targeting LpxC in the lipid A biosynthetic pathway in Gram-negative bacteria. The LpxC inhibitor head groups, tail groups and morpholine moiety (if present) are highlighted in grey.

Table 1. Activities of LPC-058, LPC-011, LPC-087 and the reference compound CHIR-90 against 234 clinical isolates of Enterobacteriaceae and 135 clinical isolates of non-fermentative Gram-negative bacilli

Organism group (number of isolates tested)	MIC (mg/L)	LPC-058	LPC-011	LPC-087	CHIR-090
Enterobacteriaceae (234)	MIC_{50}	0.06	0.12	0.25	0.5
	MIC_{90}	0.12	0.25	0.5	2
	range	≤0.015–0.5	≤0.015–1	≤0.015–2	0.03–8
P. aeruginosa (51)	MIC_{50}	0.25	0.5	2	2
	MIC_{90}	0.5	1	4	4
	range	0.06–0.5	0.12–1	0.25–8	0.5–8
A. baumannii (26)	MIC_{50}	0.25	≥64	1	≥64
	MIC_{90}	1	≥64	32	≥64
	range	0.12–4	—	0.5 to ≥64	—
B. cepacia (22)	MIC_{50}	0.5	4	≥64	≥64
	MIC_{90}	1	8	≥64	≥64
	range	0.12–2	1–8	—	—
S. maltophilia (20)	MIC_{50}	≥64	≥64	≥64	≥64
	MIC_{90}	≥64	≥64	≥64	≥64
	range	—	—	—	—
A. xylosoxidans (16)	MIC_{50}	≥64	≥64	≥64	≥64
	MIC_{90}	≥64	≥64	≥64	≥64
	range	—	—	—	—

A similar trend in antibacterial activities was observed for clinical isolates of *P. aeruginosa* (Table 1). LPC-058 and LPC-011 were more active (MIC_{50} ≤0.5 mg/L and MIC_{90} ≤1 mg/L) than LPC-087, which had similar MIC_{50} and MIC_{90} values as the reference compound CHIR-090 (2 and 4 mg/L, respectively). Overall, the activities of the compounds against Enterobacteriaceae and *P. aeruginosa* could be ranked (in decreasing order) as follows: LPC-058 > LPC-011 > LPC-087 > CHIR-090.

Interestingly, the LpxC inhibitor activity patterns against *A. baumannii* and *Burkholderia cepacia* differed distinctly from those against Enterobacteriaceae and *P. aeruginosa*. For *A. baumannii*, only the difluoromethyl-substituted compounds (LPC-058 and LPC-087) displayed a significant level of antibiotic activity (Table 1). LPC-058 displayed a higher potency (MIC_{50} = 0.25 mg/L and MIC_{90} = 1 mg/L) against clinical isolates of *A. baumannii* than LPC-087 (MIC_{50} = 1 mg/L and MIC_{90} = 32 mg/L), a compound that has the same head group but a different tail (with an additional morpholine moiety attached to the biphenyl-diacetylene scaffold; Figure 1). For *B. cepacia*, only LPC-058 and LPC-011 were active; once again, LPC-058 was the most potent antibiotic (Table 1). Since LPC-058 and LPC-011 share the same linear *para*-amino-substituted biphenyl-diacetylene tail group and differ from LPC-087 and CHIR-090 in terms of the absence of the morpholine unit in the tail, their activity against *B. cepacia* indicates that the morpholine substitution may adversely affect the activity of LpxC inhibitors. None of the LpxC inhibitors evaluated here was effective against *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*; this lack of activity was unexpected, given the presence of LpxC enzymes in these species.

Overall, the results of MIC assays with a large collection of Gram-negative clinical strains indicated that LPC-058 (with the difluoromethyl-*allo*-threonyl-hydroxamate head group and a linear biphenyl-diacetylene tail group) was the most active LpxC inhibitor and had the broadest spectrum of activity.

Inhibitory activity of LpxC inhibitors against defined efflux mutants

We next examined the effect of non-specific drug-resistant mechanisms (such as the overexpression of RND efflux pumps) on the antibiotic activity of the biphenyl-diacetylene derivatives LPC-058, LPC-011 and LPC-087. For this, MIC values were determined for selective WT strains of *E. aerogenes*, *P. aeruginosa* and *A. baumannii* and their isogenic mutants overexpressing efflux pumps (Table 2). The MICs of LPC-058 and LPC-011 (which have different head groups but the same tail group) were moderately higher (by a factor of four) for the AcrAB-TolC overproduction mutant of *E. aerogenes* than for the WT strain. The MICs of the two compounds were only slightly affected by overexpression of MexAB-OprM or MexCD-OprJ efflux pumps in *P. aeruginosa* and the AdeABC efflux pump in *A. baumannii* CIP7010-1. In contrast, the difference in MICs was greater for LPC-087 (which bears the same morpholine moiety in the tail as CHIR-090) than LPC-058 and LPC-011. The MICs of LPC-087 for the efflux pump mutant strains of *E. aerogenes* and *P. aeruginosa* were, respectively, 8- and 4-fold greater than those for the isogenic parental strain. It has already been reported that CHIR-090 is a substrate of the RND efflux pumps in *P. aeruginosa*.[10,20] These observations suggest that the morpholine group in LPC-087 and CHIR-090 may be preferentially recognized (relative to the linear *para*-amino-substituted biphenyl-diacetylene tail group in LPC-058 and LPC-011) by the RND efflux pump family.

Table 2. MICs of LPC-058, LPC-011, LPC-087 and CHIR-090 for *E. aerogenes*, *P. aeruginosa* and *A. baumannii* mutant strains overexpressing RND pump efflux systems and their respective parental strains

Strain	Efflux pump status	MIC (mg/L)			
		LPC-058	LPC-011	LPC-087	CHIR-090
E. aerogenes					
ATCC 13048	WT	0.12	0.25	0.5	0.5
CM64	AcrAB-TolC overexpressed	0.5	1	4	4
P. aeruginosa					
PAO1	WT	0.12	0.25	2	2
PT629	MexAB-OprM overexpressed	0.12	0.25	8	8
ERYR	MexCD-OprJ overexpressed	0.25	0.5	8	8
A. baumannii					
CIP7010	WT	4	ND	ND	ND
CIP7010-1	AdeABC overexpressed	8	ND	ND	ND

WT, isogenic, antibiotic-susceptible parent; ND, not determined.

Table 3. Activities of LpxC inhibitors as a function of the resistance phenotype in Enterobacteriaceae, *P. aeruginosa* and *A. baumannii*

Organism group (number tested)	MIC (mg/L)	LPC-058	LPC-011	LPC-087	CHIR-090
Enterobacteriaceae non-MDR/XDR (196)	MIC_{50}	0.06	0.12	0.25	0.5
	MIC_{90}	0.12	0.25	0.5	1
	range	≤0.015–0.25	≤0.015–1	≤0.015–1	0.03–2
Enterobacteriaceae MDR or XDR (38[a])	MIC_{50}	0.06	0.12	0.25	0.5
	MIC_{90}	0.25	0.5	1	2
	range	≤0.015–0.25	0.06–1	0.25–1	0.25–8
P. aeruginosa non-MDR/XDR (31)	MIC_{50}	0.25	0.5	2	2
	MIC_{90}	0.5	1	4	4
	range	0.06–0.5	0.25–2	0.25–4	0.5–8
P. aeruginosa MDR or XDR (20[b])	MIC_{50}	0.25	0.5	4	4
	MIC_{90}	0.5	1	8	8
	range	0.12–0.5	0.25–1	1–8	2–8
A. baumannii non-MDR/XDR (6)	MIC_{50}	0.25	≥64	1	≥64
	MIC_{90}	0.5	≥64	2	≥64
	range	0.12–0.5	≥64	0.5–2	≥64
A. baumannii MDR or XDR (20[c])	MIC_{50}	0.25	≥64	1	≥64
	MIC_{90}	1	≥64	32	≥64
	range	0.12–4	≥64	0.5 to ≥64	≥64

[a]Includes 17 ESBL-producing and 5 carbapenemase-producing strains [OXA-48 ($n=3$), NDM ($n=1$) and KPC ($n=1$)].
[b]Includes three ESBL-producing and five carbapenemase-producing strains [VIM ($n=4$) and IMP ($n=1$)].
[c]Includes 1 ESBL-producing and 10 carbapenemase-producing strains [OXA-23 ($n=9$) and OXA-40 ($n=1$)].

Inhibitory activity of individual LpxC inhibitors against MDR and/or XDR strains

To assess the broader impact of multidrug resistance (i.e. other than efflux pump overproduction), we compared the MICs of LPC-058, LPC-011, LPC-087 and CHIR-90 when tested against MDR/XDR or non-MDR/XDR clinical isolates of Enterobacteriaceae, *P. aeruginosa* and *A. baumannii*. All the LpxC inhibitors had very similar MIC values for MDR and XDR strains (Table 3). In general, the MIC_{50} and MIC_{90} values of the biphenyl-diacetylene-based compounds (LPC-058, LPC-011 and LPC-087) for non-MDR/XDR clinical isolates of Enterobacteriaceae and *P. aeruginosa* were similar to those for MDR/XDR clinical strains (with a 2-fold difference, typically).

The two LpxC inhibitors with activity against *A. baumannii* (the difluoromethyl-substituted compounds LPC-058 and LPC-087) differed in their relative potencies against MDR/XDR isolates. The

activity of the morpholine-containing LPC-087 against MDR/XDR *A. baumannii* isolates was much lower (the MIC_{90} increased from 2 mg/L to 32 mg/L), whereas LPC-058 activity was only slightly affected (the MIC_{90} increased from 0.5 mg/L to 1 mg/L). Overall, these observations support the concept whereby LPC-058 (with a difluoromethyl-*allo*-threonyl-hydroxamate head group and a linear biphenyl-diacetylene tail group) can effectively overcome multidrug resistance in all the MDR/XDR strains tested here.

Table 4. MICs (in CAMHB) for the four MDR/XDR strains used in the time–kill assays

Strain	MIC (mg/L)					
	LPC-058	CTX	CAZ	IPM	AMK	CIP
E. coli with CTX-M-15	0.06	256	ND	0.25	32	64
K. pneumoniae with KPC-2	0.12	512	ND	16	32	16
P. aeruginosa with VIM-1	0.5	ND	64	256	4	16
A. baumannii with OXA-23	0.5	ND	128	32	1024	32

CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; AMK, amikacin; CIP, ciprofloxacin; ND, not determined.

Bactericidal effect of LPC-058 alone

Since LPC-058 (i) was the most potent LpxC inhibitor, (ii) displayed the broadest spectrum of antimicrobial activities, and (iii) was only slightly affected by the efflux-based antibiotic resistance mechanisms, we next sought to determine its bactericidal activity against two MDR strains (CTX-M-15 ESBL-producing *E. coli* and KPC-2 carbapenemase-producing *K. pneumoniae*) and two XDR strains (*P. aeruginosa* and *A. baumannii* producing VIM-1 and OXA-23 carbapenemases, respectively). All four MDR/XDR strains exhibited a high level of resistance to β-lactams, amikacin and ciprofloxacin (Table 4). At a concentration of 1× or 2× MIC, LPC-058 alone was strongly bactericidal (with a reduction of $\geq$3 $\log_{10}$ cfu/mL) against MDR/XDR *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Figure 2). The bactericidal activity of LPC-058 was more prominent for ESBL-producing *E. coli* than for carbapenemase-producing *K. pneumoniae* and *P. aeruginosa*: a full bactericidal effect was rapidly observed (within 3 h) at 1× MIC (0.06 mg/L) for ESBL-producing *E. coli*, but occurred more slowly (within 6 h) at 2× MIC (0.24 and 1 mg/L, respectively) for KPC-2 carbapenemase-producing *K. pneumoniae* and VIM-1-producing *P. aeruginosa*. In contrast, LPC-058 was bacteriostatic at $\leq$4× MIC (MIC = 0.5 mg/L) against OXA-23 carbapenemase-producing *A. baumannii* and was slowly bactericidal at 8× MIC (Figure 2).

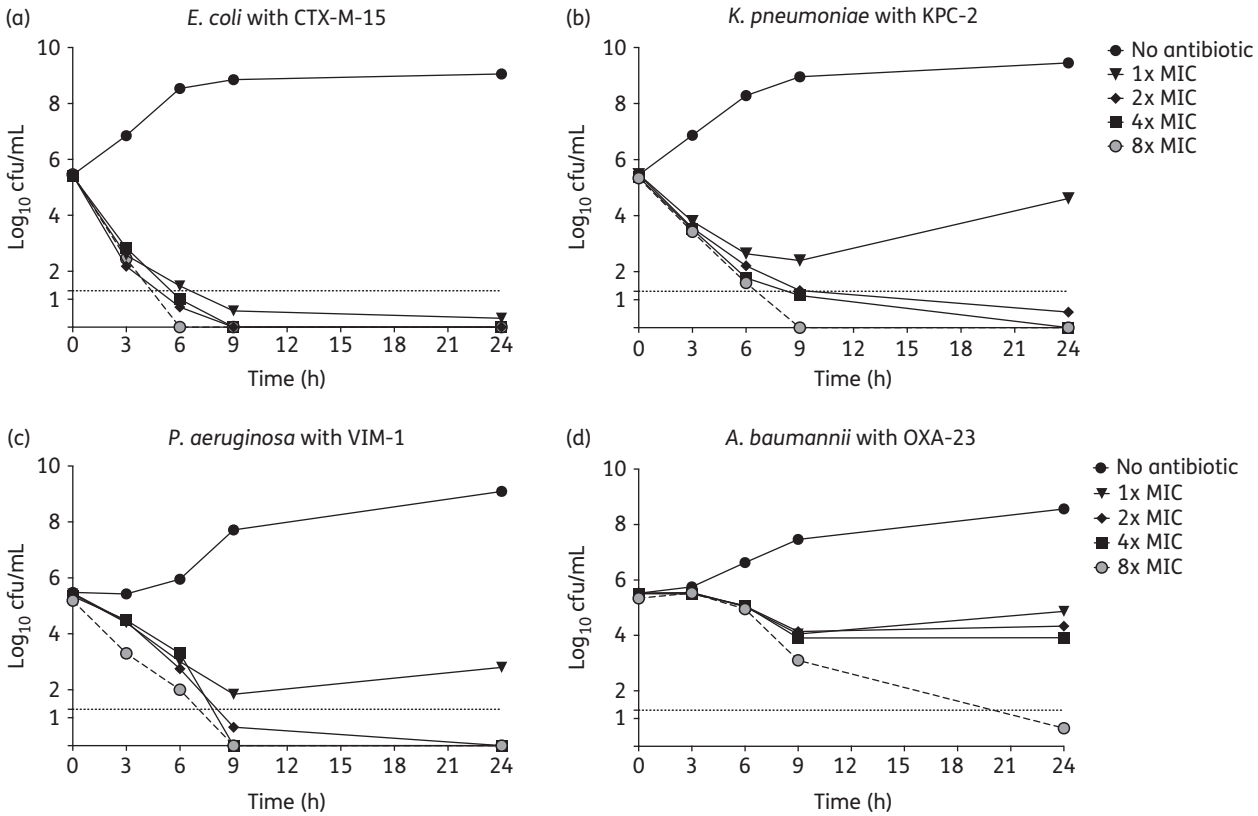

Figure 2. Twenty-four hour time–kill curves with LPC-058 alone at 1×, 2×, 4× and 8× MIC, as tested against CTX-M-15 ESBL-producing *E. coli* (a), KPC-2 carbapenemase-producing *K. pneumoniae* (b), VIM-1 carbapenemase-producing *P. aeruginosa* (c) and OXA-23 carbapenemase-producing *A. baumannii* (d). The broken horizontal line indicates the LOD ($\leq$1.3 $\log_{10}$ cfu/mL). The bacterial count corresponds to the mean of at least two independent experiments in duplicate.

Table 5. Interactions between conventional antibiotics and LPC-058 at 0.25×, 0.5× and 1× MIC against *E. coli* producing CTX-M-15 ESBL and *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* producing KPC-2, VIM-1 and OXA-23 carbapenemases, respectively

		Combination effect, time in h		
		multiple of the MIC of LPC-058		
Strain	Multiple of the MIC of conventional antibiotics	0.25×	0.5×	1×
E. coli CTX-M-15	CTX 0.5×	synergy, 9 h	synergy, 6 h	indifference
	AMK 0.25×	synergy, 9 h	synergy, 9 h	indifference
	CIP 0.5×	synergy, 6 h	synergy, 6 h	indifference
K. pneumoniae KPC-2	CTX 0.25×	indifference	synergy, 24 h	synergy, 24 h
	IPM 0.5×	synergy, 9 h	synergy, 9 h	synergy, 9 h
	AMK 0.5×	synergy, 9 h	synergy, 9 h	synergy, 9 h
	CIP 0.5×	indifference	indifference	synergy, 24 h
P. aeruginosa VIM-1	CAZ 0.5×	synergy, 6 h	synergy, 9 h	synergy, 24 h
	IPM 0.125×	synergy, 6 h[a]	synergy, 9 h	synergy, 24 h
	AMK 0.5×	synergy, 9 h	synergy, 9 h	synergy, 24 h
	CIP 0.25×	indifference	indifference	synergy, 24 h
A. baumannii OXA-23	IPM 0.5×	ND	synergy, 3 h	synergy, 3 h
	AMK 0.062×	ND	indifference	indifference
	CIP 0.5×	ND	synergy, 6 h[a]	synergy, 6 h

CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; AMK, amikacin; CIP, ciprofloxacin; ND, not determined.
[a]Bacterial regrowth at 24 h.

Synergistic effect of LPC-058 with conventional antibiotics

We next assessed the potential synergistic activity of LPC-058 with β-lactams, amikacin and ciprofloxacin against the above-mentioned MDR/XDR bacteria. We deliberately chose to use sub-inhibitory concentrations of the conventional antibiotics that could nevertheless be achieved clinically.[21–25] All combinations of LPC-058 with a β-lactam were synergistic when tested against the four MDR/XDR strains (Table 5 and Figure S1). A higher concentration of LPC-058 (1× MIC) did not further enhance the bacterial killing of the β-lactam plus LPC-058 combinations, when compared with LPC-058 at subinhibitory concentrations (0.25× and 0.5× MIC). The synergistic killing effect of the cefotaxime plus LPC-058 combination was more rapid for ESBL-producing *E. coli* (at 6 or 9 h) than for KPC-2-producing *K. pneumoniae* (at 24 h, with an LPC-058 concentration ≥0.5× MIC). At all LPC-058 concentrations, the ceftazidime plus LPC-058 combination produced a greater killing effect with VIM-1-producing *P. aeruginosa* than LPC-058 alone. Impressively, the LPC-058 plus imipenem combination was effective against all carbapenemase-producing strains; this was especially true for the OXA-23-producing *A. baumannii*, which was killed within 3 h and did not display any regrowth (Table 5 and Figure S1).

Similarly, the LPC-058 plus amikacin combination displayed synergistic activity against all MDR/XDR strains (Table 5 and Figure S1) other than the OXA-23 carbapenemase-producing *A. baumannii* (which was highly resistant: MIC of amikacin = 1024 mg/L). Hence, the synergistic improvements in the activity of LPC-058 with ciprofloxacin appear to be species-dependent. For CTX-M-15-producing *E. coli* and OXA-23-producing *A. baumannii*, the synergistic effect of LPC-058 plus ciprofloxacin occurred rapidly (within 6 h), but was weaker for carbapenemase-producing *P. aeruginosa* and *K. pneumoniae* strains (with indifference at ≤0.5× MIC of LPC-058 and prevention of bacterial regrowth at 24 h with 1× MIC of LPC-058, respectively). Overall, our results highlight the ability of LPC-058 to eradicate MDR/XDR strains when combined with conventional antibiotics usually associated with a high level of resistance.

Discussion

The worldwide rise and spread of ESBLs and carbapenemases in Gram-negative bacilli has created an urgent need to develop new antimicrobial agents.[1–3] Our present results show that the newly discovered difluoromethyl-*allo*-threonyl-hydroxamate compound LPC-058 is the most potent (*in vitro*) of all the biphenyl-diacetylene tail scaffold compounds tested here. It was ∼10 times more active than the reference compound CHIR-090 against a broad panel of Enterobacteriaceae and non-fermentative Gram-negative bacilli and effectively overcame resistance mechanisms in MDR/XDR strains. LPC-058 is the first inhibitor to have shown activity against both *A. baumannii* and *B. cepacia*.[10,11] Furthermore, LPC-058 is an effective bactericidal antibiotic (at 1× or 2× MIC) against MDR/XDR Enterobacteriaceae and *P. aeruginosa* strains. Lastly, LPC-058 displays synergy with β-lactams, amikacin and ciprofloxacin.

The generally greater potency and broader spectrum of activity displayed by LPC-058, LPC-011 and LPC-087 (relative to CHIR-90) likely reflects the presence of a biphenyl-diacetylene tail (Figure 1).[8,11] LpxC enzymes accommodate the acyl chain of the

substrate through a hydrophobic passage, which also hosts the tail group of LpxC inhibitors. As the contour of the hydrophobic passage differs in distinct LpxC enzymes, it has been shown that the diacetylene group's elasticity and its smaller footprint within the substrate-binding hydrophobic passage of LpxC orthologues contribute to the compounds' broad-spectrum antimicrobial activity (relative to CHIR-090).[8,9] However, our MIC assay data suggested that the presence of the biphenyl-diacetylene tail scaffold in LpxC inhibitors is not enough to gain activity against *A. baumannii*. In fact, LPC-011 has the same tail group as LPC-058 (Figure 1), but was ineffective against *A. baumannii*. Optimal activity of LpxC inhibitors against *A. baumannii* depends strictly on the presence of a difluoromethyl-*allo*-threonyl-hydroxamate head group (Figure 1), since only LPC-087 and LPC-058 were active against *A. baumannii*. Addition of the difluoromethyl moiety to the LPC-011 threonyl head strengthens the compound's electrostatic interactions with the amino acids in the LpxC active site.[11] We speculate that the introduction of fluorine atoms might also increase the compound's ability to cross lipid membranes, as has previously been demonstrated for fluoroquinolone compounds.[26]

It is noteworthy that LPC-087 (a compound bearing the same head group as LPC-058, but with an additional morpholine moiety in the tail) was generally less active than LPC-058. It is possible that the presence of this morpholine group weakened the inhibitor–LpxC interaction or diminished the compound's ability to cross the bacterial membrane. In the present study, both LPC-087 and CHIR-090 (which also has a morpholine group) were more affected by the overexpression of efflux pumps than LPC-058 (which lacks the morpholine unit). This observation suggests that LPC-087's low activity (relative to LPC-058) may reflect the greater ability of target bacterial cells to excrete compounds containing a morpholine group.

We were very surprised to find that the broad-spectrum LpxC inhibitor LPC-058 was inactive against *S. maltophilia* and *A. xylosoxidans*, despite the presence of a high level (~55%) of sequence homology between these LpxC orthologues and that of *E. coli* (Figure S2). Further biochemical studies are now required to establish whether or not the existing LpxC inhibitors can: (i) inhibit the target enzyme's activity; (ii) effectively penetrate the membrane barrier; or (iii) avoid efficient extrusion by the various efflux pumps present in these species.[27,28]

In conclusion, we found that LPC-058 (bearing a difluoromethyl-*allo*-threonyl-hydroxamate head group and a *para*-amino substituted biphenyl-diacetylene tail group) is highly effective *in vitro* against a broad range of Enterobacteriaceae and non-fermentative Gram-negative clinical strains. The compound retains potent activity against MDR/XDR strains. Moreover, our results showed that LPC-058 was synergistic with several classes of antibiotics against carbapenemase-producing isolates, suggesting that an LPC-058-containing combination regimen might hold promise for the clinical treatment of drug-resistant Gram-negative pathogens.

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Transparency declarations

P. Z. and E. J. T. declare a competing financial interest: a patent on difluoromethyl-*allo*-threonyl-hydroxamate was awarded to P. Z. and E. J. T. All other authors: none to declare.

Supplementary data

Supplementary methods, Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org).

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