



Mutations Reducing *In Vitro* Susceptibility to Novel LpxC Inhibitors in *Pseudomonas aeruginosa* and Interplay of Efflux and Nonefflux Mechanisms

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ABSTRACT Upregulated expression of efflux pumps, *lpxC* target mutations, LpxC protein overexpression, and mutations in *fabG* were previously shown to mediate single-step resistance to the LpxC inhibitor CHIR-090 in *P. aeruginosa*. Single-step selection experiments using three recently described LpxC inhibitors (compounds 2, 3, and 4) and mutant characterization showed that these mechanisms affect susceptibility to additional novel LpxC inhibitors. Serial passaging of *P. aeruginosa* wild-type and efflux pump-defective strains using the LpxC inhibitor CHIR-090 or compound 1 generated substantial shifts in susceptibility and underscored the interplay of efflux and nonefflux mechanisms. Whole-genome sequencing of CHIR-090 passage mutants identified efflux pump overexpression, *fabG* mutations, and novel mutations in *fabF1* and in PA4465 as determinants of reduced susceptibility. Two new *lpxC* mutations, encoding A214V and G208S, that reduce susceptibility to certain LpxC inhibitors were identified in these studies, and we show that these and other target mutations differentially affect different LpxC inhibitor scaffolds. Lastly, the combination of target alteration (LpxC_{A214V}) and upregulated expression of LpxC was shown to be tolerated in *P. aeruginosa* and could mediate significant decreases in susceptibility.

KEYWORDS LpxC inhibitor, *Pseudomonas aeruginosa*, cumulative resistance, decreased susceptibility, efflux, target mutation

Success in the identification and development of novel antibacterial agents for treating serious Gram-negative infections has remained elusive. This is in part due to the inability of potent small molecule inhibitors of specific intracellular targets to accumulate adequately in the cell to fully engage their target. Gram-negative pathogens can exclude toxic molecules through the combination of a highly impermeable outer membrane (OM) and active efflux (1, 2).

The genome of *P. aeruginosa* encodes at least 12 members of the resistance nodulation cell division (RND) family efflux pumps, along with many pumps of other families (3). Of the RND family, the MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN pumps have been clearly associated with resistance to antibacterial compounds (4). RND family pumps are tripartite in structure with an inner membrane pump (e.g., MexB), an outer membrane channel (e.g., OprM), and a membrane fusion protein that links these (e.g., MexA) (5). The RND outer membrane channel component traverses the Gram-negative outer membrane, and therefore RND family pumps can expel toxic molecules that are entering cells back out across the OM to outside the cells. The OM is an asymmetrical bilayer with a phospholipid inner leaflet and an outer leaflet

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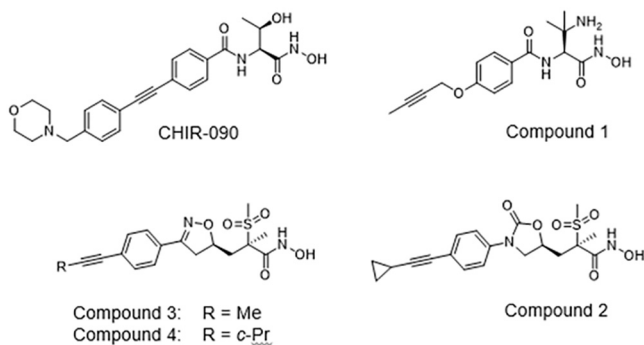


FIG 1 Chemical structures of LpxC inhibitors in this study.

made up primarily of lipopolysaccharide (LPS), also referred to as endotoxin. LPS is an amphipathic molecule composed of a relatively conserved lipid A region that forms the outer leaflet of the outer membrane bilayer to which is attached a core oligosaccharide and highly variable O-antigen polysaccharide repeating units that extend outward from the cell (6). The OM permeability barrier reduces the influx of many toxic molecules, which enhances the effectiveness of efflux in preventing intracellular accumulation of antibacterial compounds (1).

Along with being a key factor in establishing the permeability barrier of the OM, LPS is also essential for growth and virulence in many Gram-negative pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Targets in the lipid A biosynthetic pathway, in particular LpxC (7–12), or in the machinery that transports LPS and assembles it in the OM (13) have been the focus of intense drug discovery efforts. Early efforts to identify LpxC inhibitors yielded compounds that had activity against *E. coli* but not *P. aeruginosa* (14), raising the possibility that these inhibitors did not accumulate sufficiently in *P. aeruginosa* (as is often the case) or that LpxC was not essential in this pathogen. Further studies showed that the differential activity was related to differences in the LpxC amino acid sequences between these pathogens (15, 16). Efforts taking the *P. aeruginosa* LpxC protein into account initially yielded the LpxC inhibitor CHIR-090 that had potent activity against this pathogen (17) (Fig. 1), and since then many others have been reported (7, 18, 19). Recent examples from our group include compound 1 (9) and compound 2 (oxazolidinone 13f), compound 3 (isoxazoline 25d), and compound 4 (isoxazoline 25e) described previously (12) (Fig. 1) that have potent *in vitro* activity against *P. aeruginosa*. These compounds are somewhat atypical in terms of novel antibacterial discovery for their ability to potently kill *P. aeruginosa* wild-type cells, which are notoriously recalcitrant to most novel target inhibitors. Their antibacterial activity may relate in part to a currently not well understood ability to accumulate in Gram-negative cells.

Several mechanisms are known to reduce the *in vitro* susceptibility of *P. aeruginosa* to CHIR-090, including upregulated efflux by MexAB-OprM (via mutations in the repressor gene *mexR*), MexCD-OprJ (via mutations in the repressor gene *nfxB*), or MexEF-OprN (via mutations in *mexS*), mutations in the fatty acid pathway gene *fabG* and *lpxC* structural gene mutations (encoding L18V), and upstream mutations causing LpxC overexpression (20). We also noted that *in vitro* passaging of cells in the presence of CHIR-090 can lead to cumulative and substantial reductions in susceptibility. Here, we used single-step mutant selections and passaging approaches, along with targeted and genome sequencing, to examine the role of efflux and nonefflux mechanisms in mediating decreased susceptibility to CHIR-090 and the more recently described LpxC inhibitors compound 1 (9), compound 2 (oxazolidinone 13f), compound 3 (isoxazoline 25d), and compound 4 (isoxazoline 25e) (12) (Fig. 1) that have potent *in vitro* activity against *P. aeruginosa*.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid ^a	Relevant characteristics ^b	Source or reference
Strains		
<i>P. aeruginosa</i>		
K767	PAO1 prototroph (described in reference 48)	K. Poole
K1454	K767 <i>mexR</i> (<i>nalB</i>), upregulated MexAB-OprM	49
K767-CDA0033	K767 <i>nfxB</i> , upregulated MexCD-OprJ	This study
K767-CDR0026	K767 overexpressing LpxC	20
K767-LpxC _{G208S}	K767 engineered to encode LpxC _{G208S}	This study
K767-CDJ0011	K767 engineered to encode LpxC _{L18V}	20
K767-CDJ0037	K767 encoding LpxC _{A214V} , selected on compound 3	This study
K767-CDR0061	K767 <i>fabG</i> (C494T), selected on CHIR-090	20
K2732	PAO1 prototroph	K. Poole
K2732-P2	K2732 FabF1 _{T306A} , passage 2, selected on CHIR-090	This study
K2732-P6	K3732 <i>nfxB</i> stop TGA-Cys; FabG _{D190G} ; PA4465 _{N193T} , passage 6, selected on CHIR-090	This study
K2732-P13	K2732 MexR _{T130P} ; <i>nfxB</i> stop TGA-Cys; FabG _{D190G} ; PA4465 _{N193T} , passage 13, selected on CHIR-090	This study
K2733	K2733 (K2732 Δ <i>mexB</i> Δ <i>mexX</i> Δ <i>mexCD-oprJ</i> Δ <i>mexEF-oprN</i>)	K. Poole
K2733-P6a (NC)	K2733 PA4465 _{N193T} , passage 6, selected on CHIR-090	This study
K2733-P6b (SC)	K2733 FabG _{A159V} , passage 6, selected on CHIR-090	This study
K2733-P13a (NC)	K2733 FabG _{A167V} ; PA4465 _{N193T} , passage 13, selected on CHIR-090	This study
K2733-P13b (SC)	K2733 FabG _{A167V} ; PA4465 _{N193T} , passage 13, selected on CHIR-090	This study
K2733-PA4465 _{N193T}	K2732 engineered to encode PA4465 _{N193T}	This study
PAO1V	PAO1 prototroph	50
PAO1V-CDJ0042	PAO1V encoding LpxC _{A214V} , selected on compound 2	This study
K2153	Clinical isolate, MexXY upregulated, active <i>mexT</i>	25
K2376	K2153 <i>mexS</i> in-frame deletion, upregulated MexEF-OprN expression	51
K2153-CDR0063	K2153 <i>mexS</i> , upregulated MexEF-OprN expression	20
<i>E. coli</i>		
Stellar	High transformation efficiency HST08 strain	Clontech
Plasmids		
pRK2013	Helper plasmid	52
pAK1900	<i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector; Ap ^r	A. Kropinski
pAK-lpxC	pAK1900 with <i>lpxC</i> and 100 bp upstream sequence from K767	20
pAK-lpxC WT	pAK1900 with WT <i>lpxC</i> and 100 bp upstream sequence from K767	20
pAK-lpxC WT UP	pAK-lpxC WT with C→A substitution 11 bp upstream of <i>lpxC</i> ; LpxC upregulated)	20
pAK-LpxC _{A214V}	pAK-lpxC WT altered so that <i>lpxC</i> encodes A214V	This study
pAK-LpxC _{A214V} UP	pAK-LpxC _{A214V} with C→A substitution 11 bp upstream of <i>lpxC</i> ; LpxC _{A214V} upregulated)	This study
pMMB-10	Gateway adapted pMMB low-copy-number expression vector; Ap ^r Cb ^r ; IPTG inducible	53
pMM-10-fabF1	IPTG inducible <i>fabF1</i> expression vector	This study
pMM-10-PA4465	IPTG inducible PA4465 expression vector	This study
pEX18ApGW	Gateway adapted gene replacement vector, Ap ^r Cb ^r , <i>sacB</i>	54
pEX18ApGW-lpxC-G622A	Gene replacement vector with <i>lpxC</i> G622A allele	This study
pEX18ApGW-PA4465-A578C	Gene replacement vector with PA4465 A578C allele	This study

^aAdditional mutants are summarized in Table S1 in the supplemental material. NC, normal-size colony; SC, small colony.

^bCb^r, carbenicillin resistance; Ap^r, ampicillin resistance.

RESULTS

Selection of single-step *P. aeruginosa* mutants using novel LpxC inhibitors.

Single-step mutants of two different *P. aeruginosa* PAO1 lineages (K767 and PAO1V) or the clinical isolate K2153 (Table 1) were selected using novel LpxC inhibitors shown in Fig. 1. Different PAO1 lineages were used at different times over the course of several years to isolate mutants to survey mechanisms reducing susceptibility. The mutant frequencies obtained for these selections are shown in Table 2 and are consistent with what would be expected given the multiplicity of mechanisms likely to affect these compounds. Of the two PAO1 lineages used, PAO1V appeared to have a higher frequency of mutant selection for compounds 2 and 3 at lower multiples of the MIC than did K767. Different lineages of PAO1 are known to diverge over time (21), so this is perhaps not surprising, but we did not investigate this further for these two strains. The likely resistance loci *lpxC*, *fabG*, *mexR* (repressor of MexAB-OprM efflux pump expression), and *nfxB* (repressor of MexCD-OprJ efflux pump expression) genes were then PCR amplified and sequenced from a set of selected mutants. The identified mutations are summarized in Table S1 in the supplemental material. Mutations in *mexR* and *nfxB* were

TABLE 2 Frequencies of selecting *P. aeruginosa* single-step mutants with decreased susceptibility to LpxC inhibitors

Strain	Compound	Frequencies ^a at:			
		2× MIC	4× MIC	8× MIC	16× MIC
K767	2	2.16×10^{-8}	3.6×10^{-10}	$<1.79 \times 10^{-10}$	$<1.79 \times 10^{-10}$
K767	3	7.27×10^{-8}	3.16×10^{-9}	3.51×10^{-10}	$<7 \times 10^{-10}$
PAO1V	2	1.98×10^{-7}	1×10^{-8}	4.77×10^{-10}	$<9.55 \times 10^{-10}$
PAO1V	3	3.77×10^{-7}	2.28×10^{-8}	$<1.14 \times 10^{-9}$	$<1.14 \times 10^{-9}$
PAO1V	4	TNTC	3.97×10^{-10}	7.93×10^{-11}	ND
K2153	4	TNTC	1.13×10^{-7}	3.31×10^{-9}	ND

^aTNTC, too numerous to count; ND, not determined.

identified among the isolates, consistent with the involvement of MexAB-OprM and MexCD-OprJ in reducing *P. aeruginosa* susceptibility to other LpxC inhibitors (20, 22). Some *P. aeruginosa* PAO1 lineages have inactive variants of the *mexT* gene that encodes the positive activator of *mexEF-oprN* efflux pump gene expression (23), and selection of MexEF-OprN pump upregulated mutants can be infrequent in such strains. These include PAO1 strain K767 (Table 1), which has an inactive *mexT* gene due to the presence of one or both of two point mutations (24), and PAO1V, which has an inactive *mexT* presumably due to an 8-bp insertion in *mexT* (3). The clinical isolate K2153 (Table 1) has an active *mexT* allele, and MexEF-OprN pump upregulated mutants can be selected via loss-of-function mutations in the *mexS* gene whose product suppresses MexT (20, 25). The LpxC inhibitors CHIR-090 and LpxC-4 selected for upregulated expression of MexEF-OprN in an appropriate strain background (20, 22), so we tested this for compound 4 using strain K2153. Mutants were selected at a frequency of approximately 1×10^{-7} at 4× MIC (Table 2) and of 9 mutants examined, 5 harbored mutations in *mexS* (Table S1). Along with these efflux related mutations, several mutations in *fabG* were found, as well as the upstream *lpxC* mutation causing overexpression of LpxC (20, 22) (PAO1V-CDJ0028) and a novel *lpxC* structural gene mutation (encoding LpxC A214V) selected by compounds 3 and 4 (K767-CDJ0037 and PAO1V-CDJ0042).

Table 3 compares the shift in susceptibility to CHIR-090 and compounds 2 to 4 in a representative *fabG* mutant and in mutants upregulated for MexAB-OprM, MexCD-OprJ, or MexEF-OprN efflux pump expression (mutations related to *lpxC* are discussed in later sections). The *fabG* mutant K767-CDA0033 was 4- to 8-fold less susceptible to all four compounds. Upregulation of MexAB-OprM expression (engineered strain K1454) also mediated a 4- to 8-fold decrease in susceptibility to these compounds. Upregulated expression of MexCD-OprJ (K767-CDA0033) reduced susceptibility to CHIR-090 and compound 3 by 16-fold but had little impact on susceptibility to compounds 2 or 4. This suggested that these are weak substrates for MexCD-OprJ, however, several *nfxB* mutants were selected using these compounds (Table S1), suggesting that different *nfxB* mutations may lead to a range of susceptibility shifts. Two previously characterized mutants upregulated for MexEF-OprN expression (K2376 and K2153-CDR0063) were >4-fold less susceptible to all compounds.

TABLE 3 Impact of *fabG* mutation and efflux pumps on susceptibility to LpxC inhibitor compounds in *P. aeruginosa*

Strain	Relevant factor	MIC (μg/ml) ^a			
		CHIR-090	Compound 4	Compound 3	Compound 2
K767	Wild type	1	0.25	0.25	0.25
K767-CDR0061	<i>fabG</i> mutation	8	2	2	1
K1454	MexAB-OprM up	8	2	2	2
K767-CDA0033	MexCD-OprJ up	16	0.25	4	0.5
K2153	Wild type	2	0.5	1	0.5
K2376	MexEF-OprN up	16	8	4	4
K2153-CDR0063	MexEF-OprN up	>16	16	16	>4

^aSusceptibility shifts ≥ 4 -fold are indicated in boldface.

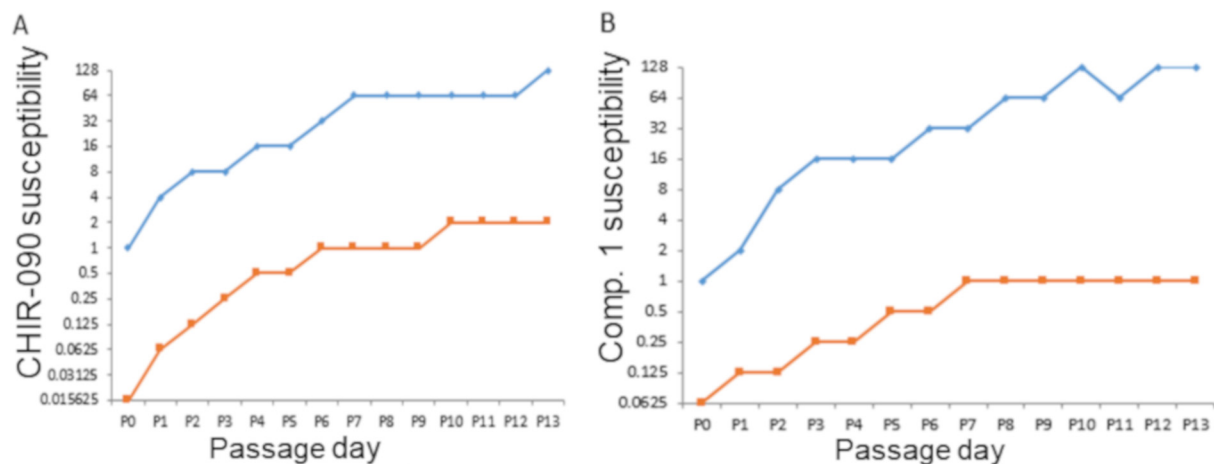


FIG 2 Cumulative decrease in the susceptibility of *P. aeruginosa* wild type (K2732, blue lines) and an efflux-deficient mutant (K2733, orange lines) to CHIR-090 (A) or compound 1 (B) during serial passaging. The y axes show the susceptibility (MIC, $\mu\text{g/ml}$) of mixed populations, as described previously (20).

Cumulative effect of mechanisms reducing susceptibility to LpxC inhibitors that emerge during serial passage.

Consistent with the range of mechanisms that individually reduce susceptibility to CHIR-090 and other LpxC inhibitors, *in vitro* serial passaging experiments using CHIR-090 led to substantial cumulative decreases in susceptibility in *P. aeruginosa* (MIC shifts to 128 $\mu\text{g/ml}$) (20). Here, we undertook serial passage studies using a wild-type *P. aeruginosa* PAO1 strain K2732 and its efflux-deficient mutant (lacking MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN function), K2733 to ascertain the impact of efflux on the magnitude of decreased susceptibility and to isolate and characterize additional mutants. Like K767, this parent strain K2732 has an inactive *mexT* gene, and as such the emergence of MexEF-OprNM upregulation was not anticipated during passaging. Susceptibility to CHIR-090 decreased steadily over 13 passages for K2732, with the MIC of CHIR-090 starting at 1 $\mu\text{g/ml}$ and reaching 128 $\mu\text{g/ml}$ (Fig. 2A). The susceptibility of the efflux defective strain K2733 also decreased in a stepwise fashion, but the lack of efflux appeared to limit the ultimate level of insusceptibility reached over a similar time frame. CHIR-090 was much more potent against the efflux defective mutant culture at the beginning of the experiment (MIC 0.0156 $\mu\text{g/ml}$) and ultimately the CHIR-090 MIC stabilized at 1 to 2 $\mu\text{g/ml}$ from passages 6 to 13 (Fig. 2A). Therefore, there was a similar circa 64-fold shift in susceptibility whether efflux was active or not, but with efflux the ultimate level of susceptibility was about 32- to 64-fold lower. A similar pattern was seen when experiments were conducted using compound 1 (9) except that there was only a 16-fold decrease in susceptibility over 13 passages in the pump defective strain (Fig. 2B).

Interplay of mechanisms leads to substantially reduced susceptibility to LpxC inhibitors.

Several mutants were isolated from different stages of the CHIR-090 serial passaging experiment and genome sequenced (Table 4). Qualitatively there appeared to be some differences in colony sizes on some of the selection plates, and this is noted in Table 1. As expected, mutations in the regulators of efflux appeared in isolates derived from the wild-type strain (*nfxB* in K2732-P6 and *mexR/nfxB* in K2732-P13; Table 4). Intriguingly, the combination of *nfxB* and *mexR* mutations in K2732-P13 suggests that MexAB-OprM and MexCD-OprJ can work together to increase efflux of CHIR-090. As anticipated, no mutations in the regulatory genes controlling expression of the four pumps that are inactivated in strain K2733 emerged. In isolates selected from both the parent and efflux deficient mutant, mutations in *fabG* were identified (K2732-P6, K2732-P13, K2733-P6b, and K2733-P13ab, Table 5), which are known to reduce susceptibility to LpxC inhibition (20). We did not find mutations in *lpxC* or the region upstream of *lpxC* in the mutants studied here, but two new mutations were identified in genes

TABLE 4 Mutations identified in mutants isolated during serial passage in CHIR-090

Strain ^a	Passage step (CHIR-090 MIC in $\mu\text{g/ml}$)	Mutation or amino acid substitution(s)
K2732 (WT efflux)	Parent (1)	
K2732-P2	2 (4)	FabF1 _{T306A}
K2732-P6	6 (32)	<i>nfxB</i> stop TGA-Cys; FabG _{D190G} ; PA4465 _{N193T}
K2732-P13	13 (64)	MexR _{T130P} ; <i>nfxB</i> stop TGA-Cys; FabG _{D190G} ; PA4465 _{N193T}
K2733 (efflux deficient)	Parent (0.0313)	
K2733-P6a (nc)	6 (0.25)	PA4465 _{N193T}
K2733-P6b (sc)	6 (0.5)	FabG _{A159V}
K2733-P13a (nc)	13 (0.5)	FabG _{A167V} ; PA4465 _{N193T}
K2733-P13b (sc)	13 (1)	FabG _{A167V} ; PA4465 _{N193T}

^anc, normal colony size; sc, small colony size.

not previously associated with decreased susceptibility to LpxC inhibitors. One of these was in *fabF1* (encoding FabF1_{T306A}) and occurred in one isolate derived from the wild-type strain at passage 2 (K2732-P2, Table 4). This conferred a 4-fold decrease in susceptibility (CHIR-090 MIC shift from 1 to 4 $\mu\text{g/ml}$, Table 4). The wild-type *fabF1* gene was then cloned into an inducible plasmid and expressed in *trans* in the passage-selected *fabF1* mutant K2732-P2. As expected, the mutant containing empty vector or the uninduced *fabF1* plasmid grew fairly well in the presence of 1 $\mu\text{g/ml}$ of CHIR-090, whereas growth of the parent K2732 containing empty vector was much less (Fig. S1). Induction of *fabF1* expression (complementation) reduced growth of K2732-P2 to levels comparable to the parent strain, consistent with a role for FabF1_{T306A}. The other new mutation was found in the hypothetical gene PA4465 (3) (encoding N193T), and this occurred together with mutations in *nfxB*, *mexR*, and *fabG* in (K2732-P6 or K2732-P13, Table 4) or singly and with *fabG* mutations in mutants from K2733 (K2733-P6ab and K2733-P13ab, Table 4). The single mutation in K2733-P6a mediated an \sim 8-fold decrease in susceptibility. This mutation was engineered singly back onto the genome of the pump plus parent K2732 (designated K2732-PA4465_{N193T}). These cells were complemented in *trans* with an inducible plasmid-borne copy of wild-type PA4456 (Fig. S2). K2732-PA4465_{N193T} harboring either the empty control vector or the uninduced PA4465 vector grew in 0.5 $\mu\text{g/ml}$ of CHIR-090, whereas K2732 containing empty vector did not, consistent with this engineered mutation reducing susceptibility. Induction of PA4465 expression strongly reduced growth of K2732-PA4465_{N193T} consistent with a role for PA4465_{N193T} (Fig. S2).

An *lpxC* mutation encoding LpxC_{G208S} reduces susceptibility to CHIR-090. A serial passage experiment in CHIR-090 using a *P. aeruginosa* *mutS* hypermutator strain (CDR0017) generated two mutants with upregulated expression of LpxC (20). Here, we examined a third mutant from this experiment that did not have upregulated LpxC expression and lacked the associated *lpxC* upstream mutation, which revealed a mutation within the structural *lpxC* gene encoding LpxC_{G208S}. For further study, we engineered it into the *P. aeruginosa* nonhypermutator parent PAO1 strain K767 (K767-LpxC_{G208S}, Table 1), where it shifted susceptibility to CHIR-090 4-fold (MIC from 0.5 to 2 $\mu\text{g/ml}$, Table 5). Purified LpxC_{G208S} was \sim 9-fold less active than the wild-type LpxC (k_{cat}/K_m of 1.64×10^4 compared to $1.51 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$, Table 5) but also bound CHIR-090 \sim 74-fold less than did the wild-type protein (K_i app of 9.64 ± 2.08 compared to $0.13 \pm 0.01 \text{ nM}$, Table 5). Therefore, the G208S substitution decreased binding to CHIR-090. Although the LpxC_{G208S} protein was less catalytically active, no difference in growth curves of cells expressing LpxC_{G208S} (Mueller-Hinton broth) was seen compared to the parent by standard growth curve (data not shown).

TABLE 5 Impact of LpxC_{G208S} on susceptibility of *P. aeruginosa* PAO1 to CHIR-090 and on LpxC enzymatic activity and binding to CHIR-090

Strain	CHIR-090 MIC ($\mu\text{g/ml}$)	Protein	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1} \text{ M}^{-1}$)	Mean K_i app (nM) \pm SD (CHIR-090)
K767	0.5	LpxC	0.89	5.89	1.51×10^5	0.13 ± 0.01
K767-LpxC _{G208S}	2	LpxC _{G208S}	0.53	32.4	1.64×10^4	9.64 ± 2.08

TABLE 6 Impact of different LpxC target mutations on susceptibility to LpxC inhibitor scaffolds

Strain	LpxC variant	MIC ($\mu\text{g/ml}$) ^a				
		CHIR-090	Compound 3	Compound 2	MER	TOB
K767	LpxC _{WT}	1	0.50	0.25	1	0.125
K767-CDR0026	LpxC _{WT} upregulated	16	16	8	1	0.25
K767-CDJ0011	LpxC _{L18V}	8	0.5	1	1	0.25
K767-LpxC _{G208S}	LpxC _{G208S}	4	0.125	0.125	0.5	0.125
K767-CDJ0037	LpxC _{A214V}	0.5	>16	4	0.5	0.125
PAO1V	LpxC _{WT}	1	0.25	0.25	1	0.125
PAO1V-CDJ0042	LpxC _{A214V}	0.5	16	2	1	0.125

^aSusceptibility shifts \geq 4-fold are indicated in boldface. MER, meropenem; TOB, tobramycin.

Different LpxC protein variants have differential impacts on susceptibility to LpxC inhibitor scaffolds.

To date, we have selected three different LpxC target alterations across this and previous studies (L18V, G208S, and A214V) using different compounds. We examined the specificity of these changes for different LpxC inhibitor scaffolds (Table 6). As a control, strain K767-CDR0026, which demonstrates constitutive upregulation of wild-type LpxC, was included (20). Since the impact of LpxC upregulation on susceptibility to LpxC inhibitors is presumably mediated by a compound titration effect, it should impact all LpxC inhibitors regardless of scaffold. As expected, susceptibility to CHIR-090, compound 3 and compound 2 are all reduced by 16- to 32-fold against K767-CDR0026 compared to its parent (Table 6). In contrast, the specific LpxC variants tested here had differential impacts that correlated generally with which compound was used to select the mutation. The engineered strain (K767-CDJ0011) expressing LpxC_{L18V} (20) was 8-fold less susceptible to CHIR-090 (Table 6). This is consistent with the LpxC_{L18V} variant having been originally identified through a selection experiment with CHIR-090. This strain was not less susceptible to compound 3, but there was a 4-fold shift in susceptibility to compound 2. The LpxC variant of K767-LpxC_{G208S} was also originally identified from mutants selected using CHIR-090. This engineered mutant was 4-fold less susceptible to CHIR-090 but was not less susceptible, and was possibly slightly more susceptible, to both compound 3 and compound 2 (Table 6). Two separate mutants selected on compound 3 (K767-CDJ0037 and PAO1V-CDJ0042, Table 6) that express the LpxC_{A214V} variant were substantially less susceptible (\geq 16-fold) to compound 3 and to compound 2 (8- to 16-fold) but were not less susceptible to CHIR-090. Although not tested directly here, mutants expressing LpxC_{A214V} were also selected using compound 4 at 4 \times or 8 \times the MIC (Table S1), and therefore LpxC_{A214V} also impacts susceptibility to compound 4. We utilized existing crystal structures of LpxC bound to CHIR-090 (9) and compound 2 (12), and an unpublished crystal structure of compound 3 in an effort to explain the impact of A214V, L18V, and G208S amino acid substitutions on the susceptibility to these compounds. Residue A214 is located opposite the α -helix of the domain II insert that encapsulates the acyl chain of the natural substrate. This region of the protein is flexible and for CHIR-090, compound 2, and compound 3 envelops the “tail” portions of these ligands. We manually modified residue 214 from Ala to Val using PyMOL (The PyMOL molecular graphics system (v2.0; Schrödinger, LLC) in the CHIR-090 LpxCPa crystal structure and rendered the surface of the binding pocket. We then overlaid the crystal structures of ligands compound 2 and compound 3 bound to LpxCPa to visually inspect whether they can fit into the altered binding pocket. As illustrated in Fig. 3, the phenyl group of compounds 2 and 3 would clash with A214V, and a rearrangement of the flexible region of the protein would need to occur to accommodate this change. We hypothesize this causes a reduction in the activity of compounds 2 and 3 against LpxC_{A214V}. Interestingly, CHIR-090 is agnostic to this change because the acetylene substituent can tolerate this modification. In fact, this modification is a better complement to the ligand and could be the reason for the slightly improved MIC against mutants expressing this variant. These observations are consistent with previous

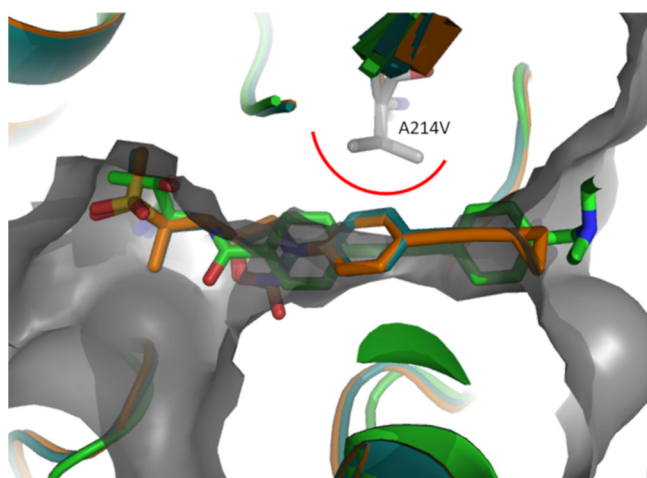


FIG 3 Overlay of the CHIR-090 structure (green, PDB 5U39) with compound 2 (orange, PDB 6MAE) and compound 3 (blue, unpublished). The A214V location is modeled on the CHIR-090 structure and highlighted by gray sticks; the protein surface with this modification is illustrated in gray.

reports that a different tunnel residue modification differentially affected susceptibility to LpxC inhibitors, depending on the position of acetylene groups (26, 27). The L18V substitution reduces the activity of CHIR-090 and to a potentially lesser degree for compound 2 but had no impact on compound 3. The L18V substitution increases the binding cavity of ligands (Fig. 4). We hypothesize that Leu18 for CHIR-090 acts as a scaffolding residue to keep the ligand in place, whereas for compounds 2 and 3, the ligand is interacting with His19 (via CH—O interaction or NH—O interaction at 3.4 Å) and is less reliant on Leu18 to maintain the binding mode (Fig. 4). Therefore, the more rigid compounds 2 and 3 are not as impacted by this transformation as is CHIR-090. The crystal structure information does not provide a clear notion as to the impact or selectivity of G208S since it involves a side chain that points away from the binding site of the ligand.

Upregulation of LpxC expression combined with LpxC_{A214V} can substantially decrease susceptibility to compound 1. To explore the potential impact of upregulated expression of a resistant variant of LpxC, we compared the impact of LpxC_{A214V} expressed in *trans* to that of wild-type LpxC on susceptibility to compound 1 in *P.*

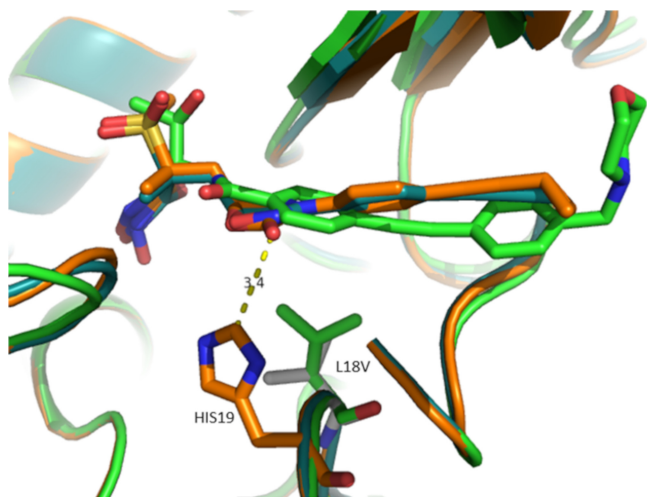


FIG 4 Overlay of the CHIR-090 structure (green, PDB 5U39) with compound 2 (orange, PDB 6MAE) and compound 3 (blue, unpublished). The L18V location is modeled on the CHIR-090 structure and highlighted in gray sticks. The distance between compound 2 and His19 (3.4 Å) is indicated by a yellow dashed line.

TABLE 7 Impact of LpxC overexpression and LpxC_{A214V} amino acid substitution on susceptibility to compound 1 and CHIR-090 in PAO1 strain K767

Strain	MIC ($\mu\text{g/ml}$)	
	Compound 1	CHIR-090
K767	1	1
K767 (pAK1900)	1	1
K767 (pAK-LpxC WT)	4	4
K767 (pAK-LpxC _{A214V})	16–32	2
K767 (pAK-LpxC WT UP)	32	64
K767 (pAK-LpxC _{A214V} UP)	>128	32

aeruginosa K767 (summarized in Table 7). The MIC of compound 1 against PAO1 harboring the empty plasmid vector pAK1900 was 1 $\mu\text{g/ml}$ and rose to 4 $\mu\text{g/ml}$ against cells containing pAK1900 with the wild-type *lpxC* gene with native upstream region (pAK-LpxC WT). This shift is likely due to the copy number of pAK1900, which is approximately 4 to 5 copies per cell. Adding the mutation encoding LpxC_{A214V} (pAK-LpxC_{A214V}) further reduced susceptibility 4- to 8-fold, confirming the direct role for A214V in reducing susceptibility to compound 1. As expected, the MIC of compound 1 against cells containing pAK1900 with the wild-type *lpxC* gene but with the upstream mutation (pAK-LpxC WT UP) was \sim 8-fold higher than against cells with the same vector lacking the upstream mutation (an MIC of 32 $\mu\text{g/ml}$ compared to an MIC of 4 $\mu\text{g/ml}$), presumably reflecting further increased expression of LpxC. Adding the A214V-encoding *lpxC* variant (pAK-LpxC_{A214V} UP) decreased susceptibility at least an additional 8-fold, providing further confirmation of the role of A214V but also showing that LpxC_{A214V} and protein overexpression together is tolerated and generates substantial decreases in susceptibility to compound 1. This also extends the specificity of this mutation to include compound 1 and, consistent with the data in Table 6, the A214V mutation did not affect CHIR-090, even upon overexpression of LpxC_{A214V}.

DISCUSSION

In this study, we show that previously identified mechanisms decreasing the *in vitro* susceptibility to the LpxC inhibitor CHIR-090 also impact the susceptibility to several newer LpxC inhibitor scaffolds. Upregulation of efflux pumps MexAB-OprM, MexCD-OprJ, or MexEF-OprN reduced the susceptibility to CHIR-090 (20), and we show that these pumps affect the newer LpxC inhibitors under study here. Single-step resistance selections with another inhibitor, LpxC-4, yielded mutants upregulated for MexEF-OprN expression (*nfxC* mutants) and MexAB-OprM expression (*nalB* mutants) but did not select mutants upregulated for MexCD-OprJ (*nfxB* mutants) (22). Very recently reported single-step and passage selection experiments with another series of newer LpxC inhibitors, typified by ACHN-975, yielded mutants upregulated for MexCD-OprJ and MexEF-OprN but not MexAB-OprM (19). Although not definitive, this suggests that there may be substantial differences in pump recognition profiles across different LpxC structures. Consistent with this, we also saw a differential impact of MexCD-OprJ upregulation on the different compounds studied here. A thorough evaluation of the impact of these pumps on activity across a broad range of LpxC inhibitors might therefore yield insights into pump evasion. As is the case for many antibacterial compounds, efflux can work together with nonefflux mechanisms to generate significant levels of resistance, and this was shown here for CHIR-090 and compound 1.

Among the nonefflux mechanisms identified here are novel alterations in the LpxC protein target, and we show that these can be scaffold specific, as might be expected. The remaining mechanisms of upregulated LpxC protein expression, as well as mutations in *fabG* and the newly identified mutations in *fabF1* and PA4465, could differ from specific target mutations in that they may be more scaffold independent. We and others (19) have found that upregulation of LpxC mediated a generally consistent shift in susceptibility to a range of LpxC inhibitors, as expected. The fatty acid biosynthetic cycle, within which FabG resides, is intricately related to lipid A biosynthesis, and

selection of *fabG* mutants is reminiscent of the selection of *fabZ* mutants by LpxC inhibitors in *Escherichia coli* (28) and *Klebsiella pneumoniae* (22, 29). Alterations in *fabZ* may mediate resistance in *E. coli* by altering intermediate flux between pathways (28) and/or via intricate regulatory pathways controlling phospholipid and lipid A homeostasis (30); however, the interrelationships of these pathways and the mechanism(s) by which *fabG* mutations mediate resistance in *P. aeruginosa* are not currently well understood. There appears to be a large number of *fabG* mutations that are tolerated and, in keeping with this, they were consistently selected in studies with CHIR-090 (20) and other compounds in this study, as well as by the ACHN series of LpxC inhibitors (19). Intriguingly, although LpxC-4 did select *fabZ* mutants in *K. pneumoniae*, no *fabG* mutations were reported for *P. aeruginosa* (22), which could suggest that some scaffolds are less impacted by mechanisms mediated by fatty acid pathway mutations, but this remains to be more fully explored.

Two new mutations in *fabF1* (encoding beta-ketoacyl-acyl carrier protein synthase II [3]) and the hypothetical gene PA4465 (3) were identified in this study. Cells lacking FabF1 are defective in the production of *cis*-vaccenic acid (31) and become nonmotile (32), but an understanding of the specific function of FabF1 in *P. aeruginosa* awaits more study (33). Although the effect of the alteration in FabF1 on susceptibility to CHIR-090 appears to be subtle, the identification of a mutation in a gene related to fatty acid biosynthesis is consistent with the role of *fabG* mutations in *P. aeruginosa* and *fabZ* mutations in *E. coli* (28, 30, 34) and *K. pneumoniae* (22, 29) in decreasing susceptibility to LpxC inhibitors. Mutations in *fabZ* in *E. coli* may increase metabolic flux into the lipid A pathway and/or rebalance phospholipid and lipid A homeostasis to confer resistance, underscoring the intricate relationship between these pathways (28, 30, 35). This notion has been further supported by the finding that the majority of proteins in the *E. coli* fatty acid elongation cycle were found as part of a broad LpxC interactome (36). The gene PA4465 encodes a hypothetical protein that appears to be an analog (50% identity, 65% similarity) to *E. coli* RapZ (37). RapZ is important for controlling the cellular levels of GlmS (glucosamine-6-phosphate synthase) and therefore the cellular levels of glucosamine-6-phosphate (GlcN6P), an important precursor for the synthesis of both the peptidoglycan cell wall and LPS. GlmS is controlled directly by the small RNA GlmZ, which binds and activates *glmS* mRNA. GlmZ levels are controlled by RapZ, an RNase adaptor protein that targets GlmZ to RNase E for degradation. Another small RNA, GlmY, can act as an antiadaptor and sequester RapZ under conditions of GlcN6P depletion, thereby stabilizing the GlmS mRNA (3, 37–42). Therefore, this regulatory circuit is important in controlling GlcN6P homeostasis through GlmS. The involvement of the putative RapZ homolog of *P. aeruginosa*, which may link these cell envelope biosynthetic pathways through a common pathway intermediate, is reminiscent of the interrelationship between the lipid A and fatty acid biosynthesis pathways mentioned above. Indeed, several proteins related to cell wall biosynthesis, including RapZ, GlmS, GlmU (bifunctional glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridylyltransferase), and GlmM (phosphoglucosamine mutase) were identified in an *E. coli* LpxC interactome study (36). It was also recently shown that *E. coli* cells remodel their peptidoglycan in response to defects in LPS transport to the OM, which provides protection against cell lysis (43). It is interesting to note that alterations in FabF1 and PA4465 have so far only emerged from serial passage studies. FabF1 is not essential for growth of *P. aeruginosa* (31), RapZ is not essential in *E. coli* (44), and transposon insertions have been reported in PA4465 (*Pseudomonas* Genome DB [www.pseudomonas.com]), suggesting that these functions could presumably be lost at high frequency. Their lack of appearance during single-step experiments could possibly reflect the subtle effect such mutations have on susceptibility, making them less likely to survive single-step selections at multiples of the MIC. Nonetheless, the identification of multiple-pathway interrelated mutations indicates that adjustments of these interrelationships contributes to survival upon LpxC inhibition. This notion is further expanded by the recent finding that mutations in *acpP*, which encodes acyl carrier protein required for many steps of the lipid biosynthetic pathway and which interacts with

FabG, reduced the susceptibility to ACHN-95 and related molecules (19). The exact mechanism(s) by which these mutations mediate decreased susceptibility warrants further study.

Relating to the intricate pathway interrelationships described above, overexpression of LpxC in *E. coli* can be toxic, and therefore LpxC levels are tightly controlled via proteolytic degradation by FtsH and other mechanisms (36). This suggested that target upregulation might not be readily selected by LpxC inhibitors, and this has not to our knowledge been reported in *E. coli*. *P. aeruginosa* LpxC lacks this proteolytic control by FtsH (45) and, perhaps reflecting this, we found that overexpression of LpxC is well tolerated in *P. aeruginosa* and that upregulated expression mediates a substantial decrease in susceptibility to LpxC inhibitors (20). This ability to tolerate upregulated LpxC could therefore combine with alterations of the LpxC protein to mediate more substantial decreases in susceptibility, and indeed we show this to be the case using LpxC_{A214V}, although the contribution of target mutations can be scaffold specific. Although we did not find an instance of upregulated expression of LpxC_{A214V} in our passaging studies, we did identify the LpxC_{G208S} variant from passaging and upon reviewing historical data from studies with compound 1, we had a *P. aeruginosa* mutant that emerged during a hollow-fiber model experiment that harbored the *lpxC* upstream mutation, overexpressed LpxC, and had the mutation encoding LpxC_{A214V}, all suggesting that this combination does emerge under selection pressure.

In summary, we have assembled a range of some available information on mechanisms and interactions that may decrease susceptibility to LpxC inhibitors from studies over several years with different LpxC inhibitor compounds. An understanding of which *in vitro* selected mechanisms would ultimately prove to emerge during use of an LpxC inhibitor in the clinic of course awaits further progress in this area. These studies can, however, inform our understanding of antibacterial targets and pathways and provide a framework for establishing dosing requirements intended offset the development of resistance.

MATERIALS AND METHODS

Strains, growth conditions, and susceptibility testing. The strains and plasmids used in this study are listed in Table 1. Media for routine genetic manipulations was lysogeny broth (LB) or agar unless specified otherwise and media for mutant selections and susceptibility testing was cation-adjusted Mueller-Hinton broth or agar. Single-step mutant selections, serial passage studies and MIC determinations were carried out as previously described (20). Compound CHIR-090 was previously described (20). Compound 1 (9) and compounds 2 to 4 (12) were synthesized at Novartis. Other antibiotics and IPTG (isopropyl- β -D-thiogalactopyranoside) were purchased commercially. Susceptibility testing was carried out as previously described (20).

Whole-genome sequencing. Genomic DNA isolation, fragment library preparation, Illumina sequencing, and variant calling were performed as described previously (20).

DNA methods. The *lpxC* G622A mutation encoding LpxC_{G208S} and the PA4465 A578C mutation encoding PA4465_{N193T} were introduced onto the genome of *P. aeruginosa* using the sac-based gene exchange vector pEX18ApGW as follows. The *lpxC* gene harboring the A578C mutation was PCR amplified from the genome of serial passage mutant CDR0017 (colony 3 described in reference 20) using the Gateway adapted primers *lpxC*-5' (TACAAAAAAGCAGGCTCAGGAGTAGAGATGTGATTGGTG) and *lpxC*-3' (TACAAGAAAGCTGGGTGAGATCGTCGGCAATCCACGCCTG). Gateway adapters were added with a second round of PCR using the attB1 (GGGGACAAGTTTGTACAAAAAAGCAGGCT)/attB2 (GGGGACCACTTGTACAAGAAAGCTGGGT) primer pair, and then this fragment was cloned into pDON221 using Gateway cloning (Invitrogen). A sequence-confirmed pDON221-*lpxC* A578C construct was then used to create pEX18ApGW-*lpxC*-G622A using Gateway cloning following the supplied instructions. The PA4465 gene harboring the A578C mutation was PCR amplified from the genome of K732-6 using Gateway-adapted primers PA4465-5' (GGCCAGTGCCAAAGCTTgaccgaagaacatctcgaac) and PA4465-3' (ACGAATTCGAGCTCGGTA Ccgtctgctctctgttatggg). Gateway adapters were added in a second round of PCR using the attB1/attB2 primers, and this fragment was cloned into pDON221 using Gateway technology. A sequence-confirmed pDON221-PA4465-A578C construct was then used to create pEX18ApGW-PA4465-A578C using Gateway cloning. Plasmids pEX18ApGW-*lpxC*-G622A and pEX18ApGW-PA4465-A578C were then mated into *P. aeruginosa* PAO1 strains K767 and K2732, respectively, using the *E. coli* Stellar strain (Clontech) that harbored the helper plasmid pRK2013 (46). *P. aeruginosa* merodiploids were selected on L-agar containing 25 μ g/ml Irgasan and 150 μ g/ml carbenicillin, and double crossovers were then selected on L-agar with 5% sucrose. Sucrose-resistant, carbenicillin-sensitive resolvants were then screened for growth on L-agar containing 2 μ g/ml CHIR-090, and mutations were confirmed on the genome by PCR amplification and sequencing. IPTG-inducible complementation plasmids were made as follows. The *fabF1* gene was PCR amplified from *P. aeruginosa* wild-type PAO1 using the Gateway-adapted primers 5' *fabF1* GW (TCGAGGAGGATATTCgtgtcgcgtagacgcgtcattactg) and 3' *fabF1* GW (CAAGAAAGCTGGTTtcagtcggcg

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