SHORT-FORM PAPER

# Inhibition of LpxC increases antibiotic susceptibility in *Acinetobacter baumannii*

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**Key Words:** *Acinetobacter baumannii*; antibiotic resistance; lipopolysaccharide; LpxC

26 **Abstract**

27 LpxC inhibitors have generally shown poor in vitro activity against
28 *Acinetobacter baumannii*. We show that the LpxC inhibitor PF-5081090 inhibits lipid
29 A biosynthesis, as determined by silver staining and measurements of endotoxin levels,
30 and significantly increases cell permeability. The presence of PF-5081090 at 32 mg/L
31 increased susceptibility to rifampicin, vancomycin, azithromycin, imipenem and
32 amikacin, but had no effect on susceptibility to ciprofloxacin and tigecycline.
33 Potentiating existing antibiotics with LpxC inhibitors may represent an alternative
34 treatment strategy for multidrug resistant *A. baumannii*.

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37 The increasing number of infections caused by multidrug resistant *Acinetobacter*
38 *baumannii* warrants that novel treatment strategies are explored (1). Inhibitors of LpxC,
39 a zinc-dependent deacetylase that catalyzes the first committed step in the biosynthesis
40 of lipid A, have been identified and are currently being developed for use as
41 antimicrobial agents (2, 3). LpxC inhibitors have demonstrated potent antibacterial
42 activity against a number of Gram negative species including, *Pseudomonas*
43 *aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (3-6). However, these
44 inhibitors have been reported to have poor antimicrobial activity against *A. baumannii*
45 strains in vitro (3, 6, 7), which may be explained by the finding that lipopolysaccharide
46 (LPS) biosynthesis is not essential for viability in *A. baumannii* (8, 9). In spite of a lack
47 of in vitro antibacterial activity, a recent study has reported that LpxC inhibition can
48 contribute to *A. baumannii* clearance in vivo by enhancing bacterial
49 opsonophagocytosis and reducing inflammation (7). Importantly, in this study mice that
50 were treated with an LpxC inhibitor were completed protected from *A. baumannii*
51 infection (7). We recently reported that *A. baumannii* clinical isolates that acquire
52 resistance to colistin via the loss of LPS due to mutations in the lipid A biosynthesis
53 genes *lpxA*, *lpxC* and *lpxD* demonstrate increased cell permeability and increased
54 susceptibility to certain antibiotics (10). Based on these findings, we hypothesized that
55 pharmacologic inhibition of LPS biosynthesis in *A. baumannii* with an LpxC inhibitor
56 may similarly increase antibiotic susceptibility.
57 The *A. baumannii* reference strain ATCC 19606 and six clonally distinct,
58 multidrug resistant clinical isolates (Ab-84, Ab-108, Ab-167, Ab-176, Ab-7 and Ab-31)
59 that have been previously characterized were used (11, 12). IB010 is a colistin-
60 resistant, LPS-deficient derivative of *A. baumannii* ATCC 19606 which contains a
61 deletion (nucleotides 104-565) in the *lpxD* gene (13). PF-5081090 (also called LpxC-4;

62 Sigma) is a pyridone methylsulfone hydroxamate-based LpxC inhibitor that is
63 commercially available (3, 6).
64 Checkerboard assays were performed in order to determine the concentrations
65 PF-5081090 in combination with azithromycin, rifampicin, and vancomycin that
66 inhibited growth of all seven strains included in the study. These antibiotics were
67 chosen for checkerboard assays as mutant strains deficient in LPS biosynthesis were
68 previously shown to have increased susceptibility to vancomycin, rifampicin, and
69 azithromycin (10). Fractional inhibitory concentration indices (FICI) were calculated as
70 previously described (14), and results were interpreted as follows: synergistic effect is
71 defined as FICI of ≤0.5; partial synergism as FICI > 0.5 < 1; additivity as FICI = 1;
72 indifference as FICI >1≤4; and antagonism as FICI of more than 4. As shown in Table
73 1, PF-5081090 was synergistic with all antibiotic combinations for all strains tested.
74 Furthermore, the presence of 32 mg/L of PF-5081090 reduced MIC values for the
75 antibiotic tested ≥ 8 fold for all strains and antibiotics, except in the case of
76 azithromycin for strain Ab-84, which was diminished just two fold. Based on these
77 results, 32 mg/L of PF-5081090 was used for all subsequent studies.
78 We next wanted to characterize the effect of PF-5081090 on LPS biosynthesis in
79 *A. baumannii*. Overnight cultures of ATCC 19606 were diluted 50-fold in Mueller
80 Hinton broth in the absence and presence of 32 mg/L of PF-5081090. Growth was
81 monitored at 37 ºC with shaking until reaching an $OD_{600}$ of 0.5. LPS was extracted from
82 the bacteria resulting from 5 mL of each culture using the LPS Extraction Kit (iNtRON
83 biotechnology) following the manufacturer's instructions. A carbohydrate-specific
84 silver staining protocol was used to visualize LPS after separation of samples by SDS-
85 PAGE as described previously (15). As can be seen in Figure 1A, untreated ATCC
86 19606 cells showed abundant lipid A (lane 1), whereas ATCC 19606 grown in the

87 presence of 32 mg/L of PF-5081090 demonstrated a dramatic reduction in the presence
88 of lipid A (lane 2). IB010, an LPS- deficient derivative of ATCC 19606 which contains
89 a mutation in the *lpxD* gene (13), was used as a control and did not contain lipid A, as
90 expected (lane 3). To further characterize the ability of PF-5081090 to inhibit LPS
91 biosynthesis, endotoxin levels were measured in ATCC 19606 strain and four of the
92 multidrug resistant clinical isolates that had been grown in the presence (32 mg/L) or
93 absence of PF-5081090 using the QCL-1000 Limulus Amebocyte Lysate assay (Lonza)
94 according to the manufacturer's instructions. All strains demonstrated a reduction in
95 endotoxin levels after treatment with PF-5081090 compared to untreated cells (Table 2).
96 We previously demonstrated that *A. baumannii* mutants deficient in LPS due to
97 mutations in *lpxA*, *lpxC* or *lpxD* demonstrated increased membrane permeability (10).
98 To determine if a similar phenotype was produced with pharmacologic inhibition of
99 LpxC, cell permeability was characterized in the ATCC 19606 strain and four multidrug
100 resistant clinical isolates in the presence (32 mg/L) or absence of PF-5081090 using an
101 ethidium bromide accumulation assay as previously described (10). Briefly, bacterial
102 cells were treated as described above for measuring endotoxin levels, and aliquots of 95
103 µL of cells adjusted to an $OD_{600}$ of 0.2 were incubated for 10 min at 37 ºC.
104 Fluorescence ($\lambda_{excite}$, 530 nm; $\lambda_{emit}$, 600 nm) was measured at 60 min after the addition
105 of 5 µL of ethidium bromide (final concentration 2 mg/L). The assay was performed in
106 triplicate samples. All strains showed significant increases in cell permeability in the
107 presence of PF-5081090 (Figure 1B).
108 We next determined if pharmacologic inhibition of LpxC with 32 mg/L of PF-
109 5081090 affected susceptibility to amikacin, azithromycin, ciprofloxacin, colistin,
110 imipenem, rifampicin, tigecycline and vancomycin for all strains included in the study
111 by determining MIC values by broth microdultion according to the recommendations of

112 the Clinical and Laboratory Standards Institute (16). As shown in Table 2, 32 mg/L of
113 PF-5081090 resulted in increased susceptibility to rifampicin in all strains, with all
114 strains demonstrating an MIC of $\leq$ 0.03 in the presence of the LpxC inhibitor,
115 representing a more than 1,000-fold reduction in the case of two strains demonstrating
116 rifampicin MICs of 32 mg/L in the absence of inhibitor. PF-5081090 also increased
117 susceptibility to vancomycin and azithromycin, resulting in reductions in MICs of
118 between 8- and 16-fold for all strains except Ab-84, which did not demonstrate
119 increased susceptibility to azithromycin in the presence of PF-5081090 (Table 2). A
120 mechanistic explanation for the lack of increased susceptibility to azithromycin
121 observed with strain Ab-84 is, at present, not clear. A more moderate effect was
122 observed with amikacin and imipenem, with all strains showing 2- to 8-fold increased
123 susceptibility. No changes in MIC values were observed with ciprofloxacin and
124 tigecycline. Interestingly, all strains were more resistant to colistin in the presence of
125 PF-5081090, presumably due to reduce levels of LPS, the major bacterial target for
126 colistin. Importantly, the MIC of PF-5081090 alone was 256 mg/L for all strains tested,
127 which is in agreement with results from previous studies describing high MIC values for
128 *A. baumannii* with LpxC inhibitors (3, 6, 7).
129 To further characterize the ability of PF-5081090 to increase susceptibility to
130 rifampicin, vancomycin and azithromycin, time-kill assays were carried out using the
131 ATCC 19606 strain. Overnight cultures of the ATCC 19606 strain were adjusted to a
132 concentration of $5 \times 10^5$ cfu/mL in Mueller-Hinton broth with the indicated
133 concentrations of rifampicin, vancomycin or azithromycin both in the presence or
134 absence of 32 mg/L of PF-5081090. Growth at 37 ºC was monitored by quantitative
135 plating of aliquots taken at 0, 2h, 4h, 8h, 12h, 24h and 48h. The assay was performed in
136 triplicate with independent cultures.As can be seen in Figure 1C, ATCC 19606 cultured

137 in the presence of subinhibitory concentrations of rifampicin (0.03 mg/L), vancomycin
138 (32 mg/L), and azithromycing (0.125 mg/L) grew similarly to untreated cultures.
139 However, when PF-5091080 (32 mg/L) was included in the cultures together with these
140 antibiotics at the same concentrations, significant bacterial killing was observed.
141 Notably, in the case of azithromycin, regrowth was observed after the initial bactericidal
142 effect (Figure 1C). The presence of 32 mg/L of PF-5081090 alone did not affect the
143 growth of ATCC 19606 (data not shown).

144 The results presented here indicate that, although LpxC inhibition alone does not
145 result in high level in vitro antibacterial activity in *A. baumannii*, it can increase
146 susceptibility to certain antibiotics. Given a recent study demonstrating that treatment
147 with an LpxC inhibitor alone can afford protection against *A. baumannii* infection (7),
148 characterizing the effect LpxC inhibition in combination with other antibiotics in
149 experimental models of infection may be of interest. The increased susceptibility to
150 rifampicin that is observed upon inhibition of LpxC was especially dramatic, as MIC
151 values for all strains were at the lower limit of concentrations used in the study (≤ 0.03
152 mg/L). The results obtained with vancomycin are also of potential interest since this
153 antibiotic is traditionally used for the treatment of Gram positive bacterial infections
154 due to the intrinsic resistance to vancomycin seen with most Gram negative species.
155 These results are in line with previous findings describing synergism between
156 vancomycin and colistin, an antibiotic that also produces bacterial membrane damage,
157 in *A. baumannii* (17). The increased cell permeability observed upon inhibition of
158 LpxC (Figure 1B) make it tempting to speculate that the increased antibiotic
159 susceptibility is, at least in part, due to the increased permeability of the bacterial
160 membrane. The results presented here are in agreement with our recently published
161 findings demonstrating that strains deficient in LPS biosynthesis due to mutations in

162 *lpxA*, *lpxC* and *lpxD* have increased susceptibility to certain antibiotics (10). Taken
163 together, these studies indicate that LPS loss, whether it is due to mutation or
164 pharmacologic inhibition, can increase antimicrobial susceptibility to certain antibiotics
165 in *A. baumannii*.
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257 **Table 1.** Fractional inhibitory concentration indices for azithromycin, rifampicin and

258 vancomycin in combination with the LpxC inhibitor PF-5081090.

| Strain | FICI AZM + In | FICI RIF + In | FICI VAN +In |
|---|---|---|---|
| **ATCC 19606** | 0.25 (1/8 MIC AZM + 1/8 MIC PF) | 0.15 (1/8 MIC AZM + 1/32 MIC PF) | 0.18 (1/8 MIC AZM + 1/16 MIC PF) |
| **Ab-84** | 0.37 (1/8 MIC AZM + 1/4 MIC PF) | 0.15 (1/8 MIC AZM + 1/32 MIC PF) | 0.25 (1/8 MIC AZM + 1/8 MIC PF) |
| **Ab-108** | 0.25 (1/8 MIC AZM + 1/8 MIC PF) | 0.14 (1/8 MIC AZM + 1/64 MIC PF) | 0.25 (1/8 MIC AZM + 1/8 MIC PF) |
| **Ab-167** | 0.25 (1/8 MIC AZM + 1/8 MIC PF) | 0.15 (1/8 MIC AZM + 1/32 MIC PF) | 0.25 (1/8 MIC AZM + 1/8 MIC PF) |
| **Ab-176** | 0.25 (1/8 MIC AZM + 1/8 MIC PF) | 0.14(1/8 MIC AZM + 1/64 MIC PF) | 0.25(1/8 MIC AZM + 1/8 MIC PF) |
| **Ab-7** | 0.18 (1/8 MIC AZM + 1/16 MIC PF) | 0.18 (1/8 MIC AZM + 1/16 MIC PF) | 0.18 (1/8 MIC AZM + 1/16 MIC PF) |
| **Ab-31** | 0.18 (1/8 MIC AZM + 1/16 MIC PF) | 0.15 (1/8 MIC AZM + 1/32 MIC PF) | 0.18 (1/8 MIC AZM + 1/16 MIC PF) |

259 FICI: Fractional Inhibitory Concentration Index; AZM: Azithromycin; RMP:
260 Rifampicin; VAN: Vancomycin; PF:PF-5081090; In: PF-5081090

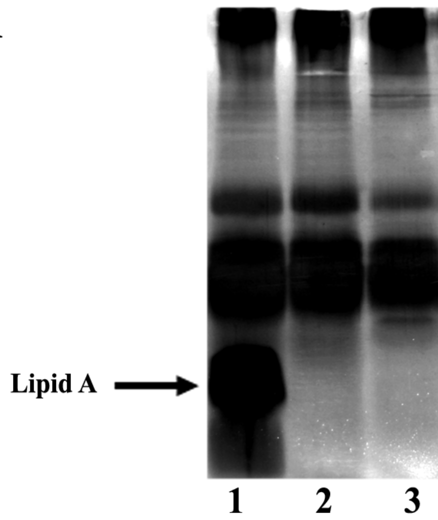
| Strain | EU Fold change | Minimum inhibitory concentration (mg/L) in the absence (-In) and presence (+In) of 32 mg/L of PF-5081090 (In) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AMK | | AZM | | CIP | | CST | | IPM | | RIF | | TGC | | VAN | | |
| | | -In | +In | -In | +In | -In | +In | -In | +In | -In | +In | -In | +In | -In | +In | -In | +In | In |
| ATCC 19606 | 8.4 | 2 | 1 | 0.5 | 0.125 | 0.125 | 0.125 | 0.125 | 2 | 1 | 0.25 | 2 | ≤0.03 | 0.06 | 0.125 | 128 | 8 | 256 |
| Ab-84 | 7.4 | 8 | 2 | 64 | 64 | 128 | 128 | 0.06 | 0.25 | 128 | 64 | 2 | ≤0.03 | 0.5 | 0.5 | 128 | 16 | 256 |
| Ab-108 | 5.5 | 256 | 64 | 16 | 2 | 32 | 32 | 0.06 | 0.5 | 256 | 64 | 2 | ≤0.03 | 0.5 | 0.5 | 128 | 16 | 256 |
| Ab-167 | 6.5 | 256 | 64 | 16 | 2 | 64 | 64 | 0.125 | 2 | 256 | 64 | 4 | ≤0.03 | 1 | 0.5 | 128 | 16 | 256 |
| Ab-176 | 3.5 | 256 | 64 | 16 | 2 | 64 | 64 | 0.25 | 0.5 | 256 | 64 | 4 | ≤0.03 | 0.5 | 0.5 | 128 | 16 | 256 |
| Ab-7 | ND | 16 | 8 | 64 | 4 | 64 | 64 | 0.125 | 1 | 16 | 4 | 32 | ≤0.03 | 0.5 | 0.5 | 256 | 32 | 256 |
| Ab-31 | ND | 64 | 8 | 16 | 1 | 32 | 32 | 0.125 | 2 | 32 | 4 | 32 | ≤0.03 | 0.25 | 0.25 | 256 | 16 | 256 |

261
262 **Table 2. Minimum Inhibitory Concentrations of *A. baumannii* strains in the absence and presence of PF-5081090.** MDR: multidrug resistant. EU:
263 Endotoxic Units. AMK: amikacin; AZM: azithromycin; CIP: ciprofloxacin; CST: colistin; IPM: imipenem; RIF: rifampicin; TGC: tigecycline; VAN:
264 vancomycin; In: PF-5081090; ND: Not Determined.
265

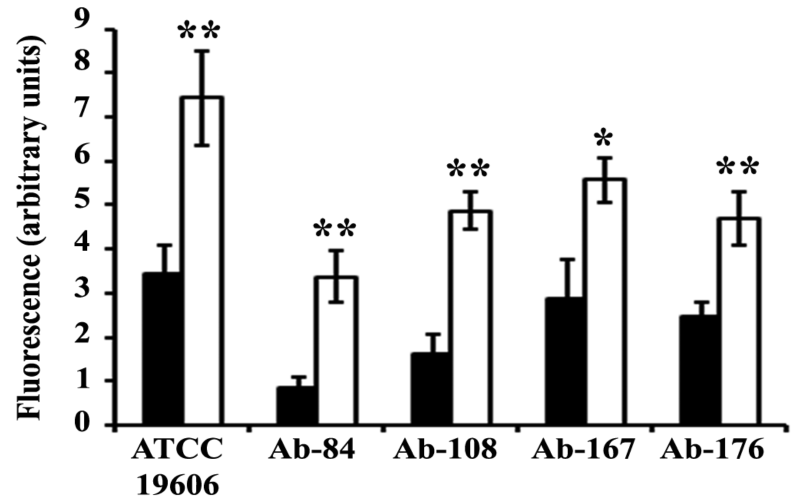
## Figure Legend

(A) Lipid A detection after silver staining. Lane 1: ATCC 19606; Lane 2: ATCC 19606 treated with 32 mg/L of PF-5081090; Lane 3: IB010. (B) Cell permeability of *A. baumannii* strains treated (white bars) with 32 mg/L of PF-5081090 or left untreated (black bars) determined by measuring accumulation of ethidium bromide into the cell. This experiment was performed by triplicate. *p=0.01; **p≤0.006; Student´s t-test. (C) Time-kill curves of combinations with PF-5081090 in ATCC 19606. Growth of ATCC 19606 was measured in MHB (circles), in the presence (squares) of the antibiotics rifampicin (0.03 mg/L), vancomycin (32 mg/L) or azithromycin (0.125 mg/L) and in the presence of the antibiotic and 32 mg/L of PF-5081090 (triangles). The assay was performed in triplicate with independent cultures.

A




B

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