Molecular Validation of LpxC as an Antibacterial Drug Target in Pseudomonas aeruginosa

Adam Barb, Duke University Medical Center
Khisimuzi E. Mdluli
Pamela R. Witte
Toni Kline
Alice L. Erwin, et al.

Available at: https://works.bepress.com/adam_barb/3/
Molecular Validation of LpxC as an Antibacterial Drug Target in Pseudomonas aeruginosa


Departments of Research Biology and Chemistry, Chiron Corporation, 201 Elliott Avenue West, Suite 150, Seattle, Washington 98119, and Departments of Biochemistry and Chemistry, Duke University Medical Center, Durham, North Carolina 27710

Received 2 February 2006/Returned for modification 2 March 2006/Accepted 24 March 2006

LpxC [UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc deacetylase] is a metalloamidase that catalyzes the first committed step in the biosynthesis of the lipid A component of lipopolysaccharide. A previous study (H. R. Onishi, B. A. Pelak, L. S. Gercken, L. L. Silver, F. M. Kahan, M. H. Chen, A. A. Patchett, S. M. Galloway, S. A. Hyland, M. S. Anderson, and C. R. H. Raetz, Science 274:980–982, 1996) identified a series of synthetic LpxC-inhibitory molecules that were bactericidal for Escherichia coli. These molecules did not inhibit the growth of Pseudomonas aeruginosa and were therefore not developed further as antibacterial drugs. The inactivity of the LpxC inhibitors for P. aeruginosa raised the possibility that LpxC activity might not be essential for all gram-negative bacteria. By placing the lpxC gene of P. aeruginosa under tight control of an arabinose-inducible promoter, we demonstrated the essentiality of LpxC activity for P. aeruginosa. It was found that compound L-161,240, the most potent inhibitor from the previous study, was active against a P. aeruginosa construct in which the endogenous lpxC gene was inactivated and in which LpxC activity was supplied by the lpxC gene from E. coli. Conversely, an E. coli construct in which growth was dependent on the P. aeruginosa lpxC gene was resistant to the compound. The differential activities of L-161,240 against the two bacterial species are thus the result primarily of greater potency toward the E. coli enzyme rather than of differences in the intrinsic resistance of the bacteria toward antibacterial compounds due to permeability or efflux. These data validate P. aeruginosa LpxC as a target for novel antibiotic drugs and should help direct the design of inhibitors against clinically important gram-negative bacteria.

Lipopolysaccharide has a critical function in gram-negative bacterial membrane integrity and resistance to host defenses, and therefore, the conserved lipopolysaccharide biosynthetic enzymes are attractive targets for novel antibacterial drugs. A drug targeting enzymes of this biosynthetic pathway would need to be active against Pseudomonas aeruginosa and other nonfermenting gram-negative bacterial species, as well as against Escherichia coli and other enteric bacteria, to be clinically useful. The P. aeruginosa outer membrane is less permeable to small molecules than that of E. coli, and P. aeruginosa has several multidrug efflux pumps. As a result of both of these factors, P. aeruginosa is less susceptible than E. coli to many antibiotics (24). Several laboratories have focused on the metalloenzyme LpxC [UDP-(3-O-acyl)-N-acetylglucosamine deacetylase], since it catalyzes the first committed step in lipid A synthesis (Fig. 1) and has been demonstrated to be essential for the growth of E. coli (3, 12, 38). P. aeruginosa LpxC is similar in sequence (Fig. 2) and catalyzes the same activity (11). While the essentiality of LpxC activity for P. aeruginosa has not been formally proven, the lpxC gene was not inactivated in a saturating transposon mutagenesis study (15). These data suggest that it might be possible to discover LpxC inhibitors active against both E. coli and P. aeruginosa. However, none of the early LpxC inhibitors, some of which showed antibacterial activity against E. coli and certain other organisms, were able to inhibit growth of P. aeruginosa (5, 12, 27–29). It was tempting to assume that the reason for this failure was the intrinsic resistance of P. aeruginosa to antibiotics. Challenging this assumption, we undertook the studies described here to evaluate the basis for the refractory nature of P. aeruginosa to LpxC inhibitors that are effective against E. coli. We focused on the compound L-161,240 (Fig. 1), the most active of the LpxC inhibitors reported by researchers at Merck (4, 27). We found that the critical reason for the inactivity of this compound against P. aeruginosa was its failure to inhibit enzyme activ-
ity. These findings have implications for designing effective strategies to identify LpxC inhibitors that can be developed as novel antibacterial drugs.

MATERIALS AND METHODS

Reagents and bacterial cultivation. The bacterial strains and plasmids used are listed in Table 1. P. aeruginosa strains were grown at 37°C in Luria-Bertani (LB) broth (Difco) or plated on sheep blood agar (Remel). E. coli was grown in LB broth or on LB agar. EDTA, bis-Tris buffer, sucrose, arabinoase, and dimethyl sulfoxide (DMSO) were purchased from Sigma as ultrapure agents. Yeast extract and tryptone were obtained from Difco. Restriction enzymes, T4 DNA ligase, and their reaction buffers were obtained from New England Biolabs. Polymyxin B nonapeptide, tetracycline, ampicillin, carbenicillin, gentamicin, and kanamycin were all purchased from Sigma. Compound L-161,240 was synthesized as described previously (4). Antibacterial compounds were dissolved in DMSO to make stock solutions of polymyxin B nonapeptide at 3 mg/ml, L-161,240 at 10 mg/ml, and tetracycline at 125 mg/ml. For growth curves, DMSO was added to control tubes as needed so that DMSO concentrations were the same in all cultures within each experiment.

Enzyme inhibition assays. LpxC activity was measured as previously described (13, 20), using either crude cell extracts (36) of E. coli W3110 or P. aeruginosa PAO1 or purified enzyme from E. coli BL21/DE3/pLysS/pJEJ1 (14) or P. aerugi-

nosa PAO1 (16) as the enzyme source. Assays were done in 25 mM phosphate buffer at pH 7.4 with 5 mM substrate at 30°C, with enzyme concentrations (typically 0.5 to 10 nM) adjusted to keep the conversion below 10% over the time course of the assays.

DNA manipulations. Standard recombinant DNA procedures were used (30). The primers for amplification of the coding region of the lpxC genes included Ndel and EcoRI restriction sites for subsequent cloning. For the E. coli gene, the primers were 5'-GGGAATTCATAATGCTTCACAAACAAAGGACACTTAAAC GT-3' and 5'-CCGGGAATTCATTTAGTCGTCAGCTGAAAGGCGCT-3', and for the P. aeruginosa gene, they were 5'-GGGAATTCATAATGCTTCACAAACAAAGGACACTTAAAC GT-3' and 5'-CCGGGAATTCATTTAGTCGTCAGCTGAAAGGCGCT-3', and for the P. aeruginosa gene, they were 5'-GGGAATTCATAATGCTTCACAAACAAAGGACACTTAAAC GT-3' and 5'-CCGGGAATTCATTTAGTCGTCAGCTGAAAGGCGCT-3'. These primers were used in a PCR mixture containing the template either 10 to 50 µg P. aeruginosa genomic DNA or 1 µg plasmid pKD6 containing the E. coli lpxC gene (34). The lpxC genes were amplified using Pwo DNA polymerase (Roche) in a 100-µl reaction mixture containing a 200 µM concentration of each deoxynucleoside triphosphate and a 0.5 µM concentration of each primer for 30 cycles (94°C denaturation, 55°C annealing, and 72°C polymerization). The PCR products were purified with the QiAquick PCR purification kit from QIAGEN and digested with NdeI and EcoRI restriction enzymes. The bands of the sizes predicted for the lpxC genes were identified following gel electrophoresis and excised from the gel. The excised DNA was purified using the QiAquick gel extraction kit from QIAGEN. The purified DNA was cut with NdeI and EcoRI and ligated into the T vector expression vector (36) pET21b (Novagen) that had been cut in the multiple cloning site with NdeI and EcoRI. The ligation mixture was transformed into DH5α, which was plated on LB agar containing ampicillin (250 µg/ml). The inserts in the resulting clones were sequenced before being subcloned. The E. coli and P. aeruginosa inserts were subcloned into pDN19 to produce plasmids pEC-lpxC1 and pPA-lpxC1, respectively, for low-copy-number complementation of E. coli and P. aeruginosa. The inserts in the resulting clones were sequenced before being subcloned. The E. coli and P. aeruginosa inserts were subcloned into pDN19 to produce plasmids pEC-lpxC2 and pPA-lpxC2, respectively, for complementation of the P. aeruginosa promoter replacement mutation.

Construction of a P. aeruginosa strain with lpxC expression tightly controlled by the araBAD promoter. Promoter replacement was carried out using a homologous recombination strategy, whereby recombination of pBEM10 with P. aeruginosa removed the native lpxC promoter and placed the tightly regulated araBAD promoter upstream of lpxC on the chromosome (Fig. 3). In preliminary experiments (data not shown) in which this promoter controlled expression of the luciferase gene lux (9), it was found that in P. aeruginosa, there was a low background level of expression in the absence of arabinoase and that this expression was not eliminated by the addition of glucose as it is in E. coli (22). Background levels of promoter expression were successfully reduced by altering the sequence of the ribosome binding site from its original sequence of AGGAQG to TTCTCT. Plasmid pPW101 was constructed by ligating the RP4 origin of transfer oriT into pSP72. oriT was amplified from plasmid pEX100T (33) with an introduction of NdeI and AatII restriction sites. To create pBEM10, the following DNA pieces were amplified and sequentially ligated into pPW101: the tetracycline resistance marker from plasmid pUCP26 (26), the araBAD promoter (8) from the plasmid pBAD HisB (Invitrogen), with an altered ribosome binding site, the...
an optical density at 600 nm (OD600) of 0.1 in 5 ml of LB. The inhibitor L-161,240 designated PAO1-PBAD-lpxC, was dependent on arabinose. and grown in LB at 37°C, with shaking for approximately 4 h, and then each just upstream of the chromosomal copy of lpxC/H11032, and the reverse primer introduced an HindIII site (5'-GAATCTCAAGGTTGTTTGCAGGA-3'). A primer set was used to alter the ribosome binding site and introduce an upstream BssHII site (5'-GCGCGCGGAAGCTTATTG-3'). The forward primer introduced an XhoI site (5'-CTCGAGGCATGCATAATGTGCCTGT-3'), and the reverse primer introduced a HindIII site (5'-AAAGCTTTCTCTCTGGTTAGCCCAAAAAACGGG-3').

FIG. 2. Comparison of predicted LpxC sequences from E. coli and P. aeruginosa. An 82% similarity and 57% identity is shared between the two sequences over the entire length of the protein. Key residues are indicated by color and also by symbols above the residues, as follows. The novel zinc binding motif characteristic of all known LpxC enzymes, HKXXD, is shown in red (stars). Four other conserved histidines are shown in blue (■), and two E. coli histidines that are absent from P. aeruginosa are shown in cyan (○). The conserved phenylalanines providing a hydrophobic patch (*) are shown in orange (▲). Analogous to A. aeolicus, in E. coli histidine 265 may stabilize the oxyanion intermediate of the transition state. Significant differences in enzyme fold or participating residues are suggested by the differences in key residues, for example, the two nonconserved histidines in E. coli.

araC repressor gene (17, 31), also from pBAD HisB, and the first 340 base pairs of P. aeruginosa lpxC were amplified from PAO1 genomic DNA. The tetracycline resistance marker was amplified using a forward primer that introduced a BglII site (5'-GAATCTCAAGGTTGTTTGCAGGA-3') and a reverse primer that introduced an EcoRI site (5'-GAATCTCAATTCTCTAGTTGACA-3'). The araBAD promoter and araC gene were amplified as one piece from the pBAD HisB vector. The forward primer introduced an XhoI site (5'-CTCGAGGCATGCATAATGTG CCTGT-3'), and the reverse primer introduced a HindIII site (5'-AAAGCTTTCTCTCTGGTTAGCCCAAAAAACGGG-3').

RESULTS

Compound L-161,240, the most potent of the LpxC inhibitors described by Onishi et al., is active against E. coli, with an MIC of 1 µg/ml, but has no activity against P. aeruginosa (MIC > 50 µg/ml) (27). We found that this compound was inactive against wild-type strains of P. aeruginosa (PAO1 and ATCC 27853), strain PAO200, in which genes mexAB and oprM, encoding the major multidrug efflux pump, are deleted (32), and the hypersusceptible strain Z61 (ATCC 35151) (2). Treatment of PAO1 and PAO200 with polymyxin B nonapeptide (3 µg/ml) to increase the permeability of the membrane (23) failed to render these strains susceptible to L-161,240 (data not shown). These observations suggested that the failure of L-161,240 to reach its target within P. aeruginosa cells might not be the primary reason for its failure to inhibit growth of P. aeruginosa.

L-161,240 was 38 times more potent toward E. coli than toward P. aeruginosa in an in vitro assay of the LpxC activity in crude bacterial extracts (Table 2). For E. coli, the inhibitor was
equally as active toward purified LpxC as it was toward the LpxC in a bacterial extract. However, for *P. aeruginosa*, purification of LpxC increased its susceptibility to L-161,240.

These observations indicate that *P. aeruginosa* LpxC is more resistant to L-161,240 than the LpxC from *E. coli* and that reducing the effect of intrinsic resistance mechanisms does not render *P. aeruginosa* susceptible to growth inhibition by L-161,240. These data do not allow us to determine the relative contributions of these two aspects of *P. aeruginosa*’s resistance to the compound. In order to assess the effect of L-161,240 on *P. aeruginosa* LpxC in a bacterial cell that is known to allow entry of the compound, we made use of an *E. coli* construct in which growth at 42°C was dependent on the presence of a functional LpxC. In such a system, the LpxC gene from *E. coli* or *P. aeruginosa* could be expressed in the bacterial cell that is known to allow entry of the compound. We transformed *E. coli* with a plasmid containing either an inducible promoter (Fig. 3). Modification of the *E. coli* araBAD promoter to render it tightly regulated in *P. aeruginosa* is described in Materials and Methods. The resulting *P. aeruginosa* mutant, designated PAO1-araBAD-lpxC, was fully capable of growth at the presence of arabinose but did not grow at all in the absence of this inducer. This confirmed that *P. aeruginosa* is similar to *E. coli* in that it contains only one functional copy of lpxC and in that its activity is essential for growth. PAO1-araBAD-lpxC was transformed with a plasmid containing either *P. aeruginosa* lpxC on the *E. coli* chromosome or the lpxC gene cloned into pUCP30T. The transformants were then incubated in various concentrations of the LpxC inhibitor. In the absence of arabinose, *P. aeruginosa* strains expressing only *E. coli* lpxC were nearly as resistant as was the parent *P. aeruginosa* strain PAO1 (Fig. 5).

When the experiment was carried out in the presence of 0.2% arabinose to induce expression of the chromosomal lpxC gene, neither transformant was susceptible to L-161,240 (Fig. 5). This confirmed that the relative insensitivity of the *P. aeruginosa* enzyme to the inhibitor is sufficient to confer resistance to growth inhibition.

We constructed a mutant of *P. aeruginosa* in which the native promoter of lpxC was replaced by an inducible promoter (Fig. 3). Modification of the *E. coli* araBAD promoter to render it tightly regulated in *P. aeruginosa* is described in Materials and Methods. The resulting *P. aeruginosa* mutant, designated PAO1-ARA-lpxC, was fully capable of growth at the presence of arabinose but did not grow at all in the absence of this inducer. This confirmed that *P. aeruginosa* is similar to *E. coli* in that it contains only one functional copy of lpxC and that its activity is essential for growth. PAO1-ARA-lpxC was transformed with a plasmid containing either *P. aeruginosa* lpxC or *E. coli* lpxC (pPA-lpxC2 or pEC-lpxC2, respectively). The transformants were then incubated in various concentrations of the LpxC inhibitor. In the absence of arabinose, *P. aeruginosa* strains expressing only *E. coli* lpxC were nearly as susceptible to L-161,240 as was the parent *P. aeruginosa* strain PAO1 (Fig. 5). When the experiment was carried out in the presence of 0.2% arabinose to induce expression of the chromosomal lpxC gene, neither transformant was susceptible to L-161,240 (Fig. 5). This confirmed that the *P. aeruginosa* lpxC enzyme itself is the primary factor in the resistance of *P. aeruginosa* to L-161,240. The intrinsic resistance mechanisms of *P. aeruginosa* are more resistant to the LpxC inhibitor at this temperature (Fig. 4). This indicated that the relative insensitivity of the *P. aeruginosa* enzyme to the inhibitor is sufficient to confer resistance to growth inhibition.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> strains</td>
<td>Wild type; sequenced strain</td>
<td>10, 35</td>
</tr>
<tr>
<td>PAO1</td>
<td>meAB-oprM</td>
<td>32</td>
</tr>
<tr>
<td>PA0200</td>
<td>Chemically mutagenized strain hypersusceptible to antibiotics</td>
<td>18, 39</td>
</tr>
<tr>
<td>ATCC 35151 (Z61)</td>
<td>lpxC promoter replaced by araBAD promoter</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1-PLBAD-lpxC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>K-12 derivative</td>
<td>E. coli Genetic Stock Center</td>
</tr>
<tr>
<td>DH5α</td>
<td>Cloning host</td>
<td>Gibco/Life Technologies</td>
</tr>
<tr>
<td>BL221/DE3/pLysS/pJEJ1</td>
<td>T7 RNA polymerase-driven expression system for <em>E. coli</em> lpxC</td>
<td>13</td>
</tr>
<tr>
<td>JBK-1/pKD6</td>
<td>Chromosomal lpxC disrupted with Km cassette; lpxC on temp-sensitive plasmid; Amp</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET21b</td>
<td>Expression plasmid; Amp</td>
<td>Novagen</td>
</tr>
<tr>
<td>pDN19</td>
<td>Low-copy-number plasmid; Tet</td>
<td>25</td>
</tr>
<tr>
<td>pUCP26</td>
<td>Tet</td>
<td>26</td>
</tr>
<tr>
<td>pUCP30T</td>
<td>Replicates in both <em>E. coli</em> and <em>P. aeruginosa</em>; Gm</td>
<td>33</td>
</tr>
<tr>
<td>pSP72</td>
<td>Amp</td>
<td>Promega</td>
</tr>
<tr>
<td>pBAD/His B</td>
<td>araC ; P. aeruginosa araBAD</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEC-lpxC1</td>
<td>lpxC from <em>E. coli</em> cloned into pDN19</td>
<td>This work</td>
</tr>
<tr>
<td>pPA-lpxC1</td>
<td>lpxC from <em>P. aeruginosa</em> cloned into pDN19</td>
<td>This work</td>
</tr>
<tr>
<td>pEC-lpxC2</td>
<td>lpxC from <em>E. coli</em> cloned into pUCP30T</td>
<td>This work</td>
</tr>
<tr>
<td>pPA-lpxC2</td>
<td>lpxC from <em>P. aeruginosa</em> cloned into pUCP30T</td>
<td>This work</td>
</tr>
<tr>
<td>pPW10l</td>
<td>Derived from pSP72; oriT</td>
<td>This work</td>
</tr>
<tr>
<td>pBEM10</td>
<td>Derived from pPW10l; recombination with <em>P. aeruginosa</em> chromosome</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Antibiotic resistance markers: Amp, ampicillin; Gm, gentamicin; Km, kanamycin; Tet, tetracycline.
DISCUSSION

Our data indicate that the LpxC of *P. aeruginosa* is refractory to inhibition by compound L-161,240 at concentrations 10-fold higher than those that completely inhibit the LpxC of *E. coli*. This was demonstrated in both in vitro assays of enzyme activity and bacterial growth experiments using a *P. aeruginosa* construct in which the only active lpxC gene was from *E. coli* and a complementary construct in which growth of *E. coli* depended on the lpxC gene from *P. aeruginosa*. Thus, it is clearly the difference in enzymes, not differences between species in membrane structure or specificity of efflux pumps, that is the primary mediator of the differential susceptibilities of the two bacterial species to this inhibitor.

There remains, however, some cytoplasmic process in *P. aeruginosa* that further reduces the activity of L-161,240 (Table 2). Our experiments reveal the existence of, but do not characterize, this process. The inhibitor could be sequestered or inactivated within *P. aeruginosa*. Alternatively, *P. aeruginosa* LpxC could itself be complexed with other molecules in such a way as to limit access of the inhibitor to the enzyme. Nonetheless, the different potencies of L-161,240 against the two enzymes appear to be the single factor that contributes most to the lack of activity of the inhibitor against *P. aeruginosa*.

Our current knowledge of the structure and biochemistry of

TABLE 2. L-161,240 inhibition of the LpxC enzyme from either purified or crude sources

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>IC₅₀ (nM) of L-161,240 against:</th>
<th>IC₅₀ ratio (P. aeruginosa/E. coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli DH5α</td>
<td><em>P. aeruginosa</em> PAO1</td>
</tr>
<tr>
<td>Crude extracts</td>
<td>0.037 ± 0.002</td>
<td>1.40 ± 0.07</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>0.023 ± 0.003</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

* IC₅₀, 50% inhibitory concentration. Data are means ± standard deviations from three replicate assays.
the LpxC enzymes is limited to very few species. The relatively high degree of primary sequence similarity between the E. coli and P. aeruginosa enzymes (Fig. 2) demonstrates the inadequacy of primary sequences for accurate prediction of functional similarity between proteins. Subtle differences, such as the two nonconserved histidines in the E. coli sequence, may have significant consequences in the overall fold and binding properties. The unexpected differences between the two species’ LpxC enzymes highlight the importance of protein structure in drug design. Nuclear magnetic resonance (6) and X-ray (37) structures of LpxC from Aquifex aeolicus have been determined, and the zinc sites of the A. aeolicus and P. aeruginosa enzymes have been studied using extended X-ray absorption fine-structure spectroscopy (21). Structural studies of LpxC from species in addition to A. aeolicus may provide the structural rationale for the differential susceptibilities of these species to a given inhibitor and thereby facilitate the design of inhibitors with much broader specificity than has previously been possible.

Our data suggest that strategies to identify broad-spectrum LpxC inhibitors could be challenging and must take into account the structural differences in LpxC enzymes from different gram-negative bacterial species. The finding that nonenzyme components of cell extracts affect the activity of inhibitors within the bacterial cell adds an additional level of complexity. Target-based antibiotic discovery will be most successful when it is possible to evaluate separately each of the factors that contribute to bacterial growth inhibition: penetration of the cell wall, resistance to efflux, inactivation or sequestration by intracytoplasmic components, and potency toward the target. The use of multiple molecular and biochemical approaches allowed us to evaluate each of these for the LpxC inhibitor L-161,240 and to show that in this case, potency toward the target was the primary factor limiting activity for P. aeruginosa. This conclusion was critical to the discovery of potent small-molecule LpxC inhibitors with antibacterial activity toward P. aeruginosa and other gram-negative pathogens, the details of which have been presented elsewhere (1, 16, 19).

**ACKNOWLEDGMENTS**

This work was supported by PathoGenesis Corporation, Seattle, WA (later merged with Chiron Corporation). C.R.H.R. was supported by NIH grant GM-51310.

**REFERENCES**