Antibacterial Agents That Target Lipid A Biosynthesis in Gram-negative Bacteria

INHIBITION OF DIVERSE UDP-3-O-(R-3-HYDROXYMYRISTOYL)-N-ACETYLGLUCOSAMINE DEACETYLASES BY SUBSTRATE ANALOGS CONTAINING ZINC BINDING MOTIFS*

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UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC) catalyzes the second step in the biosynthesis of lipid A, a unique amphiphilic molecule found in the outer membranes of virtually all Gram-negative bacteria. Since lipid A biosynthesis is required for bacterial growth, inhibitors of LpxC have potential utility as antibiotics. The enzymes of lipid A biosynthesis, including LpxC, are encoded by single copy genes in all sequenced Gram-negative genomes. We have now cloned, overexpressed, and purified LpxC from the hyperthermophile Aquifex aeolicus. This heat-stable LpxC variant (the most divergent of all known LpxCs) displays 32% identity and 51% similarity over 277 amino acid residues out of the 305 in Escherichia coli LpxC. Although A. aeolicus LpxC deacylates the substrate UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine at a rate comparable with E. coli LpxC, a phenyloxazoline-based hydroxamate that inhibits E. coli LpxC with \( K_{i} \) of ~50 nM (Onishi, H. R., Pelak, B. A., Gerckens, L. S., Silver, L. L., Kahan, F. M., Chen, M. H., Patchett, A. A., Galloway, S. M., Hyland, S. A., Anderson, M. S., and Raetz, C. R. H. (1996) Science 274, 980–982) does not inhibit A. aeolicus LpxC. To determine whether or not broad-spectrum deacetylase inhibitors can be found, we have designed a new class of hydroxamate-containing inhibitors of LpxC, starting with the structure of the physiological substrate. Several of these compounds inhibit both E. coli and A. aeolicus LpxC at similar concentrations. We have also identified a phosphate-containing substrate analog that inhibits both E. coli and A. aeolicus LpxC, suggesting that the LpxC reaction proceeds by a mechanism similar to that described for other zinc metalloamidases, like carboxypeptidase A and thermolysin. The differences between the phenyloxazoline and the substrate-based LpxC inhibitors might be exploited for developing novel antibiotics targeted either against some or all Gram-negative strains. We suggest that LpxC inhibitors with antibacterial activity be termed “deacytins.”

Lipid A from Escherichia coli and Salmonella typhimurium consists of a \( \beta,1'6 \)-linked disaccharide of glucosamine that is phosphorylated at the 1- and 4'-positions and is derivatized with up to seven fatty acyl chains (1–5). All of the enzymes that synthesize lipid A and the genes encoding them have been identified in E. coli (3, 6). Single copy genes encoding homologues of the E. coli lipid A enzymes are also found in most other sequenced Gram-negative organisms. The biosynthesis of lipid A is essential for E. coli growth (7–11), possibly because it is required for outer membrane protein folding (12), and lipid A is unique to Gram-negative outer membranes. The enzymes of lipid A biosynthesis are therefore ideal targets for the development of new antibiotics (10). With the recent re-emergence of antibiotic-resistant bacteria (13, 14), there is an urgent need for drugs that act on targets different from those inhibited by the available antibiotics.

The second and committed enzyme of lipid A biosynthesis in E. coli (LpxC)\(^1\) catalyzes the N-deacytlation of UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine, yielding a free amino group that is immediately acylated with another R-3-hydroxy-myristoyl chain by LpxD (Scheme 1) (8, 15, 16). The LpxC deacetylase has been purified and characterized from both E. coli and Pseudomonas aeruginosa (17, 18). The two proteins display 57% identity and 79% similarity over their entire length (19). E. coli LpxC contains a bound zinc ion that is required for catalytic activity (20). Chiral phenyloxazoline-based hydroxamates (Fig. 1), which are presumed to coordinate the active site metal of LpxC, have recently been described (10). Some of these compounds display significant antibacterial activity against E. coli cells, but they are not active against P. aeruginosa (10). P. aeruginosa LpxC is ~20 times more resistant to the phenyloxazoline-based inhibitors than is E. coli LpxC (see below). Despite the limitations in the spectrum of the available inhibitors, the zinc ion-dependent mechanism of LpxC is very attractive from the perspective of medicinal chemistry, since extraordinarily potent inhibitors of other zinc metalloamidases have been described, including the blood pressure-lowering drugs captoril and enalapril (21, 22). The question of whether deacetylase inhibitors could be designed that effectively inhibit LpxCs from all Gram-negative bacteria has not been explored.

The recent publication of the complete genome sequence of the hyperthermophilic Gram-negative bacterium Aquifex aeolicus (23) affords a unique opportunity to study a thermostable

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\(^1\) The abbreviations used are: LpxC, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase; BSA, bovine serum albumin; bis-tris, bis(2-hydroxyethyl)iminomethylhydroxymethylmethane; DTT, dithiothreitol; FPLC, fast protein liquid chromatography.
Kinetics and Inhibition of E. coli and A. aeolicus LpxC

LpxC variant that might be amenable to x-ray crystallography studies. A. aeolicus grows at 95 °C, making it one of the most thermophilic bacteria yet described (23). As an obligate chemolithoautotroph, it grows in an atmosphere of hydrogen, carbon dioxide, and oxygen, solely in the presence of inorganic salts (23, 24). From a comparison of 16S rRNA sequences, A. aeolicus is one of the earliest diverging eu-bacteria on the evolutionary tree (23). This observation is consistent with the fact that A. aeolicus LpxC is the least homologous to E. coli LpxC (32% identity and 51% similarity over 277 amino acid residues) of the ~30 LpxCs identified so far (19). Therefore, a comparison of A. aeolicus LpxC and E. coli LpxC might reveal conserved mechanistic features and facilitate the design of broad-spectrum LpxC inhibitors.

We now report the cloning, overexpression, and purification to homogeneity of LpxC from A. aeolicus. This hyperthermophilic enzyme efficiently deacetylates the physiological E. coli LpxC substrate UDP-3-O-(R-3-hydroxy-myristoyl)-N-acetyl-glucosamine at 30 °C. However, A. aeolicus LpxC is optimally active at much higher temperatures (70 °C) than is the E. coli enzyme (40 °C). Interestingly, the phenylloxazoline-based hydroxamates that are potent inhibitors (K_i ~ 50 nM) of the E. coli deacetylase (10) have no activity against A. aeolicus LpxC, even at concentrations as high as 1 mg/ml (3–5 mM). We have therefore designed a new class of LpxC inhibitors that incorporate part of the structure of the natural substrate. Some of these new inhibitors are equally active against A. aeolicus, E. coli, and P. aeruginosa LpxCs, with IC_{50} values in the low micromolar range. These inhibitors should serve as leads for the development of novel antibiotics with activity against the entire spectrum of Gram-negative organisms.

MATERIALS AND METHODS

Buffers and Reagents—[α-32P]UTP was purchased from NEN Life Science Products. Polyethyleneimine-cellulose TLC plates were obtained from E. Merck (Darmstadt, Germany). Bis-tris buffer (ultrapure reagent) and bovine serum albumin (BSA), essentially fatty acid-free, were purchased from Sigma. Restriction enzymes were purchased from New England Biolabs, and Pfu DNA polymerase was from Stratagene. Deacetylase inhibitor L-573,655 was obtained from Dr. A. Patchett (Merck) (10). The substrate analog LpxC inhibitors TU-514, TU-517, TU-521, TU-519, and 6HTT55 were synthesized in the Department of Chemistry at the University of Alberta with the support of Glycobiotics Inc. (Athens, GA) (39) and will be described in detail in a subsequent paper. The R,S and S isomers of L-161,240 (10) were synthesized in the Department of Chemistry at Duke University by minor modifications of published procedures (25).

Cloning of A. aeolicus lpxC—The A. aeolicus lpxC gene was identified by a BLASTp search (19) using the E. coli LpxC sequence against the complete genome sequence of A. aeolicus (23). Primers were designed to the N- and C-terminal regions of the A. aeolicus lpxC gene (5′-CTGAGCAGGATCCATTCTCAGAGAGACTTGGACGG-3′ and 5′-CGAGCAGGCTATCGGGATTGAAAAAGACGG-3′, respectively). These primers were used in a polymerase chain reaction containing genomic A. aeolicus DNA (50–100 μg) as the template, kindly provided by Dr. Karl O. Stetter (23). A. aeolicus lpxC was amplified using Pfu DNA polymerase (according to the manufacturer’s specifications) in a 100-μl reaction mixture containing a 0.2 μM concentration of each dNTP and 0.5 μM concentration of each primer for 25 polymerase chain reaction cycles (95 °C denaturation, 55 °C annealing, 72 °C polymerization). The polymerase chain reaction product was digested with BamHI and NdeI restriction enzymes at positions introduced by the primer sequences. A band of the correct size predicted for the digested lpxC gene was purified by gel electrophoresis and ligated into the T7 expression vector, pET21a (Novagen), that had been cut in the multiple cloning site with the same enzymes. The final DNA sequence of the cloned lpxC gene in the plasmid, pANLpxC, was confirmed to match the published sequence exactly (23), and this construct was used for the expression and purification of the A. aeolicus LpxC protein.

A matched expression plasmid containing the E. coli lpxC gene in the pET21a background was constructed by excising E. coli lpxC from the
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TABLE I

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<tr>
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</table>

*Not determined.

A. aeolicus LpxC activity in extracts of E. coli BL21(DE3)pLysS

Specific activity of extracts of E. coli containing the indicated plasmid were assayed for LpxC activity with 3 µM UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc in 40 mM bis-tris, pH 5.9, 30 °C or pH 5.5, 60 °C. Reactions also contained 0.5 mM AMP to inhibit CDP-diglyceride hydrolase (3) and 1 mg/ml BSA. Assays of the uninduced cell extracts, and the induced pET21a/BL21(DE3)/pLysS cell extract were performed using 0.2–0.4 mg/ml extract. Assays with the induced pEcLpxC and pAaLpxC extracts were performed using 0.01 µg/ml extract.

incubation. A diameter of 6 mm indicated that there was no visible inhibition of growth beyond the edges of the disc.

RESULTS

Cloning and Expression of Active A. aeolicus LpxC—Using the BLASTp protein search algorithm (19), we identified a distantly related homologue (32% identity and 51% similarity over 277 amino acids) to E. coli LpxC (17) in the genomic sequence of A. aeolicus. We cloned the A. aeolicus lpxC gene into the pET21a expression plasmid behind an inducible T7 RNA polymerase promoter to direct overexpression of A. aeolicus LpxC in E. coli following treatment with isopropyl-β-D-thiogalactopyranoside (26). A band of approximately the correct molecular mass, as expected for A. aeolicus LpxC (32,150 Da), was identified in induced cell-free extracts (data not shown). When assayed for LpxC activity at 30 °C using the E. coli LpxC substrate UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine, extracts made from cells containing the A. aeolicus LpxC plasmid, pAaLpxC, exhibited a 100-fold increase in LpxC activity upon induction (Table I), whereas extracts of cells containing the E. coli LpxC plasmid, pEcLpxC, exhibited a ~5000-fold stimulation under the same conditions. However, when the assay temperature was raised to 60 °C, the stimulation of A. aeolicus LpxC activity upon induction was much more pronounced (~10-fold). The observed increase in activity was dependent upon the presence of the LpxC-containing plasmid; the cell-free extracts from the pET21a vector-containing cells exhibited only background levels of LpxC activity due to endogenous E. coli deacetylase encoded by the chromosomal lpxC gene (Table I). The temperature dependence of LpxC activity in extracts made from induced E. coli cells containing either pAaLpxC or pEcLpxC demonstrates that A. aeolicus LpxC is active at much higher assay temperatures than is E. coli LpxC (Fig. 2). A. aeolicus LpxC activity measured in vitro reaches a maximum level at 70 °C, whereas the temperature optimum of the in vitro E. coli LpxC reaction is 40 °C.

Purification of A. aeolicus LpxC—To characterize the A. aeolicus LpxC enzyme and compare it to the well-studied E. coli enzyme, we purified A. aeolicus LpxC to near homogeneity using a combination of anion exchange and gel filtration chromatography. Like the E. coli enzyme, A. aeolicus LpxC adheres to anion exchange resins, such as DEAE-Sephrose and Q-Sepharose, and it can be eluted with buffers containing KC1. This property was exploited to attain a 5-fold purification (Table II). A. aeolicus LpxC was then further purified on an S-200 gel filtration column, yielding a final preparation with 6.9-fold higher specific activity than the initial cell lysate. The SDS-polyacrylamide gel electrophoresis analysis of the purified A. aeolicus LpxC (Fig. 3) demonstrates that the preparation is about 90–95% homogeneous. The concentration of purified A. aeolicus LpxC was determined using the BCA protein assay with BSA as the standard.

Steady-state Kinetic Parameters—A. aeolicus LpxC activity depends upon the concentration of the substrate, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine, in a manner that is well described by the Michaelis-Menten equation at both 30 and 60 °C. The kcat/Km values for both enzymes near their optimal assay temperatures are almost identical, and at ~10² M⁻¹ s⁻¹, they are well below the limit for a diffusion-controlled enzymatic reaction (~10⁹ M⁻¹ s⁻¹) (30). A. aeolicus LpxC also resembles E. coli LpxC in that 1 mg/ml BSA is required in the assay to maintain optimal catalytic activity at low enzyme concentrations (below 10 µM) (20). At 2–5 nM enzyme, assays in the absence of BSA showed no measurable LpxC activity (data not shown). The function of BSA in the assay at low enzyme concentrations is unknown (20).

Lack of Inhibition of A. aeolicus LpxC by Phenylloxazoline-based Hydrazonates—The phenylloxazoline-based hydrazonates L-573,655 and L-161,240 are known to inhibit E. coli LpxC with K values of 24 µM and approximately 50 nM, respectively (10) (Fig. 1). The racemic form of L-161,240 (designated LNTI-229) (21) was synthesized by a modification of the published procedures (10, 25). Inhibition of A. aeolicus and E. coli LpxC activity by 0.1 mg/ml L-573,655 or LNTI-229 was measured at 30 °C in assays containing purified enzyme (3.1 nM for A. aeolicus LpxC or 2 nM for E. coli LpxC) together with 3 µM UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc in 40 mM bis-tris buffer, pH 5.5. Under these conditions, L-573,655 and LNTI-229 both inhibit E. coli LpxC, with 7.0 and 0.2% of activity remaining, respectively, compared with an uninhibited control reaction. However, neither of these compounds inhibited purified A. aeolicus LpxC under these conditions. No inhibition of A. aeolicus LpxC activity could be observed even at concentrations of L-573,655 or LNTI-229 as high as 1 mg/ml in the assays (data not shown). Also, A. aeolicus LpxC was not inhibited by either of these compounds when assayed closer to its optimal temperature for activity at 60 °C. Based on these results,
Inhibition by Substrate Analogs Containing Hydroxamate Moteties—The phenyloxazoline compounds L-573,655 and LNTI-229 (Fig. 1) require the presence of the hydroxamate functional group for inhibition of E. coli LpxC (10). In an attempt to find inhibitors with activity against the complete spectrum of Gram-negative LpxCs, a new class of compounds was synthesized that incorporate the hydroxamate functional group into substructures of the physiologically LpxC substrate (Fig. 4). These potential LpxC inhibitors contain a tetrahydropryan ring with the hydroxamate group appended at the position analogous to the location of the N-acetyl group in the LpxC substrate (Fig. 4). The IC_{50} values for inhibition by these compounds against the purified LpxC enzymes from E. coli and A. aeolicus were determined at 30 °C, using 3 μM UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylgalcosamine, a concentration of substrate that is around the K_m for both enzymes (Fig. 5 and Table IV). Inhibition by the most potent LpxC inhibitors, TU-514 and TU-517, was not time-dependent; the observed inhibition was complete within the time required to start assays containing 0.01 mg/ml of each inhibitor (about 10 s, data not shown). In routine assays, however, reactions were initiated with enzyme that had been preincubated with inhibitor for 20–40 min on ice. The best of the hydroxamate-containing substrate analogs (TU-514 and TU-517) exhibit IC_{50} values for both the A. aeolicus and the E. coli LpxC that are in the low micromolar range when assayed at 30 °C under the same conditions (Fig. 5 and Table IV).

Substrate analog inhibitors were prepared with alternative acyl chains attached at the position corresponding to the glucosamine 3-OH on the E. coli LpxC substrate (Fig. 4). Inhibition of both E. coli and A. aeolicus LpxC is dependent upon the presence and length of the acyl substituent, as indicated by the increasing IC_{50} values observed for the compounds TU-514, TU-517, TU-521, and TU-519 (Fig. 4 and Table IV). Inhibition of E. coli LpxC gradually decreases as the acyl chain is decreased from 14 to 6 to 0 carbon atoms. Inhibition of A. aeolicus LpxC exhibits a steeper dependence on the length of the acyl chain; inhibitory activity completely disappears as the acyl chain is shortened to a 6-carbon unit in the hydroxamate series. Inhibition of A. aeolicus LpxC is slightly better with the nonhydroxylated C_{14} fatty acyl substituent in TU-514, whereas inhibition of E. coli LpxC is optimal with the hydroxylated C_{14} fatty acyl moiety in TU-517 (Fig. 4 and Table IV). TU-514 and TU-517 also inhibit P. aeruginosa LpxC with IC_{50} values similar to those observed against the E. coli enzyme (data not shown).

Additional substrate analogs were synthesized with ketone, carboxylate, or trifluoromethyl ketone groups in place of the hydroxamate moiety (structures not shown). None of these compounds potently inhibited either of the LpxC enzymes, even at concentrations as high as 1 mg/ml (data not shown). However, the phosphinate-containing substrate analog 6SHT55 (Fig. 4) inhibits both E. coli and A. aeolicus LpxC with similar IC_{50} values (115 and 192 μM, respectively) (Table IV). Inhibition by 6SHT55 was not found to be time-dependent within the interval required to start the LpxC assay (data not shown). For E. coli LpxC, the C_6-containing phosphinate 6SHT55 is not as active as the C_4-containing hydroxamate compound TU-521 (Fig. 4), suggesting that the hydroxamates are better inhibitors of E. coli LpxC than the phosphinates in this series of analogs. However, when tested in assays with A. aeolicus LpxC, the phosphinate-containing 6SHT55 is a much better inhibitor than the comparable hydroxamate TU-521 (Fig. 4 and Table IV), showing that a phosphinate substituent can sometimes broaden the range of diverse LpxCs that are inhibited.

K_i Determination for Inhibitors of E. coli LpxC—Since E. coli LpxC is inhibited both by the substrate analog hydroxamates and the phenyloxazoline-based hydroxamates, we determined the K_i values for the most potent inhibitors of E. coli LpxC: LNTI-229, TU-514, and TU-517 (Figs. 1 and 4). We demonstrated that LNTI-229 acts as a competitive inhibitor of E. coli LpxC, with K_i = 104 ± 19 nM (Fig. 6), consistent with the previous extrapolation of 50 nM for L-161,240 (the R-enantiomer of LNTI-229), which was estimated from the K_m of L-573,655 and the IC_{50} of L-161,240 (10). Using the same conditions used for the K_i determination for LNTI-229, we found that TU-514 and TU-517 are also competitive inhibitors of E. coli LpxC, with K_i values of 650 ± 280 nM for TU-514 and 190 ± 50 nM for TU-517. Importantly, the S-enantiomer of LNTI-229 is ~250 times less potent as an inhibitor of the deacylase (data not shown).

Antibacterial Activity of LpxC Inhibitors—The antibacterial
activities of the three most potent inhibitors of E. coli LpxC against the E. coli K-12 strain R477 were assessed using antibiotic susceptibility disc tests. As a control, the effect of the pure S-enantiomer of LNTI-229 on the growth of E. coli R477 was measured and compared with that of the racemic inhibitor LNTI-229. A large zone of inhibition was observed surrounding a disc containing 50 μg of the LNTI-229, whereas there was no detectable inhibition of growth surrounding a disc containing 50 μg of the pure S-enantiomer (Fig. 7, discs 1 and 2, respectively). No inhibition of bacterial growth beyond the 6-mm paper disc was observed with the same amount (50 μg) of L-573,655 against E. coli R477 (Fig. 7, disc 4). For comparison, a disc containing 25 μg of the antibiotic ampicillin was also tested (Fig. 7, disc 3). Inhibition of E. coli growth by LNTI-229 is almost comparable with that observed with ampicillin under these conditions, consistent with previous determinations of the minimal inhibitory concentration (10). Despite the significant potency of TU-514 and TU-517 (K_i values of 650 ± 280 and 190 ± 50 nM, respectively) against E. coli LpxC in vitro, neither TU-514 nor TU-517 inhibited the growth of E. coli R477 in the antibacterial disc test (data not shown). The presence of the fatty acyl chain in TU-514 and TU-517 may limit the ability of these compounds to cross the cell envelope of E. coli (Fig. 4). However, some antibacterial activity is detected with TU-514 against an E. coli R477 mutant strain harboring a single amino acid substitution in LpxC (10), which renders the cells several orders of magnitude more sensitive to all deacetylase inhibitors (data not shown).

**DISCUSSION**

In the present study, we have cloned, overexpressed, and purified to homogeneity LpxC from A. aeolicus, a Gram-negative hyperthermophile that is among the most divergent of the eubacteria sequenced to date (23). The temperature profiles of the activities of E. coli and A. aeolicus LpxC are similar in shape but are shifted from one another by ~30 °C (Fig. 2). This finding is consistent with the fact that A. aeolicus grows at very high temperatures. In fact, since A. aeolicus is able to grow at temperatures up to 95 °C, it may have been caused by contaminating E. coli proteins or precipitation of BSA at high temperatures. Nevertheless, the inherent stability of the A. aeolicus enzyme (Fig. 2) should facilitate efforts to determine the three-dimensional structure of LpxC.

The E. coli LpxC substrate (16, 20), UDP-3-O-(R-3-hydroxy-myristoyl)-N-acetylglucosamine (Scheme 1), is an excellent substrate for A. aeolicus LpxC. In fact, the k_cat/K_m values for these two enzymes near their optimal temperatures in vitro are nearly identical (Table III). For both enzymes, k_cat/K_m increases with increasing temperature as the optimum is approached. For A. aeolicus LpxC, this increase is due to an increase in the steady-state k_cat value as well as to a decrease in the steady-state K_m for UDP-3-O-(R-3-hydroxy-myristoyl)-N-acetylglucosamine. The very similar kinetic parameters suggest that the E. coli LpxC substrate may be very close in structure to the physiologically relevant substrate for A. aeolicus LpxC. Recent studies (40) of the structure of lipid A of A. aeolicus support this hypothesis. These studies have shown that the mature A. aeolicus lipid A contains predominantly hydroxymyristate residues at the 3- and 3'-positions, as would be expected if UDP-3-O-(R-3-hydroxy-myristoyl)-N-acetylglucosamine (or something very similar) is the natural A. aeolicus substrate. Interestingly, the lipid A disaccharide of A. aeolicus consists of 2,3-dideoxy-2,3-diaminoglucose instead of glucosamine, suggesting that LpxC of A. aeolicus normally deacylates a substrate in which the acyl chain at position 3 is N-linked.

Previously, screens for compounds that inhibit bacterial growth by blocking lipopolysaccharide biosynthesis led to the identification of LpxC inhibitors that contained a hydroxamate group attached to a phenylloxazoline scaffold (Fig. 1) (10). These findings suggested that LpxC might be a zinc metalloenzyme. Indeed, 1–2 mol of zinc are present per mol of purified LpxC, and zinc or cobalt ions are able to re activate the inactive E. coli apo-LpxC (20). The hydroxamate inhibitors probably interfere
Inhibitor of A. aeolicus and E. coli LpxC by substrate analog compounds

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>A. aeolicus LpxC, IC₅₀ at 30 °C (µM)</th>
<th>E. coli LpxC, IC₅₀ at 30 °C (µM)</th>
<th>IC₅₀ at 1 °C (µM)</th>
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<tr>
<td>TU-514</td>
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<tr>
<td>6SHT55</td>
<td>192 ± 38</td>
<td>115 ± 33</td>
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*IC₅₀ values were determined from measurement of initial rates of LpxC activity with UDP-3-O-(R)-3-hydroxymyristoyl)-N-acetylglucosamine in 40 mM bis-tris, pH 5.5, containing 1 mg/ml BSA. The IC₅₀ for inhibition of E. coli LpxC at 30 °C was determined using 2 nM enzyme with 3 µM substrate and 1 mg/ml BSA in 40 mM bis-tris, pH 5.5. The IC₅₀ values given were determined based on lack of inhibition observed with up to 1 mg/ml inhibitor in the assays. Not determined.

FIG. 5. Inhibition of E. coli and A. aeolicus LpxC by a substrate analog inhibitor. Purified E. coli LpxC (5 nM) or A. aeolicus LpxC (7.75 nM) was incubated with the indicated concentrations of TU-514 for 20–40 min on ice in 40 mM bis-tris, pH 5.5, containing 1 mg/ml BSA. Activity assays were initiated by dilution of the enzyme/inhibitor stock (2.5-fold) into assays containing 3 µM UDP-3-O-(R)-3-hydroxymyristoyl)-N-acetylglucosamine in 40 mM bis-tris, pH 5.5, at 30 °C. Additional inhibitor was added to the activity assay to maintain the same concentration as in the preincubation. The data were fit to Equation 1 to yield the IC₅₀ values for inhibition by TU-514 of 7.2 ± 1.9 µM for E. coli LpxC and 7.0 ± 0.5 µM for A. aeolicus LpxC.

FIG. 6. Competitive inhibition of E. coli LpxC by LNTI-229. Initial rates of E. coli LpxC activity were determined at varied concentrations of substrate (1–10 µM) and inhibitor (0 nM ( ), 25 nM ( ), 50 nM ( ), 100 nM ( ), and 500 nM ( × )) using 2 nM E. coli LpxC in 40 mM bis-tris, pH 5.5, 30 °C, containing 1 mg/ml BSA. The data were fit directly to Equation 2 using the Systat software, yielding Kᵢ = 104 ± 19 nM.

with LpxC activity by chelating the active site zinc ion, in a manner similar to that observed with other zinc metalloenzymes, such as thermolysin (31) and angiotensin-converting enzyme (21, 32). In support of this hypothesis, the recent crystallization structure of an A. aeolicus protein homologous to eukaryotic histone deacetylases (although not to the LpxC deacetylase family) reveals the presence of a single zinc ion (33). Moreover, the specific hydroxamate-containing inhibitors trichostatin A and suberoylanilide hydroxamic acid coordinate the zinc ion in a similar manner as observed with other metalloamidases (33).

An unexpected finding of the present study was the inability of the phenyloxazoline-based hydroxamates to inhibit A. aeolicus LpxC. We therefore synthesized a new series of compounds more closely related to the structure of the LpxC substrate (Fig. 4) and demonstrated that these compounds inhibit A. aeolicus, E. coli, and P. aeruginosa LpxC with comparable potency. Inhibitors containing a hydroxamate moiety at the position analogous to that occupied by the reactive N-acetyl group in the physiological LpxC substrate (Fig. 4) inhibit LpxC with IC₅₀ values in the low micromolar range (Table IV). As with the phenyloxazoline-based inhibitors (10), replacement of the hydroxamate functional group with either a carboxylate or a ketone moiety results in the complete loss of inhibition against either E. coli or A. aeolicus LpxC (data not shown). Furthermore, as had been observed both with the native substrate and the phenyloxazoline inhibitors of E. coli LpxC, the hydrophobic substituents are important for effective catalysis (20) or inhibition (10), respectively. As the length of the acyl chain attached to the position in the substrate-based inhibitors corresponding to the GlcNAC 3-OH of the LpxC substrate is decreased (Fig. 4), the IC₅₀ for inhibition of LpxC increases (Table IV). Despite the fact that the 14-carbon hydroxamates
TU-514 and TU-517 inhibit A. aeolicus and E. coli LpxC to a similar extent (Fig. 5), inhibition of A. aeolicus LpxC is somewhat more dependent upon the hydrophobic character of the 3-0-acyl substituent of the inhibitor (Table IV). Even decreasing the length of the acyl chain to 6 carbons increases the IC_{50} >200-fold, resulting in the loss of any detectable inhibition of the A. aeolicus enzyme (Table IV).

Many zinc metalloaminidases, including thermolysins, carboxypeptidase A and collagenase, are potently inhibited by compounds that contain a phosphonate functional group (22, 34). Crystal structures of the phosphonamidate compound carbobenzoxypy(P)-Leu-Ala (34). The structures of the phosphonate compounds that contain a phosphonate functional group (22, 34). The structures of the phosphonate compounds that contain a phosphonate functional group (22, 34).

We therefore synthesized and demonstrated that a substrate-based compound (6SHT55) containing a phosphate moiety at the position of the reactive N-acyl group inhibits both E. coli and A. aeolicus LpxC (Fig. 4; Table IV). When 6SHT55 is compared with the corresponding hydroxamate compound with a C_4 acyl chain at the 3-position (TU-521), the phosphate inhibitor is not quite as effective as the hydroxamate against E. coli LpxC (IC_{50,hydroxx/IC_{50,phox} = 0.4}). However, when the ratio of these activities against A. aeolicus LpxC is compared, the IC_{50,hydroxx/IC_{50,phox}} is greater than 20. The fact that both LpxC enzymes are inhibited by the phosphate-containing inhibitor 6SHT55 suggests that the transition state in LpxC catalysis may be similar to the tetrahedral transition states proposed to be involved in the deacetylation that display antibiotic activity be designated “deacetyls.”

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