Structure guided design of an antibacterial peptide that targets UDP-N-acetylglucosamine acyltransferase

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UDP-N-acetylglucosamine (UDP-GlcNAc) acyltransferase (LpxA) catalyzes the first step of lipid A biosynthesis, the transfer of an R-3-hydroxacyl chain from its acyl carrier protein (ACP) to the 3-OH group of UDP-GlcNAc. Essential in the growth of Gram-negative bacteria, LpxA is a logical target for antibiotics design. A pentadecapeptide (Peptide 920) with high affinity towards LpxA was previously identified in a phage display library. Here we created a small library of systematically designed peptides with the length of four to thirteen amino acids using Peptide 920 as a scaffold. The concentrations of these peptides at which 50% of LpxA is inhibited (IC50) range from 50 nM to >100 μM. We determined the crystal structure of E. coli LpxA in a complex with a potent inhibitor. LpxA-inhibitor interaction, solvent model and all contributing factors to inhibitor efficacy were well resolved. The peptide primarily occludes the ACP binding site of LpxA. Interactions between LpxA and the inhibitor are different from those in the structure of Peptide 920. The inhibitory peptide library and the crystal structure of inhibitor-bound LpxA described here may further assist in the rational design of inhibitors with antimicrobial activity that target LpxA and potentially other acyltransferases.

Lipid A is the hydrophobic anchor that secures the sugar components (core and O-groups) of lipopolysaccharide (LPS) to the external surface of the outer membrane2,3. The lipid A component of LPS also elicits an immune response in animal systems2–4. The minimal structure required for the viability of Gram-negative bacteria is lipid IVα5. Because lipid A is essential for the viability of Gram-negative bacteria, all the enzymes involved in its biosynthesis represent potential targets for inhibitory compounds with antibacterial activities6.

UDP-N-acetylglucosamine acyltransferase (LpxA) catalyzes the first step of lipid A biosynthesis2–3. LpxA catalyzes the transfer of an R-3-hydroxymyristoyl chain from ACP to the 3-OH glucosamine group of UDP-GlcNAc. The acylation of UDP-GlcNAc is a thermodynamically unfavorable reaction with an equilibrium constant of ~0.012,6. Consequently, the second step of lipid A biosynthesis, catalyzed by the deacetylase LpxC, is the committed and first irreversible step of this pathway. Lipid A synthesized by most Gram-negative bacteria is similar to that of E. coli. Structural differences observed in the lengths of the acyl chain in the lipid A of various Gram-negative bacteria at the 3 and 3′ positions of the disaccharide glucosamine ring can be attributed to the differences in acyl chain specificity of the various LpxA orthologs. LpxA shows specificity for acyl chain containing a 3-OH moiety and only utilizes ACP as its donor substrate5. For example, E. coli LpxA is unable to substitute myristoyl-ACP for R-3-hydroxymyristoyl-ACP2. However, myristoyl-ACP appears to be an inhibitor of LpxA. Interestingly, Chlamydia trachomatis LpxA is unique in its ability to utilize myristoyl-ACP instead of R-3-hydroxymyristoyl-ACP5. Though highly specific for a R-3-hydroxymyristoyl-ACP, E. coli LpxA is capable of utilizing a shorter R-3-hydroxydecanoyl-ACP and R-3-hydroxylauroyl-ACP, albeit far less efficiently2,10. A single amino acid change in E. coli LpxA appears to be responsible for acyl chain specificity. A G173M mutation reverses the acyl chain specificity of E. coli LpxA from a 14-carbon acyl chain dependent enzyme to a 10-carbon acyl chain dependent enzyme.

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LpxA may assist in the rational design of inhibitors with antibiotic activity. It also shed light on the residues of the peptide that contributes to inhibitor efficacy. For this purpose, the structural characterization of LpxA-peptide CR20 complex sheds light on the key residues in LpxA that are important inhibitory targets. It also shed light on the residues of the peptide that contributes to inhibitor efficacy. For example, removing a single amino acid from the C-terminus of the peptide results in a three orders of magnitude loss in the efficacy of peptide CR20. Additionally, a higher resolution the crystal structure of peptide CR20 bound to LpxA may assist in the rational design of inhibitors with antibiotic activity.

**Results**

A small library of truncated peptides. Peptide 920 was identified in phage display library, and when expressed fused to glutathione sepharose (GST) in *E. coli*, it inhibited bacterial growth and showed high specificity for its target, LpxA. Here we systematically designed and synthesized a small library of peptides using Peptide 920 as a scaffold. The peptides were assayed for acyl transferase inhibitory activity under conditions where the concentrations of UDP-GlcNAc and R-3-hydroxymyristoyl-ACP were 1 μM each. The data revealed that truncations at the N-terminus of Peptide 920 were tolerated more so than truncations at the C-terminus (Table 1). Removal of three residues at the N-terminal of Peptide 920 created an efficacious peptide (CR20) with an IC₅₀ of ~50 nM. Conversely, removal of the arginine from the C-terminus of Peptide 920 (WMLDPIAGKW) resulted in a two orders of magnitude shift in the IC₅₀ (~100 μM) albeit lower than peptide CR20. This data suggests the contacts made by the C-terminus of the peptide are crucial for inhibitor efficacy and probably stability in the active site of LpxA.

Table 1. A small library of the truncated Peptide 920 and their IC₅₀. *IC₅₀* value taken from previous publication. Error bars show standard deviations of triplicates.

| Peptide identity | Peptide sequence | IC₅₀  |
|------------------|------------------|-------|
| Peptide 920      | SSGWMLDPIAGKWRS  | 60 ± 9 nM* |
| Truncation of Peptide 920 N-terminus | CR19 GWMLDPIAGKWSR | 77 ± 3 nM |
|                  | CR20 WMMLDPIAGKWSR | 50 ± 6 nM |
| Truncation of Peptide 920 C-terminus | CR21 SSGWMLDPIAGKW | 12 ± 1.4 μM |
|                  | CR22 SSGWMLDPIAGK | 9 ± 2.2 μM |
| Truncation of Peptide 920 N and C-term | CR23 GWMLDPIAGK | 10 ± 0.3 μM |
|                  | CR24 GWMLDPIA | >100 μM |
|                  | CR25 WMMLDPI | >100 μM |
|                  | CR26 WMMLD | >100 μM |

Previously, we described Peptide 920, a 15 amino acid long potent inhibitor of *E. coli* LpxA with an IC₅₀ of 60 nM. Using Peptide 920 as a scaffold, a small peptide library was created and screened. Our goal was to search for a smaller efficacious peptide with an enhanced inhibitory potential, as well as to identify the essential residues that contribute to inhibitor potency (Table 1). The most potent inhibitor was structurally characterized to visualize carefully those interactions that contributes to inhibitor efficacy.

Here we report the successful crystallization of *E. coli* LpxA complexed with peptide CR20 (WMLDPIAGKWSR) at a resolution of 1.60 Å. Peptide CR20 is a potent inhibitor of *E. coli* LpxA with an IC₅₀ of ~50 nM. The peptide is located at the interface of each adjacent subunit and interacts with residues from both sides. It occupies part of the ACP binding site that was inferred from previous structural and mutagenesis studies. All the residues of the peptide CR20 were well resolved. The design and characterization of our small library of peptides complemented with the structural characterization of LpxA-peptide CR20 complex sheds light on the key residues in LpxA that are important inhibitory targets. It also shed light on the residues of the peptide that contributes to inhibitor efficacy. For example, removing a single amino acid from the C-terminus of the peptide results in a three orders of magnitude loss in the efficacy of peptide CR20. Additionally, a higher resolution the crystal structure of peptide CR20 bound to LpxA may assist in the rational design of inhibitors with antibiotic activity.
Figure 1. Crystal Structure of LpxA-peptide CR20 complex at 1.60 Å. The individual subunits are colored pink, green, and blue. The LpxA N termini is located at the bottom, and displays the start of the β-helix domain of each subunit. The peptide (green) is in a β-hairpin conformation with the N and C termini exposed to solvent. In the peptide, the carbons are colored green, the nitrogens blue, and the oxygens red. The free enzyme (LpxA) and LpxA-peptide CR20 reveal that there were not much global movements in the side chains except for minor perturbations of those interacting directly with the peptide. LpxA appears to be a rigid structure where bound ligands are more likely to adopt different conformations, as noted previously.

Data Collection LpxA Ligand added Peptide CR20
Data collection
Wavelength (Å) 1.5415
Resolution range (Å) 21.65–1.60 (1.69–1.60)
Space group P2_1_3
a = b = c (Å) 96.73
CC1/2 (%) 94.80 (76.70)
Unique reflections 34668 (2647)
Completeness (%) 99.84 (100)
Mean I/σ(I) 14.2 (3.9)
Wilson B factor (Å²) 23.684
Rmerge 0.078 (0.085)
Refinement
Rfactor 0.1910 (0.453)
Rfree 0.2260 (0.3850)
No. of atoms 2500
No. of waters 400
No. of protein residues 274
R.m.s.d., bonds (Å) 0.007
R.m.s.d., angles (°) 1.091
Ramachandran favored (%) 98.5
Ramachandran outliers (%) 0
B factors (Å²)
Protein 23.68
Ligand 25.68
Solvent 38.90
All-atom clash score 2.85
Molprobity score 1.07 (99th percentile)

Table 2. Data-collection and refinement statistics. Values in parentheses are for the outer shell. Resolution limit was defined as the highest resolution shell where the average I/σ(I) was 2.  

\[ R_{merge} = \frac{\sum_{hkl} |F_{o}(hkl)| - <F_{o}(hkl)>}{\sum_{hkl} |F_{o}(hkl)|} \]

\[ R_{factor} = \frac{\sum_{hkl} |F_{o}(hkl)| - |F_{c}(hkl)|}{\sum_{hkl} |F_{c}(hkl)|} \]

Five percent of the reflections was used to calculate \(^5 R_{free}.\)
Peptide CR20 residues bind between adjacent subunits in the active site region. The left-handed \( \beta \)-helix fold of LpxA is not perturbed upon peptide binding (Fig. 1). An overlay of LpxA-Peptide 920 and LpxA-peptide CR20 gave a root-mean-square distance of 0.074 for all C\(\alpha\) pairs of the backbone. A comparison of the side chains from the free enzyme (LpxA), and LpxA-peptide CR20 reveal that there were not much global movements in the side chains or backbone of LpxA except for minor perturbations of those interacting directly with the peptide\(^{15}\). LpxA is a rigid structure where bound ligands are more likely to adopt different conformations.

**Interactions between peptide CR20 and LpxA.** LpxA-bound peptide CR20 is folded into a \( \beta \)-hairpin conformation. The hairpin inserts into the active site of LpxA and the N and C termini of the peptide is solvent exposed. Peptide CR20 makes extensive contacts with LpxA. An overlay of Peptide 920 and peptide CR20 reveals similarity between the buried portions of the peptide; however, there were differences observed in the residues at the end of the C and N termini (Fig. 2)\(^{13}\). In LpxA- Peptide 920 there appears to be a cation-\(\pi\) interaction between the arginine (R15) at the C-terminus of the peptide and the tryptophan (W4) at the N-terminus. In contrast, the R15 in peptide CR20, is flipped 180°, (Fig. 2) while, the tryptophan is interacting with R204 via a water-mediated interaction instead of a cation-\(\pi\) interaction (Fig. 2). This observation suggests that these bulky residues are indeed flexible and they could be manipulated for peptidomimetics.

The overall density of the peptide CR20 (all 12 residues) and the surrounding residues from LpxA was clear and continuous (Fig. 3). Peptide CR20 does not disrupt any contacts between the individual monomers of LpxA, but instead forms a bridge between the adjacent subunits. The significant interaction of peptide CR20 with LpxA is sustained by hydrogen bonds, some of which are mediated by water molecules. There is indeed an intricate hydrogen-bonding network with at least thirteen water molecules that are directly hydrogen-bonded to peptide CR20 (Fig. 4). Residues of LpxA involved in direct hydrogen bonds with the peptide are Q161 (backbone N) and R258 from one protomer and G155, G173, and M170 from the adjacent protomer (Fig. 4). These direct hydrogen bonds are also present in the LpxA-Peptide 920 complex\(^{13}\). However, there were differences, in the partners they engage with for hydrogen bond interactions. For example, R258 is directly hydrogen bonded to K9 of peptide CR20 instead of S11, and M170 is hydrogen bonded to the backbone carbonyl of G8 in peptide CR20 instead of W10 (Fig. 4). Residues that interact with peptide CR20 via water-mediated interactions are R204, R205, N198, and Q161 (side chain) from the blue chain and H160 from the pink chain. Only, Q161 water mediated interaction was previously observed in the LpxA-Peptide 920 complex\(^{13}\) (Fig. 4). It appears that there are localized deviations in the way the peptide is supported in the active site of LpxA, although peptide-binding region remains the same. There are four additional hydrogen bonds that are formed within peptide CR20. The peptide \( \beta \)-hairpin conformation may be supported by these intra-peptide hydrogen bonds.

**The importance of residues implicated in peptide binding.** Peptide CR20 is interacting with residues implicated in substrate binding as revealed by previous mutagenesis studies (H160, G173 and R204) (Fig. 5)\(^{19}\). G173 involved in acyl chain binding is not interacting with the proposed catalytic residue (H125) but could potentially block or partially occlude access to this residue. The peptide interacts with H160 via a water-mediated interaction (Fig. 4). The H160A mutant has significantly less activity than the wild-type\(^{19}\) however this residual activity was resistant to inhibition by peptide CR20 (IC\(_{50}\) \(\sim\)10,000 \(\mu\)M) (data not shown). This was surprising at first, however, H160 is interacting with K9 of the C-terminus of the peptide CR20, which is essential to the efficacy of the peptide interaction with LpxA.
Lipid A (endotoxin) plays an important role in bacterial growth, outer membrane integrity, and stimulation of the mammalian immune system. All the enzymes in the lipid A pathway are potential targets for inhibitors and some may be effective antibiotics. LpxA catalyzes the first step of lipid A biosynthesis and is essential for viability in most Gram-negative pathogens.

The E. coli LpxA-peptide CR20 complex solved at high resolution (1.60 Å) facilitated the identification of a dedicated space in the essential LpxA enzyme that can be targeted for inhibitors. LpxA has two substrates: UDP-GlcNAc and acyl-ACP. The binding site of both substrates have been identified in previous structural studies. Peptides CR19-CR22 represent potential starting points for the design of potent more efficacious inhibitor that target LpxA (Table 1). The most efficacious peptide CR20 binds in a region that would mainly occlude the binding site of acyl-ACP. The superposition of the CR20-bound LpxA to the UDP-3-O-(R-hydroxymyristoyl)-GlcNAc product bound structure shows that peptide CR20 overlaps with the UDP-3-O-(R-hydroxymyristoyl)-GlcNAc product in active site of LpxA (Fig. 6a–c). Peptide CR20 is a potent inhibitor of LpxA with an IC50 of approximately 50 nM (Table 1).

Peptide CR20 is more ordered in the active site of LpxA than Peptide 920. The electron densities of all 12 residues of peptide CR20 was clear and well defined. Notably, the interaction of peptide CR20 with LpxA is secured by a majority of polar interactions some of which are mediated by water molecules. There are approximately 13 water molecules involved in water-mediated interactions or supporting the peptide in the LpxA active site by hydrogen bonding (Fig. 4), suggesting that in the absence of substrate the active site is highly solvated. Delivery of the acyl chain by ACP would displace the solvent in the active site of LpxA.

Discussion

Lipid A (endotoxin) plays an important role in bacterial growth, outer membrane integrity, and stimulation of the mammalian immune system. All the enzymes in the lipid A pathway are potential targets for inhibitors and some may be effective antibiotics. LpxA catalyzes the first step of lipid A biosynthesis and is essential for viability in most Gram-negative pathogens.

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Acyl carrier protein is an important component in fatty acid and polyketide biosynthesis. Recently, crystal structures of LpxD, the second acyltransferase in the Lipid A pathway, in complex with intact acyl-ACP gave an unprecedented view of how ACP delivers acyl chain linked to prosthetic 4’-phosphopantethiene group among acyl transferases in the lipid A pathway. LpxA and LpxD share significant structural homology. A superposition of LpxA-peptide CR20 with LpxD-acyl-ACP reveals conservation of the acyl-ACP binding site. In support of previous enzymatic evidence that revealed Peptide 920 is largely competitive with ACP, it appears that Peptide 920 would block access of ACP to its binding site on LpxA. Perhaps, these basic residues (H160, R258, Q161, R204, R205, N198) play a role in ACP binding. ACP is a highly acidic protein with an isoelectric point of 4.1 and a pH solubility of roughly 3.9. It is likely that the charge complementarity between the LpxA and ACP interacting regions would be disrupted by inhibitory peptides. The binding site of CR20 and the other peptides presented herein could be targeted for rational design of antibiotics that could block acyl chain delivery by ACP.

Because small peptides do not cross membranes and are subjected to protease degradation, the use of peptide CR20 or the other inhibitory peptides as drugs, targeting cytoplasmic LpxA is unrealistic. However, we have mapped the space and location of important residues of LpxA, that if engaged by small molecules could contribute efficiently to the inhibition of LpxA. Therefore, all the structural and biochemical information garnered from this study are promising starting points for the development of antimicrobials using structure-based drug design. The results of this study should provide useful information for the further development of molecules that target the essential lipid A enzyme, LpxA.

Materials and Methods

Sample Preparation for crystallization. LpxA was overexpressed and purified as previously described. Briefly, LpxA was purified from BL21(DE3)/pLysE/pTO1. The construct pTO1 is a pET23c (Novagen) vector that contains the wild type lpxA gene. Cells were lysed by one passage through a French pressure cell at 18,000 psi and centrifuged at 10,000 x g for 20 min to remove cell debris. The supernatants were further centrifuged at 100,000 x g for 90 min to obtain soluble proteins. The purification scheme consists of Green19-Agarose (Sigma) affinity chromatography (pH 7.4), followed by Source Q ion-exchange chromatography (pH 8) and Superdex 200 gel filtration chromatography in 10 mM potassium phosphate buffer, pH 7, containing 250 mM NaCl. The purity...
of LpxA was evaluated by SDS-PAGE, LpxA activity assays, and electrospray ionization mass spectrometry. Mass spectra analyses were performed on a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) equipped with an electrospray source. Peptide CR20 (NH2-WMLDPIAGKWSR-COOH) and other truncated peptides described in this work were prepared at the University of North Carolina Peptide Synthesis Facility (Table 1). Each synthesized peptide was evaluated by MALDI mass spectrometry and was confirmed to have greater than 90% purity.

**Protein crystallization.** Co-crystals of LpxA-peptide CR20 complex were obtained by combining a concentrated LpxA solution (20 mg/ml) with a 25-fold molar excess of peptide CR20 (12.5 mM). Crystals of LpxA-peptide CR20 were obtained by hanging drop vapor diffusion method from a 1:1 mixture of peptide and protein. Individual droplets contained 2 μl of the LpxA-peptide CR20 mixture and 2 μl of 0.8–1.8 M phosphate buffer, pH 6.3–6.9. Crystals appeared after 24 hrs and grew to approximately 1.0 mm after 2 weeks.

**Data Collection, Structure Determination and Refinement.** Crystals of the LpxA-peptide CR20 complex were cryo-protected in 1.0 M Na/K phosphate, pH 6.9, and 35% DMSO, and then were flash cooled in liquid nitrogen. Diffraction data were collected on an R-Axis IV image plate detector. Diffraction images were processed and scaled using HKL2000. Crystals diffract to 1.60 Å and belong to the cubic space group P213 (a = b = c = 96.73 Å, Table 2). Phases were calculated using molecular replacement with the program MolRep in the CCP4i suite. Previously published structure of LpxA monomer was used as the search model (PDB code...
Iterative rounds of model building were performed using O and COOT, with rounds of refinement in REFMAC24–26. The quality of the final model was evaluated using MolProbity and PROCHECK (PDB ID: 6HY2)27,28. The figures were drawn using PyMOL (DeLano Scientific, San Carlos, CA). Data collection and refinement statistics are presented in Table 2.

**Inhibition of LpxA activity by Peptide 920.** The LpxA enzymatic reaction monitors the conversion of $[^{32}P]UDPGlcNAC$ to $[^{32}P]UDP$-3-O-(R-3-hydroxymyristoyl)-GlcNAC. The assay components consist of 40 mM HEPES buffer, pH 8, 1 mg/ml BSA, 1 μM R-3-hydroxymyristoyl-ACP, and 1 μM $[^{32}P]UDPGlcNAC$ (2 × 10^6 cpm/nmol). Truncated peptides (Table 1) was dissolved in DMSO and pre-incubated with the reaction mixture at 30 °C for 3 minutes in the absence of enzyme at concentrations ranging from 1 nM to 10 μM. The final concentration of DMSO was adjusted to 10% to match the peptide solvent in all enzymatic assays.

The reactions were started by the addition of 1 nM of LpxA and incubated at 30 °C for a maximum of 10 min. Three or four time points were collected during this time. Termination of the reaction was accomplished by the addition of 1 μl portions onto a silica thin layer chromatography (TLC) plate. The TLC plates were air dried for 10 min before developing in chloroform/methanol/water/acetic acid (25:15:4:2, v/v). Plates were exposed to PhosphorImager screens overnight, and the data were evaluated with Molecular Dynamics PhosphorImager equipped with ImageQuant software. Inhibition of LpxA by Peptide 920 and truncated peptides was analyzed by plotting the initial velocities as a function of the inhibitor concentration. To determine the IC50 at 30 °C, the data were fit to the following equations,

\[
\frac{v_i}{v_c} = \text{IC}_{50}/(I + \text{IC}_{50})
\]

or

\[
\text{% activity} = 100/(1 + I/\text{IC}_{50})
\]

where $v_i$ represents the initial rate, at given concentrations of inhibitor, $v_c$ represents the initial velocity of the control reaction without inhibitor, and I represents the inhibitor concentration. The IC50 determination represents the concentration of inhibitor needed to inhibit 50% of the enzyme activity.
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Author Contributions

A.H.W. and C.R.H.R. designed research. M.D. and A.H.W. conducted experiments, collected and analyzed data. A.H.W. wrote the manuscript. M.D. prepared figures. C.R.H.R. and A.H.W. supervised the work.

Additional Information

Competing Interests: The authors declare no competing interests.

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