Case Study

Driving Protein Crystallization and Rational Drug Design *via* Thermal Shift Assay

Structural Biology Services

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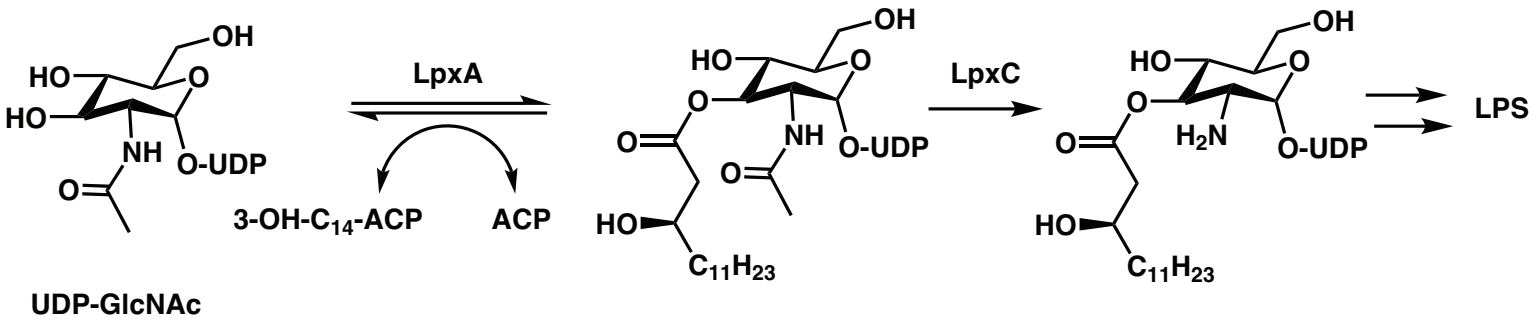
Overview

- Cayman's Structural Biology service group worked with a client to perform crystallization studies that delivered multiple high-resolution crystal structures of LpxC, an enzyme involved in Gram-negative bacteria pathogenicity, bound with lead inhibitors.
- Thermal shift assay (TSA) protein formulation and cryoprotectant screening showed Mg^{2+} and tartrate to be effectively stabilizing for *P. aeruginosa* LpxC. Improved crystallization space and novel crystallization conditions were identified by TSA that enabled the co-crystallization of *P. aeruginosa* LpxC with inhibitor compounds.
- Crystal structures determined the key interactions of the inhibitors in the *P. aeruginosa* LpxC binding pocket. These findings led to the discovery of a prodrug with high solubility and rapid conversion to active drug upon i.v. administration to treat *P. aeruginosa* infections.
- The TSA-driven crystallization platform offered by Cayman enabled the client to rapidly advance their *P. aeruginosa* drug discovery program.

Background

With the rise of antibacterial resistance, especially amongst the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) pathogens, there has been an upsurge in targeting a number of antibacterial pathways.[1] In Gram-negative species, the lipopolysaccharide (LPS) biosynthetic pathway has been shown to feature a number of viable targets for both antibacterials and antivirulence drugs. The lipid A moiety of LPS, the main constituent of the outer leaflet of Gram-negative bacterial outer membrane, has been shown to be essential for survival in multiple pathogens. UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC) is a crucial Zn^{2+}-dependent enzyme that catalyzes the deacetylation of UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine to UDP-3-O-(R-3-hydroxymyristoyl) glucosamine early in the biosynthesis of lipid A. This is an attractive target that has been pursued by a number of pharmaceutical companies.[2-4]



Experimental Need

A biopharmaceutical company approached Cayman to obtain multiple co-crystal structures of LpxC, an enzyme involved in Gram-negative bacteria pathogenicity, bound with their lead inhibitors. The structural studies of these drug-protein complexes could help drive drug discovery for new antibacterial compounds to treat multidrug-resistant Gram-negative infections.

Thermal Shift Assay Screening

Cayman's structural biologists conducted a protein formulation and cryoprotection screen using TSA. TSA is a powerful tool for identifying stabilizing conditions for proteins that have been shown to correlate with a protein's crystallization ability.[5] The information developed from this TSA was used to build a crystallization platform for the rapid co-crystallization and structure determination of *P. aeruginosa* LpxC with the client's compounds.

Purified *P. aeruginosa* LpxC was provided by the client and screened to determine essential protein formulation *via* TSA. Material was set up at approximately 0.5 mg/ml using 1:1,000 Sypro Orange Dye (ThermoFisher) over a temperature gradient of 4-100°C within step cycles of 0.3°C. A formulation screen was used to test various buffers, salts, additives, and cryoprotectants to determine the optimal formulation condition. Follow-up screens were conducted to validate conditions using the same protein and dye concentrations. Titrations of various additives were carried out to determine optimal concentrations in the presence of specific compounds for subsequent co-crystallization experiments.

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Thermal Shift Assay Results

The formulation and cryoprotectant screening yielded multiple stabilizing hits. Multiple divalent cations were observed to act as stabilizing agents. Mg^{2+} was observed to be extremely stabilizing. *P. aeruginosa* LpxC in its initial formulation was shown to be well-folded, with little aggregation and a T_m of 44.5°C (**Figure 1**). Titrating Mg^{2+} concentrations led to a maximal shift of 17.5°C and a T_m of 62°C (**Table 1**). Glycerol and ethylene glycol were observed to be effective cryoprotectants based on further stabilization (data not shown).

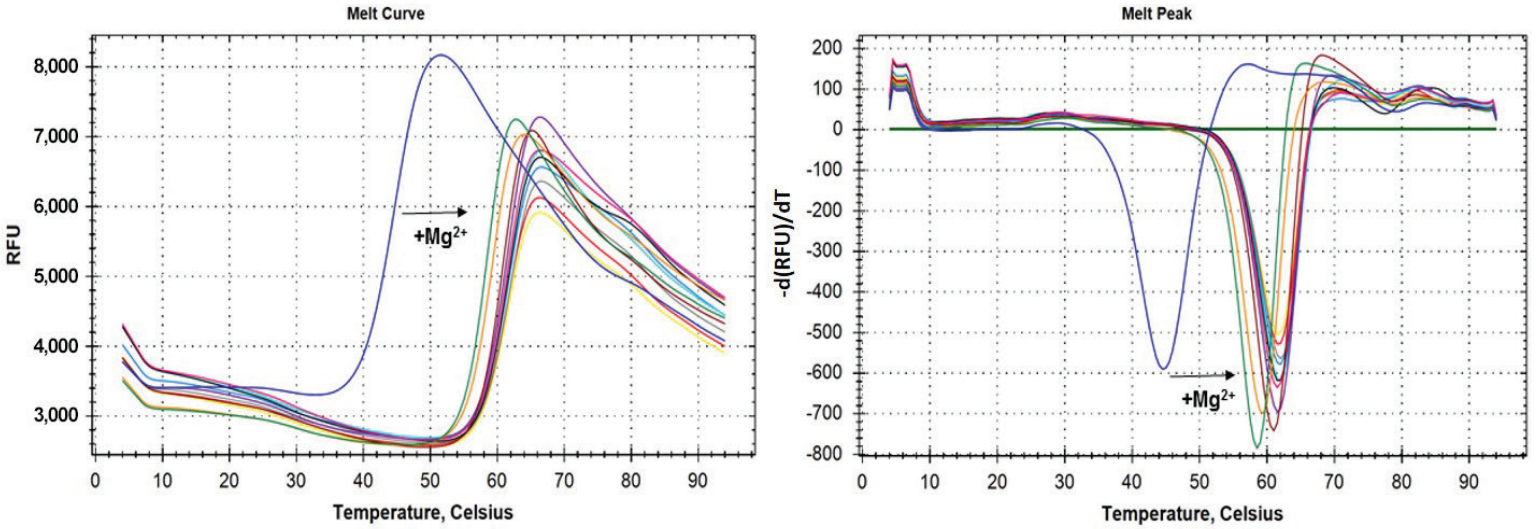

Figure 1. Magnesium TSA titration curves for *P. aeruginosa* LpxC showing RFU *versus* temperature plot and the first derivative plot.

Compound titrations were performed in the presence and absence of Mg^{2+} to determine the effect of compounds on protein *versus* apoprotein (**Figure 2**). It was possible to rank order compounds and predict which compound would have the most success within the co-crystallization experiments based on the maximal shifts. The compounds alone stabilized the protein with a range of a few degrees to 10°C. These were all further stabilized by various concentrations of $MgCl_2$. Sodium tartrate was also seen to be highly stabilizing and was added to the protein formulation, which in the presence of inhibitor could drive T_m to as high as 75°C (**Figure 3**).

[$MgCl_2$] (mM)	T_m (°C)
0	44.50
10	58.50
20	59.50
30	61.00
40	61.50
50	61.50
60	61.50
70	62.00
80	62.00
90	62.00
100	61.50
110	61.50

Table 1. Melting temperature *versus* magnesium chloride concentration.

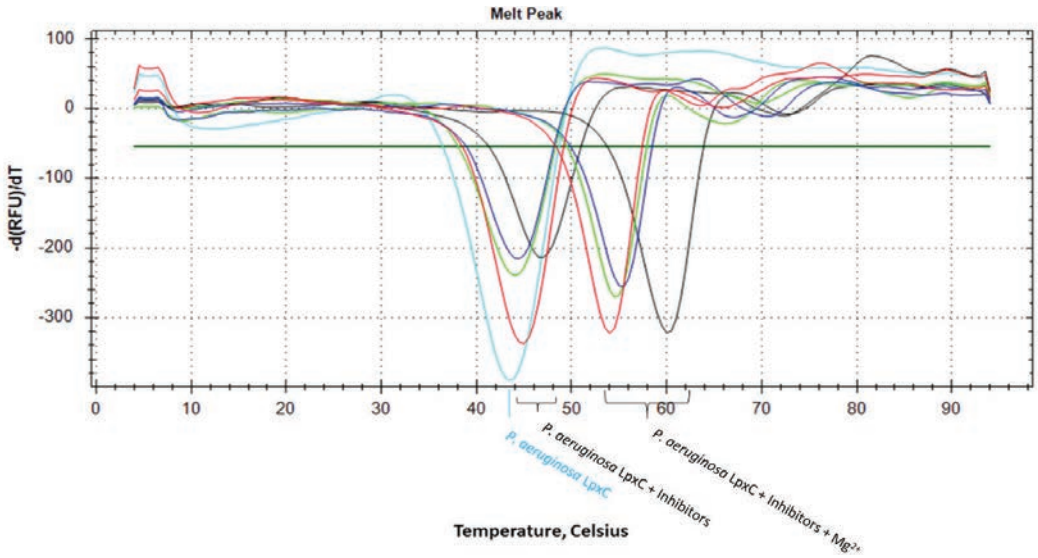

Figure 2. Compound's positive effect on protein melting profile by TSA in presence and absence of Mg^{2+}, showing first derivative of RFU *versus* temperature plot. Apoprotein is shown in cyan. Protein plus inhibitors are shown in green, red, blue, and black with a positive shift to the right with addition of Mg^{2+}.

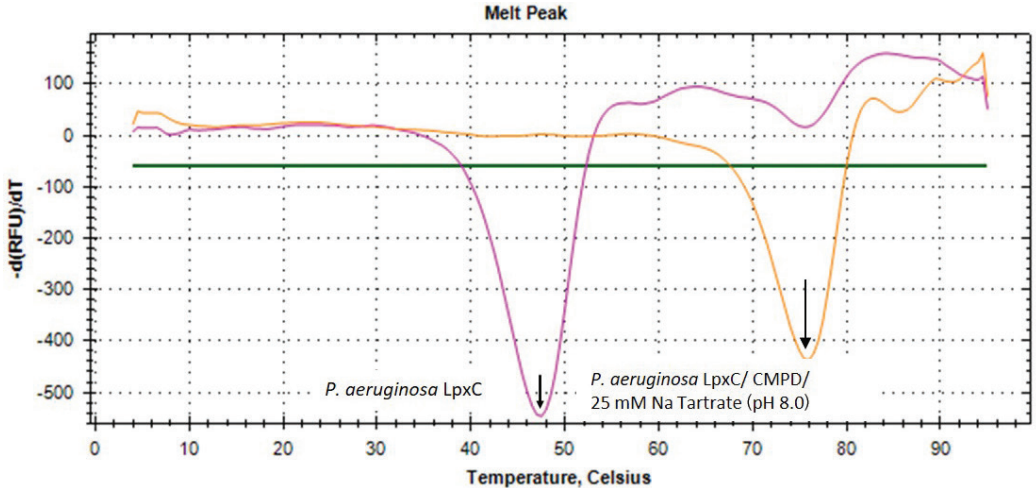

Figure 3. Sodium tartrate to *P. aeruginosa* LpxC is highly stabilizing the melting profile, showing first derivative plot of RFU *versus* temperature.

LpxC Crystallization and Structure Determination Experiments

P. aeruginosa LpxC co-crystallization was initiated utilizing using the sitting drop vapor diffusion method at 21°C. Based on the TSA results, optimal concentrations of stabilizing agents (50 mM and 25 mM of $MgCl_2$ and sodium tartrate, respectively) were added to the protein/inhibitor complex directly for crystal screening. Confirmed hits were optimized when necessary. Co-crystals of *P. aeruginosa* LpxC/ACHN-975, 50067, 50228, and 50516 were observed and transferred into cryoprotectants containing mother liquor supplemented with 15% glycerol or 15% ethylene glycol. Crystals diffracted to 1.85 Å for 50067, 50228, and 50516, and to 2.2 Å for ACHN-975 inhibitors. All data were collected at -160°C on beamline LS-CAT (21-ID) at Argonne National Laboratory. Data were integrated, scaled, and merged using HKL2000.[6] They all belong to space group $P2_1$.

Molecular replacement of LpxC datasets was performed using the scaled dataset with a previously solved *P. aeruginosa* LpxC structure as a starting model (without ligand, PDBID:4J3D) with PHASER.[7] After rigid body refinement, several rounds of model building and restrained refinement were performed in the absence of compounds using COOT.[8] After placement of the solvent molecules, each compound was modeled and refined.

Crystallization and Structure Determination Results

Together this data represents a suite of stabilizing reagents that could be leveraged to generate co-crystals of *P. aeruginosa* LpxC. For each compound, an optimum concentration of Mg^{2+} was determined and added directly to the protein sample, along with the addition of 25 mM sodium tartrate (determined through formulation screens). Co-crystallization was chosen over soaking to reduce any risk of crystal damage or cracking by conformational changes induced by inhibitor binding. A representative image of crystals obtained is shown in **Figure 4**.

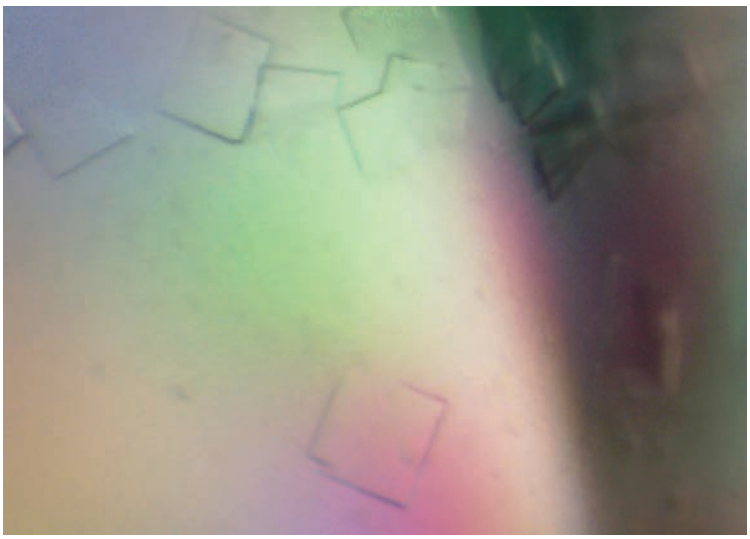

Figure 4. *P. aeruginosa* LpxC/ACHN-975 inhibitor complex crystals.

Inspection of the initial electron density maps of four solved structures showed unambiguous density for four compounds near the catalytic divalent metal. In the final refined models, the density of ACHN-975, 50067, 50228, and 50516 were well-resolved for each compound (**Figure 5**). Additionally, it was observed in those structures (except *P. aeruginosa* LpxC/ACHN-975) utilizing $MgCl_2$ that the catalytic Zn^{2+} was replaced by Mg^{2+} within the active site, since Mg^{2+} is stabilizing the enzyme.

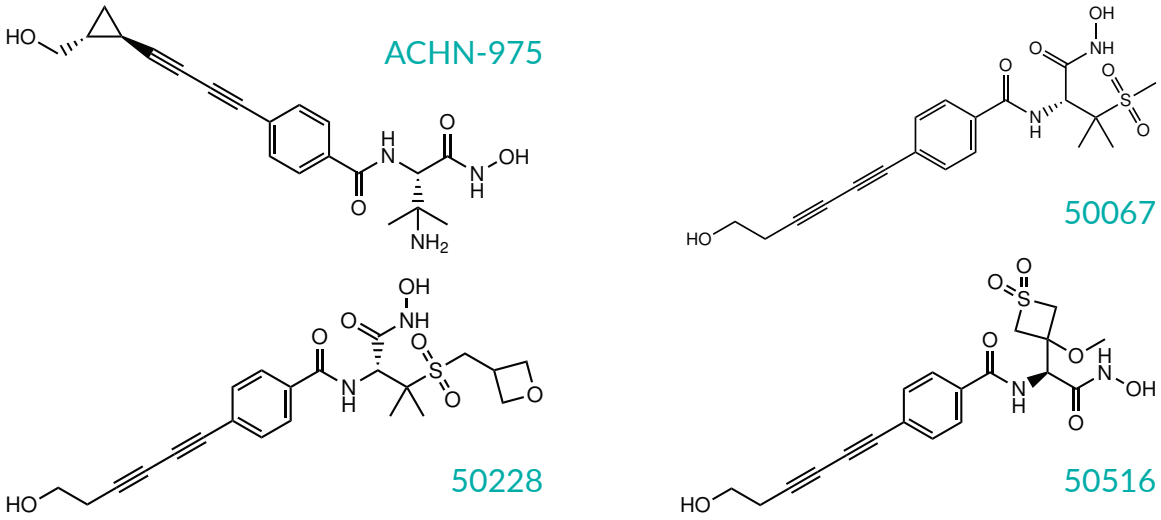

Figure 5. *P. aeruginosa* LpxC key inhibitors that were used in co-crystallization.

The structures were all well resolved. In **Figure 6**, the first determined *P. aeruginosa* LpxC co-crystal structure: *P. aeruginosa* LpxC/ACHN-975 complex is displayed. The hydroxamate moiety of ACHN-975 head group of the molecule was observed coordinating the active site Zn^{2+} ion, and the tail side of the molecule extends through a hydrophobic tunnel. The hydroxyethyl cyclopropyl tail group projects entirely out of the surface of the tunnel exit. Three crystallographic water molecules shown as red spheres make hydrogen bonds from the amine-containing head group. The inhibitor density is clearly present on the $2F_o$-F_c map, shown as gray density (**Figure 6**).

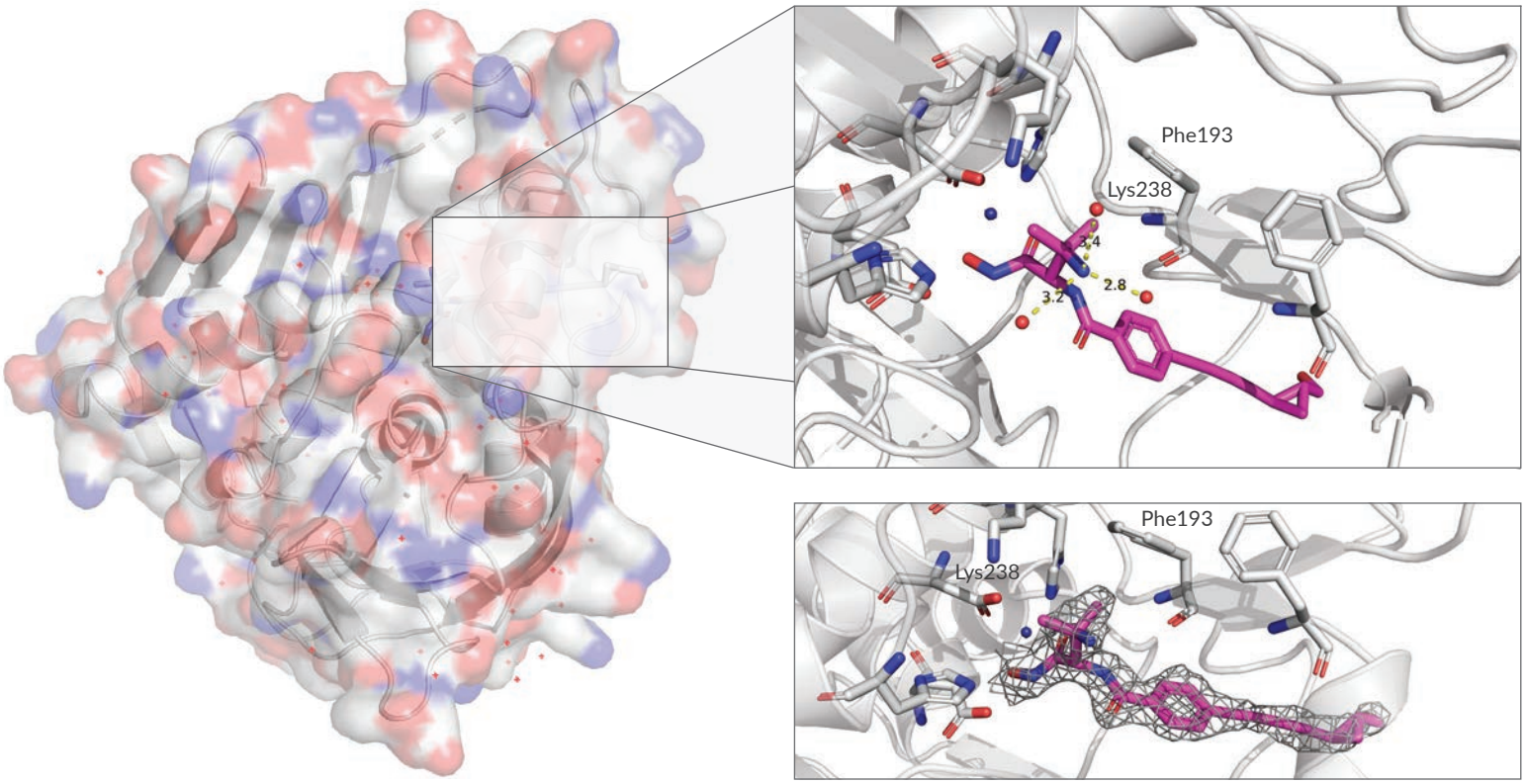

Figure 6. Structure of *P. aeruginosa* LpxC co-crystallized with ACHN-975 and its active site view. The $2F_o–F_c$ electron density map (gray) contoured at 1σ around ACHN-975 inhibitor.

Co-crystal structures of 50067, 50228, and 50516 inhibitors and *P. aeruginosa* LpxC are shown in **Figure 7**. In the LpxC/50067 structure, the amine side chain of Lys238 and backbone amide of Phe193 appeared to be in close enough proximity to serve as H-bond donors for expanded versions of the 50067 head group. The addition of the methyl oxetane moiety resulted in the oxygen atom of the oxetane positioned within hydrogen-bonding distance to the Phe193 backbone amide. In the LpxC/50516 compound structure, the presence of the thietane dioxide moiety results in a small conformational change of the Lys238 side chain. One of the oxygen atoms of the thietane dioxide moiety appears within hydrogen-bonding distance to the nitrogen atom of the re-positioned Lys238.

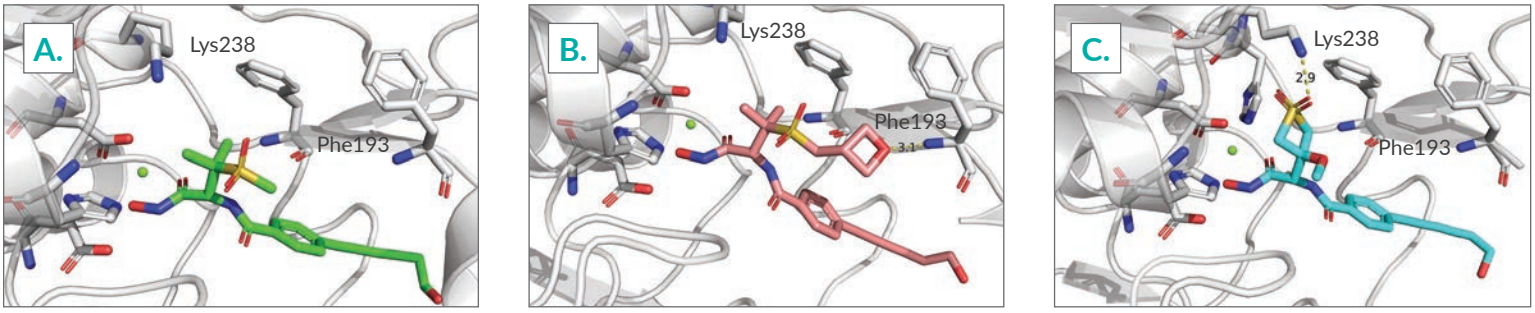

Figure 7. Structures of key inhibitors co-crystallized with *P. aeruginosa* LpxC.
A. 50067 inhibitor (green sticks). **B.** 50228 inhibitor (pink sticks). **C.** 50516 inhibitor (cyan sticks).

Summary

Cayman's Structural Biology Services group identified the necessary crystallization conditions using a thermal stability (TSA)-driven crystallization platform to provide the client with various separate high-resolution co-crystal structures of *P. aeruginosa* LpxC/inhibitor complexes. The use of TSA allowed for the prediction of conditions that would yield high-diffracting crystals. The coupled use of these stabilizing agents, which included magnesium chloride and sodium tartrate, resulted in the identification of a novel crystallization condition and advanced the program rapidly. This rational methodology was coupled with the more standard protein-sparse matrix screening approach to cover a wide area of crystallization space. These high-resolution crystal structures provided key biological data, leading to the identification of a novel clinical candidate—a prodrug that offered a wider therapeutic window for cardiovascular safety compared to the initial compounds.

Full details of this work have been published in *ChemMedChem*

Cohen, F., Aggen, J.B., Andrews, L.D., Assar, Z., *et al.* Optimization of LpxC inhibitors for antibacterial activity and cardiovascular safety. *ChemMedChem* (2019).

Additional References

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