Functional study 1

Aim: *Use structure-based inhibitor design to assess the function of bacterial immunophilins as potential targets for broad-spectrum antibiotic discovery*

Background: During the 4th year of SSGCID funding, Drs. Isobel Norville and Mitali Sarkar-Tyson from the UK Defense Science and Technology Laboratory (DSTL) requested a Fragments-of-Life screen on a cis-trans peptidylprolyl isomerase (BpML1) from *Burkholderia pseudomallei*, for which SSGCID had solved the structure by NMR (SSGCID ID: BupsA.00130.a.A1; PDB # 2KE0). BpML1 is a member of the MIP (macrophage infectivity potentiators) family of immunophilins. MIP proteins are inhibited by small molecule drugs, including rapamycin and tacrolimus, and DSTL wished to collaborate with SSGCID to design new inhibitors targeting BpML1 and its homologs from other bacterial pathogens. However, the inter-molecular crystal packing arrangement of the existing BpML1 crystal structure, revealed a packing arrangement where a helical peptide from one BpML1 molecule was bound to the active-site of the next BpML1 [125]. This made the existing crystal form of BpML1 unsuitable for soaking of crystals with fragment cocktails to identify new small molecule binding events to the active site. Therefore, Emerald developed a strategy for obtaining new crystal forms with open active sites of BpML1 that entails the engineering of mutations in both non-conserved residues on the surface of the protein and conserved residues in the active site, in order to rationally alter the crystal packing interactions to produce either "apo" crystal forms of BpML1 with an open active site region, or ligand-bound co-crystals. This strategy has proven effective, allowing us to solve the X-ray co-crystal structure of a $G_{90}A$ mutant of BpML1 in complex with FK-506, a well-known inhibitor of MIPs (**Error! Reference source not found.**). Thus, SSGCID has generated a set of BpML1 surface mutants that can be co-crystallized with known inhibitors. These exciting results have led Drs. Norville and Sarker-Tyson to request continued efforts on BpML1 to identify small molecules (fragments) binding to BpML1, as well as other BpML1 orthologues from Category A-C bacterial species, with the goal of identifying inhibitors of BpML1. These molecules would serve as leads for the development of novel broad-spectrum antibiotics.

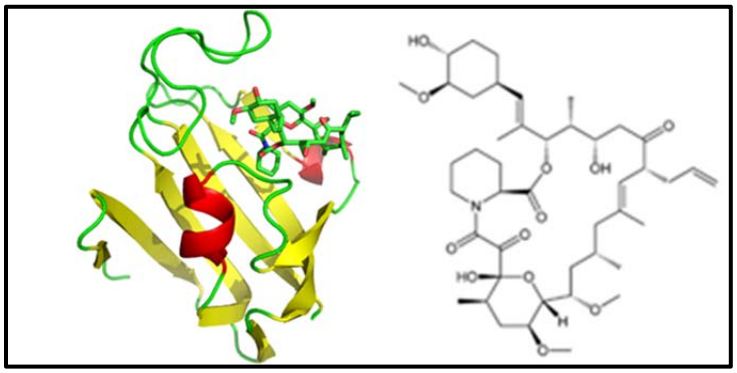

Figure 1. X-ray crystal structure of BpML1 ($G_{90}A$) in complex with FK-506
Left panel: X-ray co-crystal structure of BpML1 bound to the FK-506 compound, showing α-helices (red), β-sheets (yellow), and coils (green). **Right panel**: The chemical structure of FK-506.

Objective 1: Conduct and validate NMR-based fragment screening methods against BpML1

Solution state ligand-observe NMR spectroscopy is a versatile, robust and well-established approach for screening small molecules and fragment libraries against biologically relevant macromolecules [125-132]. Emerald BioStructures have previously published SSGCID methods for the use of their Fragments of Life™ (FOL) library to obtain fragment hits and lead molecules for drug targets [22-24, 133]. This fragment library contains a diverse set of metabolite-derived compounds and protein structural elements, and has been proven to work on a wide variety of protein targets without requiring target-based tailoring. BpML1 is highly soluble, and can be expressed with stable isotopic labels useful for protein-observe NMR studies, as demonstrated by three solution-state NMR structures deposited by SSGCID [19]. Complete peak assignments for all three NMR structures are publicly available at the Biological Magnetic Resonance Data Bank [134], and allow identification of specific sites on a target protein in the presence of a small molecule binder.

Approach: To provide starting points for new inhibitor discovery, three separate ligand-observe NMR experiments will be used to screen the (FOL) library against BpML1: saturation transfer difference (STD)-NMR, line-broadening (LB)-NMR, and two-dimensional nuclear Overhauser spectroscopy (NOESY). Compounds, such as FK-506 and cycloheximide-*N*-ethylethanoate, which are already known to demonstrate binding to BpML1 by STD-NMR and LB-NMR will serve as positive controls for the proposed NMR studies. To confirm active site binding, additional STD-NMR studies will be employed using cycloheximide-*N*-ethylethanoate and other positive control compounds as titrants against fragment hits. Known binders that displace fragment hits by STD-NMR will result in reduced binding signals for the fragment molecules, thus establishing the competitive nature of their active site association with the macromolecule. Fragment hits that are confirmed to bind the active site by STD-NMR titration will be further characterized by heteronuclear single quantum coherence (HSQC)-NMR spectroscopy. By correlating chemical shift changes for backbone residues of BpML1 which occur when a small molecule binder is mixed into the sample, HSQC data will confirm the specific binding site location for novel fragment binders. Measurement of these changes during quantitative titration can also be used to calculate binding affinities between a fragment hit and the target protein. This technique, known as "SAR-by-NMR" [132], will provide validation for fragment hits obtained through primary screening, and can be used for initial rounds of inhibitor design. This objective will be carried out at Emerald, in consultation with UW-NMR.

Objective 2: Co-crystallization and structure determination of validated fragments with BpML1

The results from Objective 1 will be used to select the best fragment leads for co-crystallization attempts with BpML1. Having previously identified conditions for ligand-dependent co-crystallization of BpML1 with FK-506 (**Error! Reference source not found.**), allows us to efficiently pursue the co-crystal structure determination of any candidate ligands found to bind to BpML1 from our proposed NMR screens.

Approach: Validated fragment hits from NMR studies will be subjected to co-crystallization at high concentrations with our panel of surface mutant forms of BpML1. Utilizing our library of crystallizable BpML1 constructs provides multiple opportunities to obtain fragment-bound co-crystals for structure determination. We anticipate solving the high resolution X-ray structures of 3-6 different fragment-bound BpML1 complexes. This objective will be carried out at Emerald.

Objective 3: Assay fragment-inspired small molecules for activity against BpML1

Information gained from the fragment-bound structures in Objective 2 will be used to enlarge, link or merge various fragments into novel lead molecules *via* structure-guided design and synthetic chemistry. These new molecules will be tested for both *in vitro* and *in vivo* activity against BpML1 from multiple pathogenic bacteria species.

Approach: Fragment-derived lead design will be used as search criteria across commercial catalogs to find cheaply available chemical analogs that resemble linked, merged, or enlarged variants of our fragment-based designs. Simple modifications of commercially available starting products will be designed and synthesized by Kalexsyn in Kalamazoo, MI (*see letter of support from Dr. Robert Gadwood*, Business Proposal Section 6). Since there are limited funds available for this proposed Functional Study, we will limit the number of purchased synthetic molecules to ~10 carefully designed novel small molecule inhibitors of BpML1 (*See Emerald's Budget Justification,* Business Proposal Section 2). The candidate small molecules will first be assayed *in vitro* by measuring inhibition of BpML1 enzyme activity. The most potent inhibitors of BpML1 will then be subjected to *in vivo* characterization by measuring their ability to inhibit cell growth in liquid culture of *Burkholderia* strains that harbor mutations making them permeable to small molecules (*see Letter of Support from Dr. Colin Manoil*, Business Proposal Section 6). The use of these strains will overcome the challenge of small molecule entry and efflux across the double cell wall of Gram-negative bacteria. Follow-up *in vivo* experiments will then be performed using *Brucella*, *Yersinia* and *Francisella*, to determine whether the inhibitors have broad-spectrum antibiotic activity. This objective will be carried out at DSTL.

Time-line:

Month 1	Conduct (STD)-NMR screening of fragment pools (Emerald)
Month 2	Verify and de-convolute fragment hits from pools *via* (LB)-NMR and NOESY (Emerald)
Month 3	Confirm fragment hits using SAR-by-NMR (Emerald)

Month 4	Co-crystallization and structure determination of validated fragment hits (Emerald)
Months 5-7	Synthesis of fragment-inspired small molecules (Kalexsyn)
Months 8-9	*In vitro* assay of inhibition of BpML1 activity (DSTL)
Months 10-12	*In vivo* assay of inhibitors against *Burkholderia, Brucella, Yersinia* and *Francisella* (DSTL)