

Functional Study 1: Use structure-based inhibitor design to assess the function of bacterial immunophilins as potential targets for broad-spectrum antibiotic discovery.

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Status: Project completed

Timeline:

ORIGINAL TIMELINE	ORIGINAL MILESTONE	REVISED MILESTONE	ACHIEVED
Quarter 1 09.2012- 11.2012	<ul style="list-style-type: none"> screen fragment pools STD-NMR (Emerald) verify fragment hits by LB-NMR and NOESY (Emerald) Confirm fragment hits using SAR-by-NMR (Emerald) 	<ul style="list-style-type: none"> screen 50% of fragment pools by HMQC-NMR (Emerald) verify fragment hits using CSA (Emerald) 	X
Quarter 2 12.2012- 02.2013	<ul style="list-style-type: none"> co-crystallization of validated fragment hits (Emerald) Initial synthesis of fragment-inspired small molecules (Kalexsyn) 	<ul style="list-style-type: none"> complete fragment screening (Emerald) SAR by catalog (Emerald) initial hit-to-lead design and synthesis (Würzburg) 	X
Quarter 3 03.2013- 05.2013	<ul style="list-style-type: none"> continued small molecule synthesis (Kalexsyn) <i>in vitro</i> assay for inhibition of BpML1 activity (DSTL) 	<ul style="list-style-type: none"> determine relative binding affinities of fragments (Emerald) continued hit-to-lead design and synthesis (Würzburg) <i>in vitro</i> activity assay (DSTL) 	X
Quarter 4 06.2013- 08.2013	<ul style="list-style-type: none"> <i>in vivo</i> assay of inhibitors against <i>Burkholderia</i>, <i>Brucella</i>, <i>Yersinia</i> and <i>Francisella</i> (DSTL) 	<ul style="list-style-type: none"> co-crystallization of fragments and top lead molecules (Emerald) continued lead optimization (Würzburg) <i>in vitro/in vivo</i> activity assays (DSTL) 	Partially

Quarter 1 (September-November, 2012): Work performed during this period was described in detail in the First Quarterly Report (2012_24). Briefly, we successfully screened 50% of the FOL library against BupsA.00130.a (BpML1) using HMQC-NMR and identified three compounds that appear to bind in the vicinity of the active site. HMQC titration experiments were then initiated to allow a quantitative assessment of Chemical Shift Anisotropy (CSA), and generate binding affinity data for use in structure activity relationship (SAR) analysis by catalog, as well as synthetic fragment derivatization.

Quarter 2 (December 2012 - February 2013): Work performed during this period was described in detail in the previous Semi-Annual Report (2013_09). Briefly, the FOL

screening campaign was completed, resulting in a total of four confirmed fragment hits. Catalog SAR was conducted, and 31 compounds purchased for testing. Three key regions of the original pipercolic acid compound series from Community Request 08 were selected for synthetic derivatization, using structural information generated by SSGCID. Synthetic pathways for these new compounds were mapped out, and those successfully synthesized were queued for testing at DSTL for PPIase activity.

Quarter 3 (March - May, 2013): Work performed during this period was described in detail in the previous Quarterly Report (2013_18). Briefly, 4 FOL fragments and 2 catalog-search variants were selected for crystallization trials based on HMQC-NMR data. Novel compound synthesis using structure-guided design began, and continued compound testing occurred for these compounds at DSTL. Crystallization attempts were initiated for the 6 NMR-based screening hits.

Quarter 4 (June - August, 2013): Work performed during this period included extensive crystallization efforts with the 4 FOL fragments and 2 SAR-by-catalog compounds identified as binders by HMQC-NMR. Multiple crystallization conditions developed optimized from the previous pipercolic acid derivative compound series were used for co-crystallization attempts with these 6 small molecules. Crystals of BupsA.00130.a were obtained from sitting drops containing the small molecules, and diffraction data was collected for over 40 crystals. Several of these crystals diffracted to ≤ 2.5 Å and a structure solution was possible using standard molecular replacement methods. Unfortunately, none of the datasets showed clear evidence of fragment binding in the electron density. To investigate this outcome, stock solutions of all 6 compounds were sent to DSTL for activity testing. All 6 compounds showed some ability to inhibit the peptidyl-prolyl isomerase activity of BupsA.00130.a *in vitro* (**Figure 1**). However, the inhibitory strength of these compounds relative to that of the lead compound series of pipercolic acid derivatives suggested that their affinity may be too weak to obtain crystallographic evidence of binding. Therefore, crystallization efforts have been halted with these materials. Instead, a concerted effort has begun at Würzburg to computationally predict the binding modes of these compounds using *in silico* methods. Due to the high structural similarity across 5 of the 6 compounds, consistency across the test set should provide a robustness score for the methods. If this can be achieved, then the predicted binding modes may be explored for structural information on how one or more of these 6 compounds may be chemically elaborated to enhance potency sufficient for further testing and experimental structural characterization.

Both structural and biological testing of the pipercolic acid compound series has continued, with a new emphasis on stereospecific output of compounds. The consistent observation of "S" enantiomers with increased potency, and a selection of this conformation to the exclusion of all "R" conformations crystallographically, indicates the importance of isomeric purity of compounds. To ensure higher potency with the lead series, stereospecific synthetic routes were planned and tested; but they still contained ~10% of the "R" conformation, requiring difficult stereospecific purification. The synthetic route for "S" conformation pipercolic acid derivatives has been optimized during this period, and is now being executed for synthesis in compound derivatization (**Figure 2**). Over 20 of the original 42 compounds designed from structural information in Q3 have now been synthesized, with ~10 passing initial synthetic tests for tractability.

These are being sent to DSTL for potency testing by PPIase activity assay, which will continue until all compounds are tested. Work conducted at DSTL included more quantitative methods to determine relative inhibitory constants of lead compounds derived from the original pipercolic acid series (**Figure 3**). Although compound CK168 still shows the strongest inhibitory strength, both SF30 and SF35 show reduced toxicity in normal human macrophages. This is likely due to insufficient host-parasite specificity in CJ168, which may bind to highly homologous human FKBP. Since SF35 is the stronger of the two lead series candidates, this compound was selected for additional testing in macrophage infectivity assays, and for further chemical diversification to improve potency.

A major revision of a manuscript summarizing work on BupsA.00130.a was initiated in Q3, with new and revised figures created before re-submission. The manuscript is currently under review at *Antimicrobial Agents & Chemotherapy*. In addition, the Würzburg group is preparing a proposal (in collaboration with Dstl, UWa, Seattle BioMed and Emerald Bio) for submission to the NATO Science for Peace and Security Programme, seeking funding to continue optimization of these Mip inhibitors and test their broad spectrum efficacy against biowarfare agents. If successful, this will mark an important milestone for our efforts to transition projects away from SSGCID's internal funding by collaboration with members of the scientific community outside the SSGCID consortium.

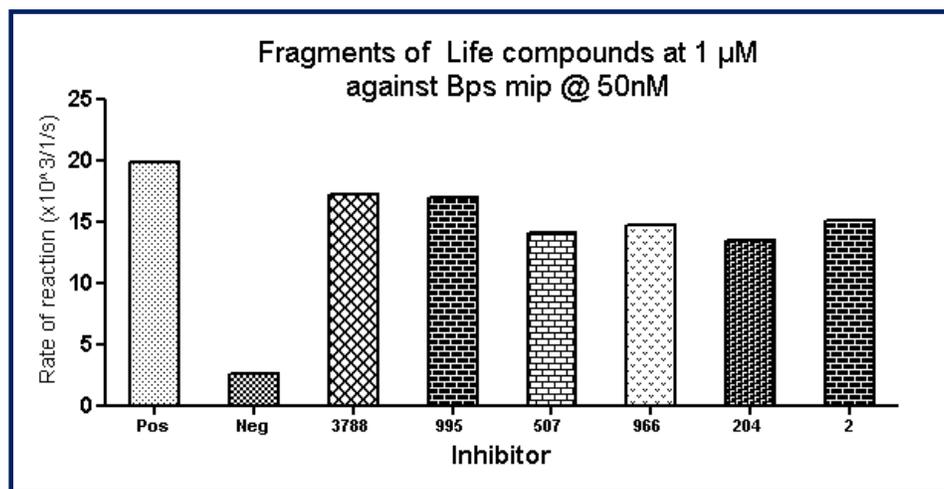


Figure 1. PPIase activity of 6 FOL-derived fragment binders.

Four FOL fragment hits (2, 204, 507, 995) and two catalog-by-SAR compounds (966, 3788) were tested for *in vitro* peptidyl-prolyl isomerase (PPIase) activity at 50 nM against BupsA.00130. All compounds showed measurable inhibition, but none were observed to be as strong as that seen for the pipercolic acid derivative lead series.

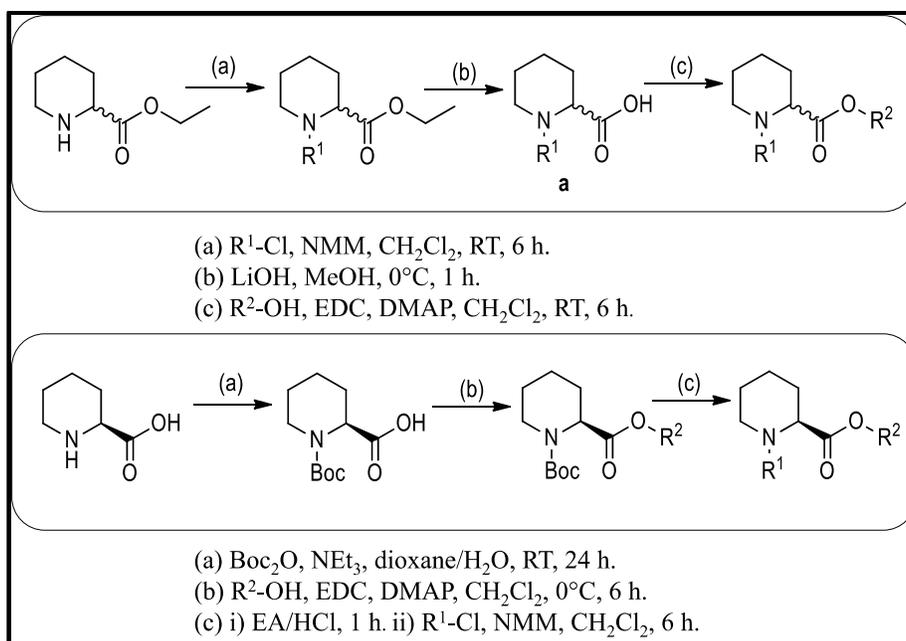


Figure 2. Synthetic routes for pipecolic acid inhibitor scaffold

Both racemic (top) and enantiomerically pure (bottom) synthetic pathways have been optimized for producing the pipecolic acids which serve as the main scaffold for lead inhibitor series development. These methods now produce ~100% "S" enantiomers for scaffold production and compound derivatization.

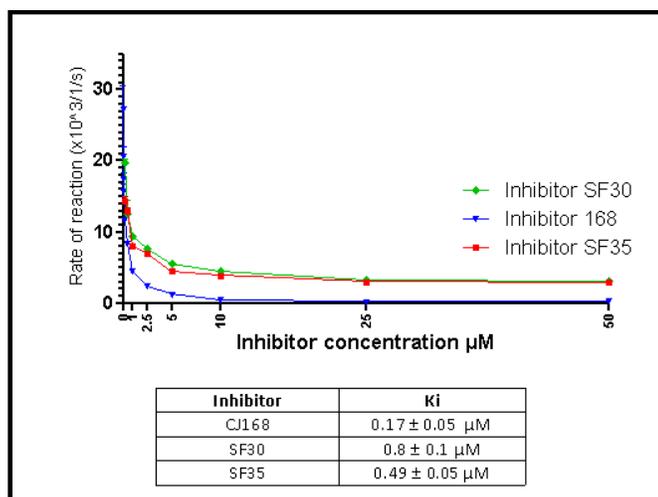


Figure 3. Inhibitory potency of lead candidates in pipecolic acid compound series

Quantitative *in vitro* activity assays were conducted to determine relative inhibitory strengths of lead candidates SF30 and SF35. Although CJ168 still shows the strongest potency, SF35 possesses more amenable chemical properties for drug design, with less toxicity in normal human macrophages. SF35 is now prioritized for whole cell macrophage infectivity testing and further chemical elaboration.