Yale University
Final Technical Report

Period Covered by the Report: 09/01/2022 through 1/03/2024

Date of Report: 4/2/2024

Project Title: Rapid Development of Bacterial Toxin-neutralizing Small Molecule Chimera
Contract Number:
Total Dollar Value: $984,098
Subcontractors: Wuxi Apptec / Proteros
Program Manager: Dr. Christopher Bettinger, DARPA, BTO
Sponsored by: Defense Advanced Research Projects Agency Biological Technologies Office (BTO)
Program: Harnessing Enzymatic Activity for Lifesaving Remedies (HEALR)
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1 High-Level Project Progress

1.1 Big Wins

During the no-cost extension period, we have conducted fragment-based and DNA-encoded library (DEL) screening efforts to identify authentic ligands for IgG1. Of these two approaches, the **DEL approach** has proven more successful, leading to the identification of *multiple ligands from diverse chemical libraries* that are *highly enriched* in the DEL screen compared to a no-target control experiment.

1.2 Go/no-go Progress

Accomplishment *Why is this significant? ID TA.*	**Month** *From Kick-off Presentation*	**Update** *What is the current status? Explain any discrepancies (behind/ahead of schedule). What is the next step?*
TA 2.1 Host ligand binding and medicinal chemistry optimization. Validate and identify ligands for the host protein	14	Behind schedule. Diverse ligand candidates identified from DNA-encoded library screening.

2 Schedule – Milestones and Deliverables

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	Duration	Start	Finished	Predecessors	11/1/2022	12/1/2022	1/1/2023	2/1/2023	3/1/2023	4/1/2023	5/1/2023	6/1/2023	7/1/2023	8/1/2023	9/1/2023	10/1/2023	11/1/2023
TA 1																	
Task 1.1. *In Silico* Toxin Screening	29	1/3/2023	2/1/2023														
Task 1.2. Toxin-binding Med Chem and Binding Optimization		2/13/2023	ongoing														
TA 2																	
Task 2.1. Host Ligand Medicinal Chemistry and Binding Optimization		12/1/2022	ongoing														
Task 2.2. X-ray crystallography of Host Protein with identified ligands																	
TA 3																	
Task 3.1. Toxin Target 1 SIDTAC Generation and Characterization				TA 1.2 + TA 2.2													
Task 3.2. SIDTAC Biological Characterization				TA 3.1													
Task 3.3. SIDTAC MoA Characterization				TA 3.1													

Team members: Saul Figueroa (SJF), John Hines (JH), Rediet Delelegne (RD)

Milestone/ Deliverable Description	**Responsible team members**	**Start Date**	**Due Date**	**Actual State Date**	**Actual End Date**	**Status**	**Dependencies** *Across tasks and teams (if applicable)*
Milestone 1.1.1.1.	SJF	12/01/2022	02/01/2023	01/03/2023	02/10/2023	Complete	
Milestone 1.1.1.2.	SJF / JH / RD / Wuxi	02/13/2023	05-07/2023	March 2023	08/04/2023	Complete	
Milestone 1.2.1.1.	SJF / JH / RD / Wuxi	05-07/2023	08-09/2023	N.A.	N.A.	N.A.	

Milestone 1.2.1.2.	SJF / JH / RD / Wuxi	08-09 /2023	09-10 /2023	07/15/ 23	10/15/ 23	Initiated. Effort suspended.	
Milestone 1.2.1.3.	SJF / JH	08-09 /2023	09-10 /2023			Not Started.	Need milestone 1.2.1.2
Milestone 2.1.1.1	SJF / JH / RD / Wuxi	12/01/ 2022	07/20 23	12/01/ 23	1/2/24	Complete	
Milestone 2.1.2.1	Proteros	12/20 22 or 01/20 23	03/20 23			N.A.	Dependent on milestone 2.1.1.1 Funds diverted for renewed screening effort.
Milestone 2.1.2.2.	Proteros	02- 03/20 23	06/20 23			N.A.	Need milestone 2.1.2.1 Funds diverted for renewed screening effort.
Milestone 3.1.1.1	SJ, Wuxi, JH, RD	06/20 23	08/20 23			N.A.	Need milestones 1.2 and 2.1 completed – unlikely to complete due to suspended efforts on milestone 1.2
Milestone 3.2.1.1.	JH, RD	09/20 23	11/20 23			N.A.	Need milestone 3.1
Milestone 3.3.1.1	JH, RD	09/20 23	11/20 23			N.A.	Need milestone 3.1

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3 Task Progress, Accomplishments, and Plans

Task #/Title	Brief Description	% Complete	Total for Task	Total Spent	Remaining to Spend	Explain Deviations between Planned vs. Actual Expenditures
1.1. *In Silico* Toxin Target Screening	*In silico* screening of 14,000 member library for potential small molecules ligands to Toxin Protein	**100**	**$6000**	**$6,000**	**$0**	
1.2. Toxin-binding Medicinal Chemistry and Binding Optimization	SAR studies will be performed on known literature compounds and HTS hits to identify features critical for Toxin binding and efforts made to increase the potency and binding specificity of lead compounds for Toxin protein.	**50**	**$117,000 (1/3 Wuxi) + $9,998.33 (consumables + analysis)**	**$91,568.86 (WuXi) + $25,970.12 (consumables + analysis) + $1,557.70 (unplanned maintenance)**	**$7,901.65 (net)**	Over budget on consumables used due to longer duration of this task. Minor unplanned maintenance. Remaining funds reallocated to task 2.1
2.1. Host Ligand Medicinal Chemistry and Binding Optimization	Medicinal Chemistry Efforts will be made to increase the binding potency and specificity of lead compounds for the Host Target protein	**50**	**$117,000 (1/3 Wuxi) + $61,200 (Proteros) + $13,996.67 (consumables + analysis)**	**$255,137.72 (WuXi) + $55,509.26 (consumables + analysis) + $1,557.70 (unplanned maintenance)**	**– $120,008.01 (net)**	Host ligand medicinal chemistry not yet complete. Increased allocation of WuXi funds from task 3.1 We have delayed actioning Proteros services until the med chem reaches an appropriate stage. Over budget on consumables used due to longer duration of this task. May need to reduce scale of collaboration with Proteros.

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						Minor unplanned maintenance.
3.1. Toxin Target 1 SIDTAC Generation and Characterization	X-ray crystallography of Host Target ligands complexed with the Host Target protein	**0**	**$117,000 (1/3 Wuxi) + $9,998.33 (consumables + analysis)**	**$0**	**$126,998.33**	Waiting on tasks 1.2 and 2.1. Have reallocated all of these WuXi dollars to completion of task 2.1
3.2. SIDTAC Biological Characterization	Host Target Ligands will be coupled to Toxin Ligands to generate SIDTACs. These will be tested for retention of Toxin binding and specificity	**0**	**$9,998.33 (consumables + analysis)**	**$0**	**$9,998.33**	Funds reallocated to task 2.1
3.3. SIDTAC MoA Characterization	SIDTACs will be characterized in cell-based assays of toxicity. Efficacy will be compared to the SoA orally available antitoxins	**0**	**$9,998.33 (consumables + analysis)**	**$0**	**$9,998.33**	Funds reallocated to task 2.1
Subtotal		–	**$472,190**	**$437,301.36**	**$34,888.66**	
Personnel		–	**$104,556**	**$126,995.50**	**–$22,439.50**	
Indirect Costs		–	**$404,876**	**$393,376.70**	**$11,499.30**	

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Technical Area 1: Toxin protein ligand enablement. Ligand discovery and medicinal chemistry optimization for PA_{83}.

Our overall goals for Technical Area 1 were the identification of authentic ligands for the Anthrax protein PA_{83} for incorporation into a neutralizing SIDTAC bifunctional molecule. The effort to identify a suitable ligand took longer than we expected and ultimately we had to suspend this effort to focus on progressing Technical Area 2. We prioritized Area 2 as it was more central to the SIDTAC *concept*, rather than a specific *application* of SIDTAC technology.

While pursuing toxin ligand enablement, we pursued two approaches towards identification of ligand candidates. Each approach utilized Surface Plasmon Resonance (SPR) binding assays to quantify binding of the ligand candidates with the toxin. In this assay, the therapeutic antibody Raxibacumab was used as a positive control – indicating that the PA_{83} protein was immobilized in a state that mimicked the biologically-active form of the protein. We note that pursuing a DEL screen (as we ultimately did in Technical Area 2) was not a feasible approach in Area 1, as *no tagged PA_{83} construct is commercially available*.

In our first approach for ligand identification, we performed in-house synthesis of four ligands reported in the scientific literature as candidate binders of PA_{83} based on computational studies. These candidates included the known PAINS compound quercetin and three other compounds that looked likely to have poor solubility (at best) or to also be spurious PAINS compounds. Indeed, after resynthesis, we confirmed that the literature candidates had extremely poor solubility properties and we were unable to validate any binding behaviour of these compounds with PA_{83}.

Second, we conducted our own *in silico* study based on a known crystal structure of PA_{83}. A commercial library of drug-like small molecules was docked to PA_{83} and scored using the MOE software package. 50 ligand candidates with the most promising docking scores were purchased and assayed against PA_{83} by SPR. As in the case of the literature compounds identified using a computational workflow, we were unable to measure any binding in the biophysical assay.

At this time, we had begun to identify ligand candidates for Technical Area 2. Before we were able to undertake a third independent approach to ligand identification in Area 1 (such as a high-throughput screening approach), we performed a counterscreen of candidate Host Protein ligands, hoping to identify ligand candidates that were *selective* for interacting with the Host protein over the Toxin. In this experiment, we were surprised to observe that each validated host protein ligand **also interacted with the toxin!**

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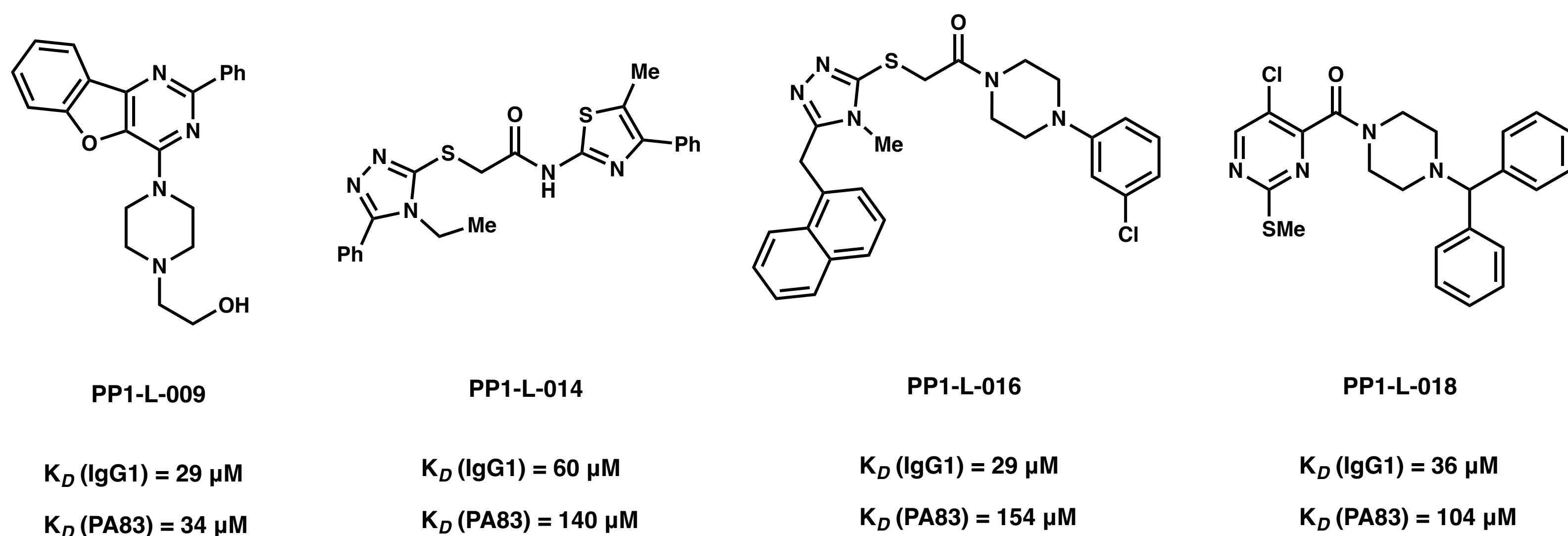

Figure 1. Counterscreen of host protein ligands shows off-target binding to PA83.

Faced with this result, we chose to pursue a *divergent* optimization of these ligand candidates, aiming to accomplish a simultaneous increase in affinity for binding to the host and toxin proteins and also an introduction of selectivity, where some compound analogues would be rendered specific binding partners for PA_{83} while others would be selected for specific binding to IgG1.

In reality, we found these ligand candidates to be intractable to optimize. While some modifications to the scaffold, particularly for **L-009** seemed to show progress for affinity and selectivity, these experiments were challenging to reproduce. Upon further investigation, these results in our SPR assay were ultimately attributed to a solubility artifact, causing us to reconsider the viability of continuing optimization of **L-009** and the other screening hits.

With this information, we renewed our screening efforts to identify viable ligand candidates via a DEL approach. Due to our remaining funds/resources, we made the difficult to choice to restrict this effort to identification of a Host Protein ligand (i.e. restricted to Technical Area 2) and discontinued our effort on Technical Area 1.

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Technical Area 2: Host protein ligand enablement. Ligand discovery for IgG1 Fc.

Our overall goals for Technical Area 2 were the identification of authentic ligands for the Host protein IgG1 (crystallizable region; Fc) for incorporation into a neutralizing SIDTAC bifunctional molecule. The initial approach used for ligand identification in the first 3 quarters seemed to lead to promising results; however, our efforts to optimize the original hits proved unfruitful and it became clear that we needed to restart our screening efforts with a different approach in the 4th quarter and during a no-cost extension period.

Our approach to ligand identification utilized SPR as a primary biophysical assay to assess and/or validate ligands for their interaction with the Fc protein. For SPR experiments, we originally utilized an immobilization strategy in which the full-length IgG1 adalimumab (Humira) was immobilized to the SPR chip via its interaction with its target TNFα. We selected this approach (rather than direct covalent immobilization of an Fc fragment) in hopes that immobilization using the antigen-binding region of the protion would 1) maximize the solvent and ligand exposure of the Fc region and 2) to minimize any conformational change to the Fc region. In this effort, we utilized the known 13-mer peptide Fc-III as a positive control (Figure 2). However, this protocol proved to be too costly, both monetarily (due to consuming significant amounts of both TNFα and Humira) and in terms of lab consumables (specifically streptavidin-functionalized SPR chips).

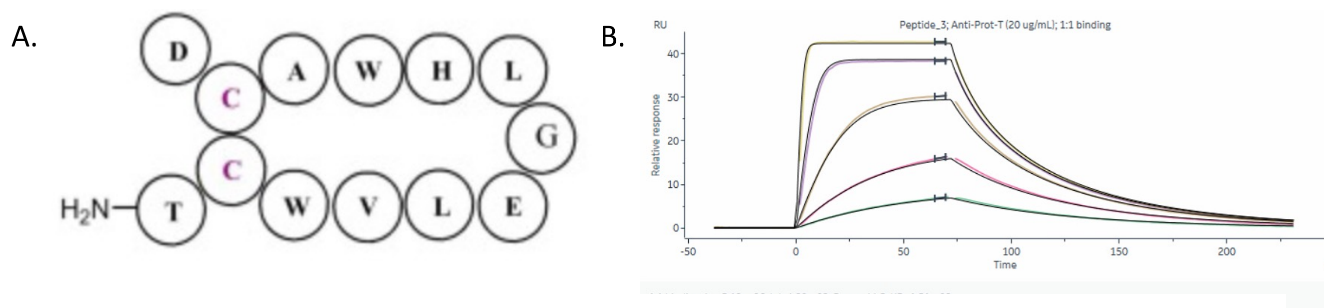

Figure 2. A. Fc-III peptide structure. B. Validated 15 nM bindin

Due in particular to inconsistent availability and shipping ... to use an alternative SPR assay. We began to express an N-terminal His-tagged c ... Herceptin) in house.

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While the yields of this protein production were not high, we were able to immobilize this His-tagged protein on Ni/NTA functionalized SPR chips, which could be regenerated and reused – eliminating our experimental bottleneck.

At the outset of the award period, we took a dual approach to identify Fc ligand candidates. As in the case of Technical Area 1, one approach was an *in silico* evaluation of commercially available ligand candidates at the dimer interface of the two heavy chain components of the Fc protein. This site was selected because it is distant from the binding site of endogenous interacting partners, such as FcRn. 50 compounds with the most promising docking scores were purchased and evaluated by SPR. Unfortunately, we were unable to detect meaningful binding of these molecules to an IgG1 protein by SPR.

In tandem, we screened a collection of compounds available through Yale in a series of high-throughput SPR experiment. This experiment was not completely unbiased with respect to the ligand identiy, as the library was initially filtered based on physicochemical properties and a “druglikeness” score. The ligands were then evaluated for interaction with Humira, Remicade (another anti-TNFα) and against the Fc fragment alone (in duplicate). ~60 screening hits were prioritized for 1) apparent potency and 2) similar behavior with each protein construct. These compounds were then resynthesized by the chemistry FTE team at WuXi Apptec.

After resynthesis at WuXi, 5 of the hit compounds still showed binding activity in SPR assays. Orthogonal validation in ITC and DSF assays was attempted without promising results. Due to the moderate-to-poor binding affinity of the unoptimized hits, we did not feel like the lack of activity in these assays was necessarily a strike against the candidate ligands. We therefore moved forward with the synthesis of chemical analogues, aiming to improve the affinity of these ligands and understand the structure-activity relationships driving interaction with IgG1. The scaffold corresponding to **L-009** (see figure 3, below) was prioritized as a primary chemotype for analogue development, while the other scaffolds were treated as backup chemotypes.

At this time, we also performed a counterscreen against the target protein PA_{83} (Technical Area 1, Figure 1). Surprisingly, we observed that each screening hit also bound to the toxin in our SPR assay. While this was concerning as a possible sign of PAINS-like activity, testing against a third (orthogonal) protein seemed to provide reassurance that the interaction was not completely unspecific. At the time we thought the non-specificity might be driven by the high lipophilicity of the screening hits. We therefore proceeded with optimization of these hit compounds, aiming to achieve increased selectivity in addition to increased potency.

While optimization of the HTS hits provided some hints of improved affinity (Figure 3), the results were somewhat irreproducible and the quality of the SPR sensorgrams was poor. These observations increased our concern that the original HTS reselts were due to PAINS-like activity, rather than indicative of a genuine binding interaction. As the hit chemotypes did not contain known PAINS motifs, we suspected aggregation/poor solubility might be responsible for the SPR results. Poor solubility of the HTS hits and their analogues was confirmed by 1) allowing solutions in aqueous buffer to stand overnight (leading to observation of a visible precipitate),

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and 2) examination of freshly-prepared solutions in aqueous buffer under a microscope (where fine particulates were observed in suspension).

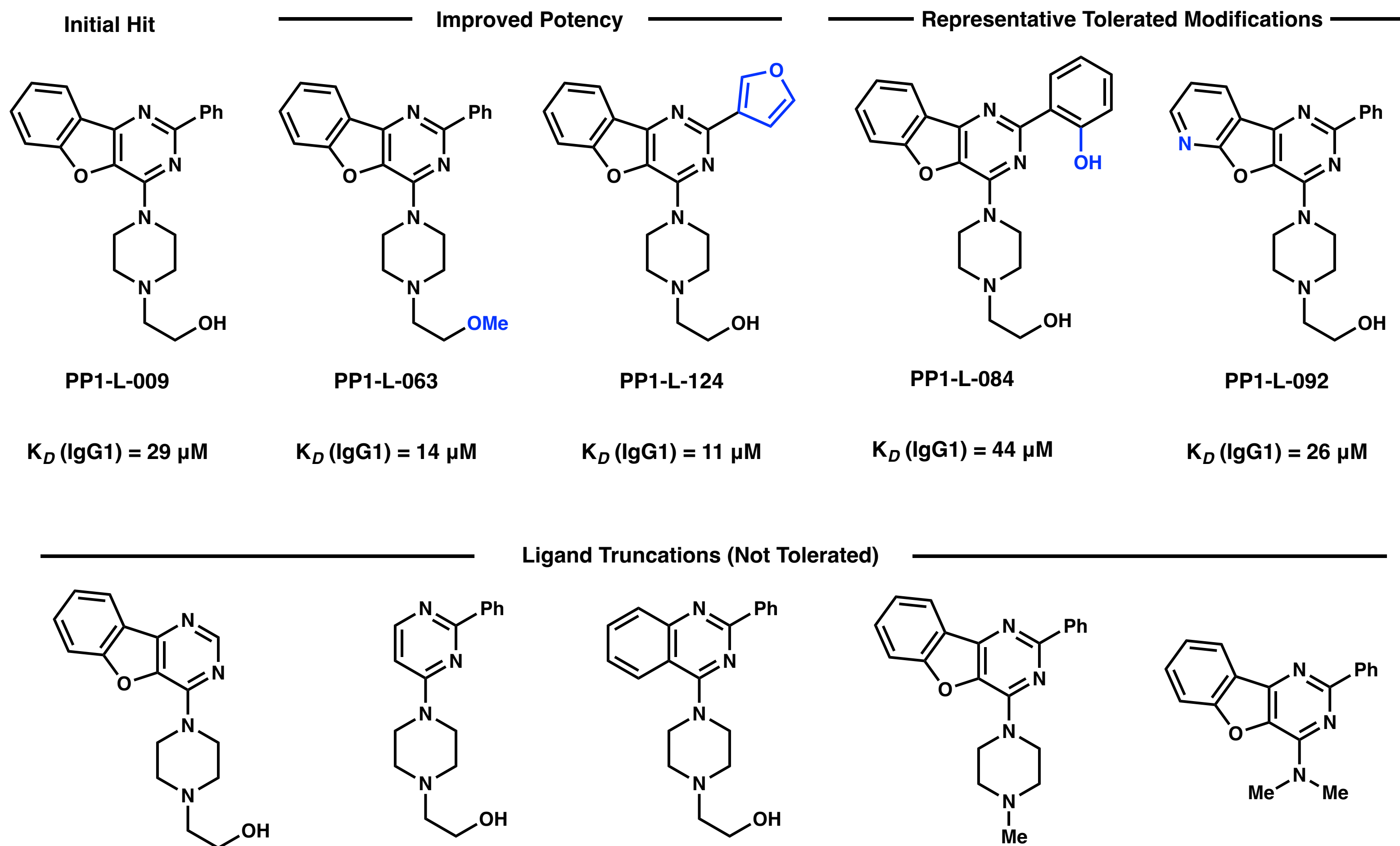

Figure 3. Representative ligand modifications leading to improved or equipotent binding as well as loss of activity.

Once it became clear that the HTS hits were not viable leads for ligand optimization, we decided to return to a renewed screening effort. At this stage, we explored 1) fragment-based screening, hoping that fragment-sized molecules would have more favorable

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solubility properties, and 2) DNA-encoded library screening. The fragment-based approach was not fruitful. Only a small number of ligands appeared to show any interaction with the Fc protein; however, these hits proved to have solubility issues upon closer inspection. Furthermore, analogues synthesized in-house did not show any binding activity.

Ultimately, the DEL approach proved the ideal approach to identifying small-molecule binders of the IgG1 Fc. We performed 3 selection rounds in our screen, incubating the small-molecules with a N-terminal His-tagged Fc construct. After the selection, analysis of the DEL kit revealed that certain small molecules were enriched up to 10,000-fold relative to a no-Fc (beads only) control sample. 9 compounds with the highest enrichment scores have been prioritized for re-synthesis in order to quantify the binding affinity of these compounds for the Fc protein (Figure 4), although we were unable to initiate this work within the funding period of the current award.

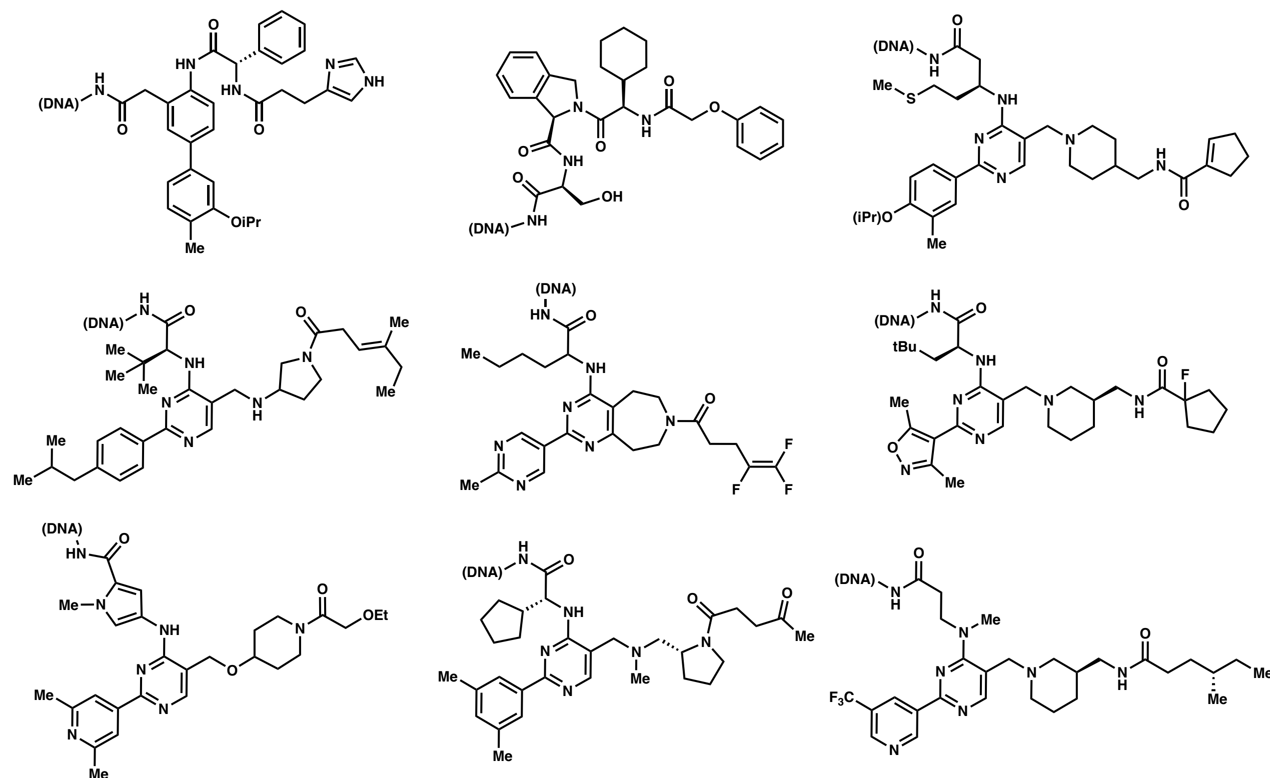

Figure 4. Highest-enrichment ligand candidates from DEL screening.

4 Project Coordination, Dissemination, and Translation Efforts

4.1 Project Coordination

The team at Yale meets on a bi-weekly basis to align on the progress of the different tasks and make the necessary adjustments based on the most recent results. All members of the Yale team are present and meet in person: Pr. Craig Crews, Dr. John Hines, Dr Saul Jaime Figueroa and Rediet Delelegne.

The Yale and Wuxi teams meet on a weekly basis, every Wednesday evening at 8:30pm. Are present Dr Figueroa from Yale and Zuo Yang from WuxiApptec. During this meeting, Wuxi updates on their progress for the week and Dr. Figueroa offers solutions if there are any chemistry roadblocks. For time sensitive matter or if major issues arise, the teams communicate by email during the week between meetings.

4.2 Dissemination and Translation (if applicable)

Non-applicable for the moment.

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5 Publications and Presentations

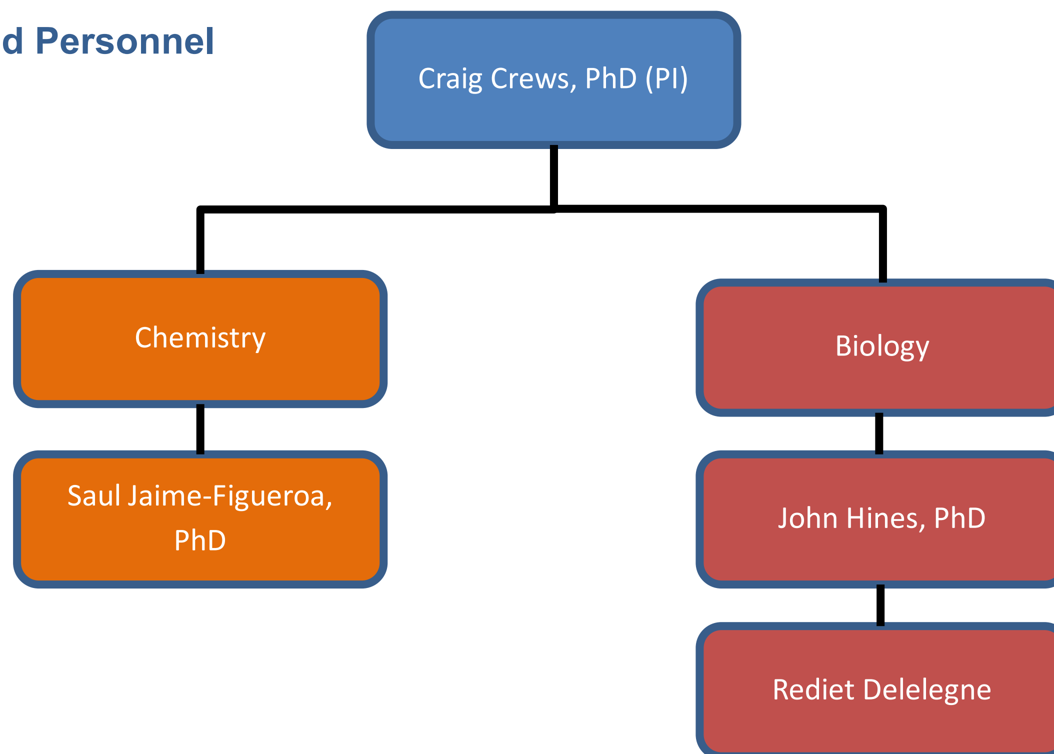
None.

6 Patents, Invention Disclosures, IDEs, etc…

None.

Appendix I – Project Context

Teaming and Personnel



Prime Team Members and Contact Information: [Name]

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Role	Full Name	Contact Information (phone and email)	Major Role(s)
PI	Craig Crews	craig.crews@yale.edu	
Associate Research Scientist in Chemistry	Saul Jaime Figueroa	saul.jaime-figueroa@yale.edu	Drive and support all chemistry efforts on the project
Associate Research Scientist in Biology	John Hines	john.hines@yale.edu	Drive all biology efforts on the project
Postbac	Rediet Delelegne	rediet.delelegne@yale.edu	Support and execute most biology efforts

Subcontract Team Members and Contact Information: [Name]

Role	Full Name	Contact Information (phone and email)	Major Role(s)
Chemistry CRO coordinator (Wuxi Apptec)	Declan Ryan	declan.ryan@wuxiapptec.com	Relations between Wuxi and Yale
Chemistry CRO team leader	Zuo Yang	zuo_yang0301@wuxiapptec.com	Leads the synthetic efforts at Wuxi.
Crystallography CRO project leader (Proteros)	Jonathan Davies	davies@proteros.de	Coordinates crystallography efforts at Proteros

Work Breakdown Structure

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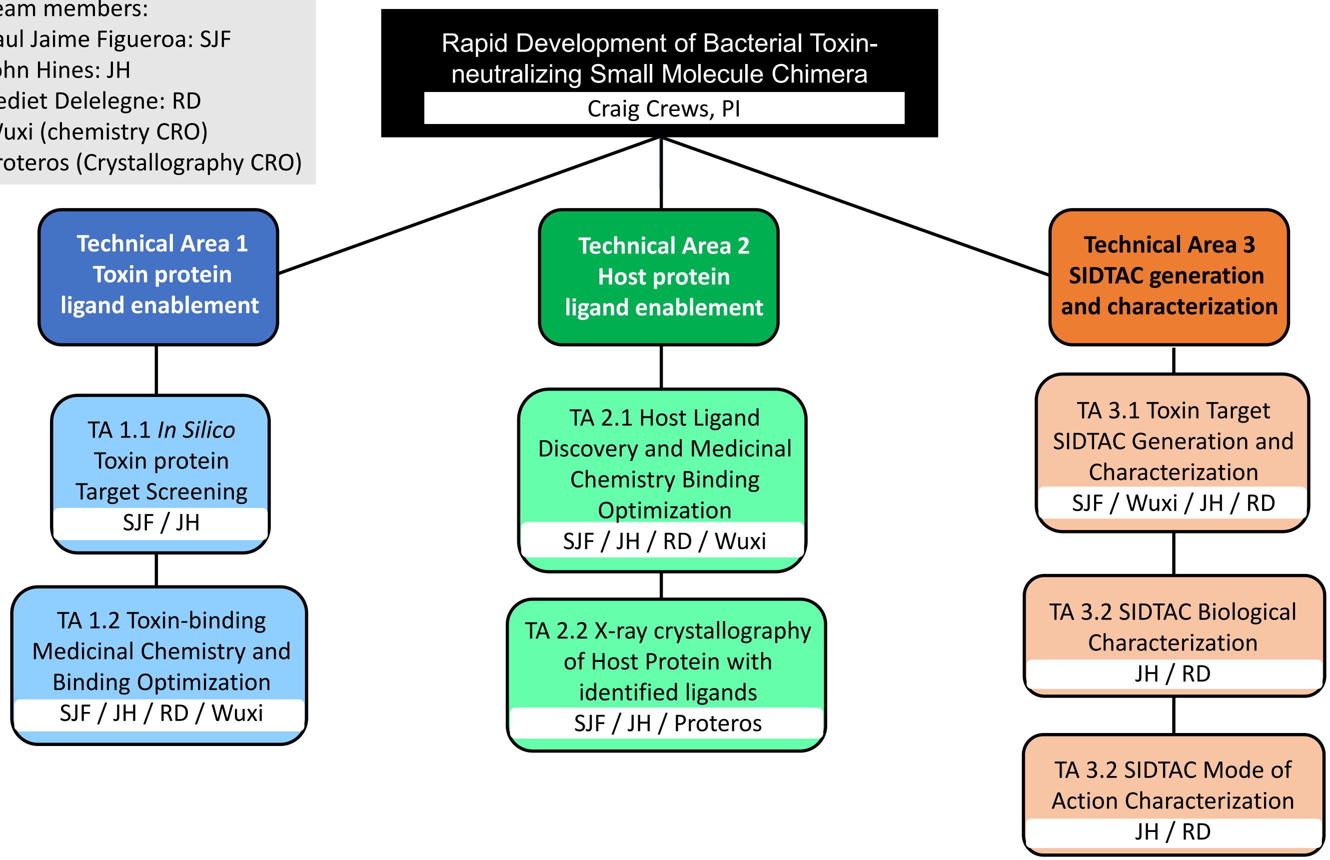

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