SAND20XX-XXXXR
LDRD PROJECT NUMBER: 215998
LDRD PROJECT TITLE: Screening for Dimerization Inhibitors for Bacterial Acetyl CoA Carboxylase
PROJECT TEAM MEMBERS: Michael Kent, Ken Sale, Bryce Ricken, Daniella Martinez, Miranda Juarros, Grover Waldrop (LSU)

ABSTRACT:
The goal of this project was to develop compounds that kill biothreat bacteria by inhibiting the activity of bacterial acetyl CoA carboxylase (ACC) by blocking dimerization of one component of that enzyme complex, biotin carboxylase (BC). The ultimate goal is to combine a BC dimerization inhibitor with an existing active site inhibitor for carboxytransferase, another component of ACC, to form a new dual inhibitor therapy for this essential enzyme to avoid onset of resistance. Developing medical countermeasures to defeat antibiotic resistance in biothreat agents is of interest for national security for protecting both warfighters and the public.

INTRODUCTION:
The goal of this work is to develop a new combination therapy against acetyl CoA carboxylase (ACC) to defeat antibiotic resistance (AR). The rapid emergence of resistance to antibiotics (ABs) in bacterial populations is occurring worldwide and reaching a crisis stage.(Ventola 2015, Ventola 2015) The recurring cycle of AB discovery, acquired resistance, synthesis of variants, and re-emergence of resistance occurs because with monotherapy a single mutation is sufficient to provide resistance to an AB.(Drlica 2003, Drlica and Zhao 2007) While improved AB management strategies can increase the time to develop resistance,(Ventola 2015) a new approach is needed to break the cycle. Combination therapies are recognized as a promising path forward.(Toleman, Bennett et al. 2007, Fischbach 2011, Munck, Gumpert et al. 2014, Walsh and Wencewicz 2014, Ventola 2015) In contrast to anti-cancer and antiviral therapies, historically monotherapy has been the default pattern for prescribing antibiotics.(Silver 2007, Walsh and Wencewicz 2014) The clinical effectiveness of combination therapy in slowing the development of resistance has been proven in the treatment of *M. tuberculosis*, the causative agent of tuberculosis.(Pokrovskaya and Baasov 2010) However, promising combination therapies to date have been limited to existing drugs for which resistance has already occurred in natural populations, sometimes limiting their effectiveness.(Toleman, Bennett et al. 2007) In addition, combinations have typically involved drugs that target different proteins and physiological processes, sometimes leading to compromising interaction effects.(Bollenbach 2015) Our hypothesis is that using two new antibiotics simultaneously targeting an essential enzyme will beat mutational statistics in bacterial populations. Evidence from the use of chloroquine against malaria,(Wootton, Feng et al. 2002, White 2004, Summers, Dave et al. 2014) antibiotics against bacteria,(Drlica and Zhao 2007, Silver 2007, East and Silver 2013) recent development of a

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vancomycin derivative that acts by multiple mechanisms in G+,(Okano, Isley et al. 2017) and modeling(Durrett and Schmidt 2008) indicates that two specific mutations will be statistically unlikely to occur within bacterial populations for many decades. If successful, our work will break from the historical monotherapy approach and will provide a new target-specific combination therapy applicable to a broad spectrum of gram-positive and gram-negative infections with low susceptibility to resistance.

ACC is an essential enzyme in both bacteria and eukaryotic cells, but differs structurally in these two domains of life. ACC catalyzes the ATP-dependent carboxylation of acetyl CoA to generate malonyl CoA, an essential step in fatty acid synthesis.(Polyak, Abell et al. 2012, Tong 2013) ACC is a new target for antibiotics (no drugs on the market yet) that has been thoroughly validated in prior work by Pfizer.(Cheng, Shipps Jr. et al. 2009, Miller, Dunham et al. 2009, Mochalkin, Miller et al. 2009, Walsh and Fischbach 2009, Polyak, Abell et al. 2012, Tong 2013) Pfizer has since severely cutback research on new ABs. Bacterial ACC is an especially target-rich enzyme complex with multiple ways to achieve a combination of two inhibitors. It is composed of two enzymes (biotin carboxylase (BC) and carboxytransferase (CT)) and biotin carboxyl carrier protein. A lead active site compound for CT is already known.(Freiberg, Pohlmann et al. 2006) Another lead compound is needed to form a combination therapy against bacterial ACC. To provide the second inhibitor, in this project we screened for an inhibitor to block dimerization of BC. Dimerization of BC is essential for activity.(Janiyani, Bordelon et al. 2001, Broussard, Price et al. 2013)

Our approach was motivated by the fact that a dimerization inhibitor for human BC was recently developed. Dimerization of BC in eukaryotic ACC is inhibited by soraphen A, a polyketide natural product fungicide produced by myxobacteria.(Tong and Harwood 2006, Cho, Lee et al. 2010) An effective dimerization inhibitor of human BC with much better pharmacological properties than soraphen A was discovered recently using a combination of structure-based computational design and experimental screens. The approach that led to discovery of ND-640 and ND-464 combined structure-based computational drug design with enzyme screens and crystallography.(Harriman, Greenwood et al. 2016, Svensson, Parker et al. 2016) Starting with the crystal structure of the BC domain of ACC2 complexed with soraphen A, a library of commercially available compounds was screened *in-silico* using displacement of high energy hydration sites as an essential criterion for hit compounds. Further screens were based on pharmacophore models of soraphen A and of pharmacophore sites. Roughly three hundred compounds were downselected and evaluated experimentally for inhibition of ACC1 and ACC2. This led to discovery of the lead compound, ND-022, which was then crystalized in complex with ACC2.(Harriman, Greenwood et al. 2016) Subsequent refinement based on the crystal structure led to ND-640 and ND-646.(Harriman, Greenwood et al. 2016, Svensson, Parker et al. 2016) The binding site of ND-640 and ND-646 on human ACC overlaps with a portion of the soraphen A binding site, but ND-640 and ND-646 are much smaller compounds with much different shapes compared to soraphen A.

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Crystal structures for bacterial BC show a pocket in the dimerization interface as for human BC, but of different shape and chemical nature.(Mochalkin, Miller et al. 2009, Polyak, Abell et al. 2012, Tong 2013) Following the prior work with human BC, in this project we combined *in-silico* and experimental screening to search for a dimerization inhibitor for bacterial BC. Grover Waldrop (LSU), who has published extensively on bacterial ACC, was an external collaborator (no-fee consultant) on this project. He provided the BC plasmid to us as well as guidance on expression in *E. coli*.

DETAILED DESCRIPTION OF EXPERIMENT/METHOD:

In-silico work.

In silico molecular docking was used to rapidly screen a large library of candidate dimerization inhibitors and to rank order compounds based on their calculated binding energy. Docking was performed using AutoDock Vina (Trott and Olson 2010) a freely available molecular docking program from UCSF (http://vina.scripps.edu/index.html). The ChemDiv3D library of ~34,000 non-redundant (structurally diverse) compounds (http://www.chemdiv.com/3d-diversity-library/) were docked to the crystal structure of the apo form of *E. coli* biotin carboxylase (pdbID:1DV1). The crystal structure of *E. coli* biotin carboxylase is for the dimer form of the protein, allowing us to identify the dimer binding domains, which were used as a docking target and allowed us to limit the search space of the *in silico* docking. As a secondary score, we calculated the number of high energy water molecules displaced by the compound upon binding for the top AutoDock Vina scoring pose of each compound. The number of waters displaced was calculated using routines in the interactive visualization and analysis of molecular structures program Chimera (Pettersen, Goddard et al. 2004) (https://www.cgl.ucsf.edu/chimera/) for determining atomic overlaps, and all waters with atoms overlapping the docked compound were counted. The structure of 1DV1, with waters added based on the results from Watermap calculations, was used as the BC structure in these calculations, and we wrote codes in Python that interfaced with Chimera to automate these calculations.

Watermap is an molecular dynamics based commercial code that generates maps of water molecules associated with proteins.(Bucher, Stouten et al. 2018) The use of Watermap was reported to be essential in prior work to find a dimerization inhibitor of human BC.(Harriman, Greenwood et al. 2016) Watermap reveals the locations of high energy waters, and prior work has shown that displacement of high energy waters is a strong predictor of successful inhibitors. A license for Watermap to perform up to 20 watermaps was purchased from Schrodinger. The executable program was downloaded and run on a Linux on the CEE – Engineering Graphics Workspace. Watermaps were generated for human BC (PDB structure 5KKN) and for *E. coli* BC (PDB structure 1DV1). The proteins were prepared for Watermap calculations using the Protein Preparation Wizard software tool of the Schrodinger suite. This tool was made available free of charge through a 30 day trial license.

Expression and purification of *E. coli* BC. Protocol for expression and purification of *E. coli* BC was provided by the Waldrop lab at LSU. The plasmid containing *E. coli* BC with Ampicillin resistance was received from the Waldrop lab and transformed into BL21 cells. An agar plate with Ampicillin was streaked and grown overnight and then LB containing ampicillin was inoculated with a colony and the culture was grown at 30 C until OD of 0.4-0.6, at which point the clls were induced with 0.05 mM IPTG. After inducing the cells were incubated at 25 C for 16 hrs and then spun down at 4 ºC. The pellet was resuspended in high salt buffer and then spun down again and then resuspended once more in high salt buffer. High salt buffer was used throughout the lysing and Ni-NTA purification steps in order to retain BC as a monomer. The high salt buffer was 500 mM KCl in 20 mM potassium phosphate pH 8. Low salt buffer used to convert the monomer to dimer was 150 mM KCl in 20 mM potassium phosphate pH 8. The cells were lysed on ice using a probe tip sonicator at 50% power, cycling on for 2 min and then off for 1 min, repeated for 15 cycles.

BC in the cell lysate was purified following protocol from the Waldrop lab. After loading the Ni-NTA column with lysate it was incubated at 4 ºC for at least 1 hr with rocking to facilitate mixing of protein with the resin and to give ample time for binding. After binding, the flow through was allowed to drip through the column by gravity and washes were performed using 15 mM imidazole, then 45 mM imidazole, and then finally elution of the sample was performed using 500 mM imidazole (each wash buffer and elution buffer contained 500 mM KCl and each was cooled on ice). BC typically eluted in the first two elution fractions.

Enzyme activity assay. To verify that the expressed protein is functional BC we performed an activity assay following protocol from the Waldrop group. The assay is a coupled enzyme assay in which BC generates ADP from ATP and then ADP is detected using pyruvate kinase and lactate dehydrogenase by following the oxidation of NADH at 340 nm.(Broussard, Price et al. 2013) BC activity is detected by a decrease in the absorbance at 340 nm. Tests were performed with BC aliquot and a blank containing buffer but no BC.

Dimerization assay. Methods to distinguish monomeric and dimeric BC, and thereby to assay for inhibition of dimerization, include size exclusion chromatography, native gels, and light scattering. We briefly explored light scattering and native gels, and concluded that SEC is the most practical and accurate method. Protein standards from Sigma-Aldrich were used to quantify the molecular weights of the peaks in the SEC chromatograms. Examples of the standards run

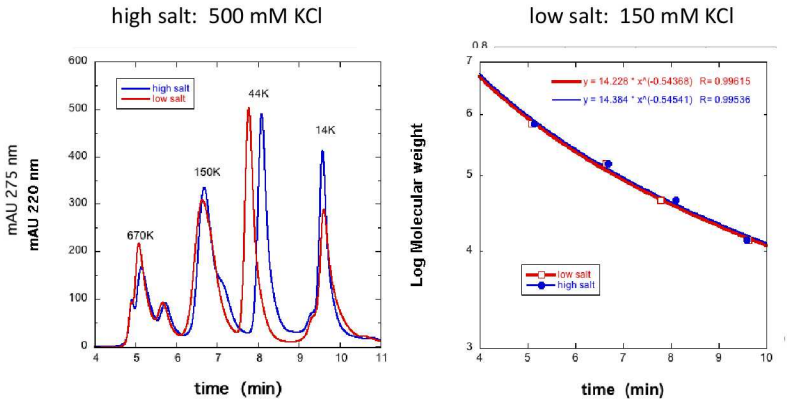

Figure 1. Example of SEC chromatograms for protein standards.

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with high salt and low salt running buffers are given in Fig 1. All standards and samples were run at 1 ml/min with injection of 10-20 ul and detection by UV absorption at 275 nm. To detect BC monomer a 100 ul aliquot of BC in high salt buffer was concentrated to 30 ul at 4 ºC using a spin concentrator (10K molecular weight cutoff), and then a 20 ul portion was injected into the SEC column with high salt running buffer. An example of a chromatogram for a sample processed in this way is shown in Figure 2 (left panel). The molecular weights of the peaks determined from the standards are indicated in the figure. To convert BC monomer to dimer, a 100 ul aliquot of BC in high salt buffer was mixed with 2 ml of low salt buffer (on ice), incubated for 10 min, and then concentrated to 30 ul at 4 ºC using a spin concentrator. The sample was then injected in the SEC column with low salt running buffer. An example chromatogram of the BC sample processed in this way is shown in Figure 2 (right panel). To perform the assay with a potential inhibitor compound, 1 mg of compound was dissolved in 50 µl of DMSO resulting in a solution of 20 mg/ml (or 50,000 µM for a compound with molecular weight of 400 g/mol). Then 1 µl of this solution was added to a 100 µl aliquot containing BC in high salt buffer on ice yielding 200 µg/ml of compound (or 500 µM for a compound with molecular weight of 400 g/mol) and 1% DMSO in high salt buffer. This solution was incubated for a minimum of 10 min to allow the compound to bind to BC. Concurrently 20 µl of the compound solution in DMSO were added to 1980 µl of low salt buffer yielding the same concentration of compound and DMSO in low salt buffer. This solution was centrifuged at 4 ºC to pellet any compound that precipitated. The supernatant was removed and mixed with the 101 µl solution containing BC and compound, incubated for 10 min, and then concentrated to 30 µl at 4 ºC using a spin concentrator. Finally, 20 ul of this solution were injected into the SEC column running with low salt buffer. In this way, the compound was present at 20 µg/ml while incubated against BC in monomeric form and was also present at 20 µg/ml during exchange to low salt buffer. Concentration to 30 µl was required to generate a detectable signal for the BC protein in the SEC. We expect that if a compound binds and inhibits BC dimerization, the peak corresponding to BC dimer will be small relative to the peak corresponding to BC monomer.

RESULTS:

Watermap analysis

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Watermap analysis was performed in the region of the BC dimerization interface to determine if high energy waters are likely to be present, and if so, to establish their locations within the binding pocket. In addition to running the Watermap analysis for E. coli BC (PDB 1DV1), we also ran the Watermap analyses for human BC (PDB 5KKN) without (apo) and with (holo) the natural ligand to gain insight from that case, since it was used on prior work to find a dimerization inhibitor for human BC. The results were tabulated in order of highest free energy and are given in Figure 3. The analysis indicates that high energy waters exist at the dimerization interface for both human BC and for bacterial BC, albeit with more higher energy waters at the dimerization interface for human BC. Figure 4 shows the positions of high energy waters calculated to interact strongly with *E. coli* BC (1DV1). Displacement of these high energy waters by compounds was a criterion used *in silico* screening of small molecule dimerization for ranking docked compounds.

E. coli BC (1DV1)

Site	Occupancy	Overlap	ΔH	-TΔS	ΔG
2	1.00	0.00	0.78	4.68	5.46
8	0.90	0.00	0.66	4.54	5.20
4	0.99	0.00	0.18	4.72	4.90
5	0.99	0.00	-0.15	4.46	4.31
12	0.60	0.00	1.39	1.86	3.25
17	0.40	0.00	2.09	1.13	3.22

Human BC (5KKN)

Site	Occupancy	Overlap	ΔH	-TΔS	ΔG
70	0.44	0.00	8.19	1.34	9.53
25	0.82	1.00	5.95	2.83	8.78
108	0.30	1.00	6.15	0.87	7.02
62	0.47	1.00	4.80	1.37	6.17
19	0.89	0.00	1.74	4.34	6.08
3	1.00	0.00	1.05	4.80	5.85
4	1.00	0.00	0.98	4.61	5.59
92	0.35	0.86	3.55	1.06	4.61
16	0.89	0.00	0.67	3.09	3.76
102	0.32	1.00	2.83	0.91	3.74
1	1.00	0.00	-0.95	4.63	3.68
97	0.32	1.00	2.73	0.94	3.67
17	0.89	0.00	0.47	2.82	3.29
51	0.57	0.00	1.54	1.70	3.24

Figure 3. High energy water molecules at the dimerization interface for E coli BC and for human BC determined by Watermap analysis.

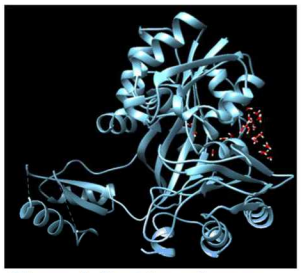

Figure 4. Position of water molecules as calculated using Watermap.

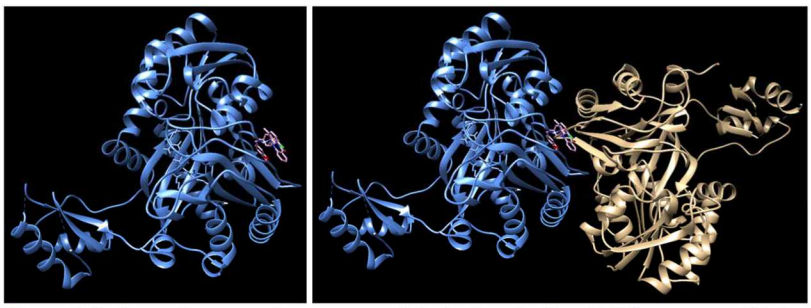

Figure 5. Left panel, top scoring compound docked to the E. coli BC monomer. Right panel, top scoring compound in the BC dimer interface showing how its propensity to disrupt dimerization.

In silico Docking

Roughly 34,000 structurally non-redundant compounds from the ChemDiv3D library were docked to the *E. coli* BC monomer, and docking was targeted to the dimerization site determined from its crystal structure. Docked compounds were ranked first by their AutoDock Vina score, which is a calculated free energy of binding, and second by the number of high energy waters the compound would displace. The top 89 in the list were purchased for testing from ChemDiv3D. Another 130 compounds were identified as desirable to test but time and funding limitations did not allow purchase of those compounds. Figure 5 shows results for the top scoring compound in both its lowest scoring docked pose in the *E. coli* BC monomer (left panel) and with the BC dimer superimposed to show how the compound likely interferes with BC dimerization.

Protein expression and purification. A total of 6 production runs of 500 ml each were performed followed by 2 production runs of 2 L each. An example of a denaturing SDS gel for the flow-through, washes, and first five elution fractions is given in Figure 6. BC was not isolated

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as a unique protein, but was present along with other proteins eluting from the Ni-NTA column as shown in Figure 6. The productions runs generated sufficient BC protein for about 300 aliquots at BC concentration sufficient for SEC analysis. The amount of BC protein produced per liter of culture is estimated at 1 mg, but is uncertain since it was not isolated as a purified protein.

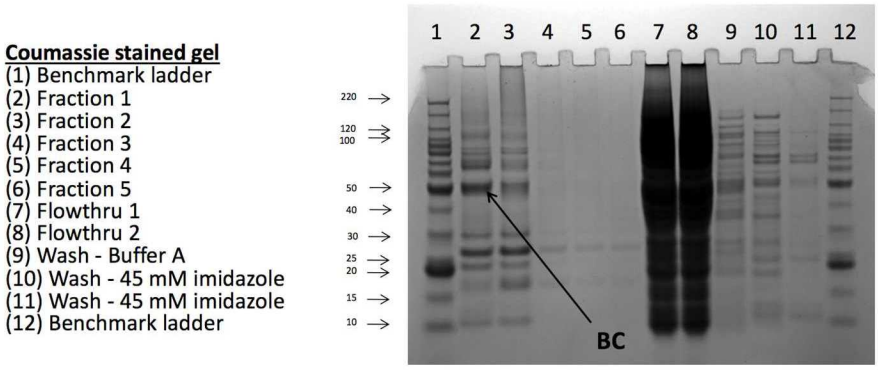

Figure 6. Denaturing gel showing result of His-tag purification with Ni-NTA column

Enzyme assay. An example of the results of the enzyme assay are shown in Figure 7. In this case an aliquot of BC in high salt buffer was diluted in a large excess of law salt buffer and then re-concentrated. The buffer exchange was performed in order to convert BC from monomer to dimer, as the dimer form has significantly greater activity than the monomeric form. These data demonstrate significant activity for partially purified BC as a dimer. This verifies that the 50K protein is indeed BC and that it is in an active form.

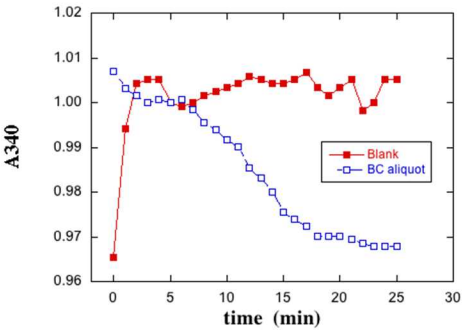

Figure 7. Assay for BC activity

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Dimerization assay. At the time of this writing, 10 of the 89 compounds have been assayed for inhibition of BC dimerization by SEC and we assign 4 as positive for inhibition although with substantially different degrees of effectiveness. Sample chromatograms are shown in Figure 8 and Figure 9, with the region of interest expanded. The data for these two figures were obtained using aliquots from different elution fractions. For each set of aliquots corresponding to different elution fractions or different protein production runs, a control is included in which the same quantity of DMSO is added but with no inhibitor compound. We assign the result as positive for inhibition if the ratio of the dimer to monomer is substantially reduced. In Figure 8, the compounds Y020-1143 and K788-8777 are assigned as negative whereas compounds K788-8584 and K786-7111 are assigned as positive for dimer inhibition, with K786-7111 showing a stronger positive result than K788-8584. In Figure 9, the compounds Y020-1388 and Y200-0701 are assigned as negative whereas compounds K806-0037 and Y020-6319 are assigned as positive for dimer inhibition, with Y020-6319 showing a stronger positive result than K806-0037.

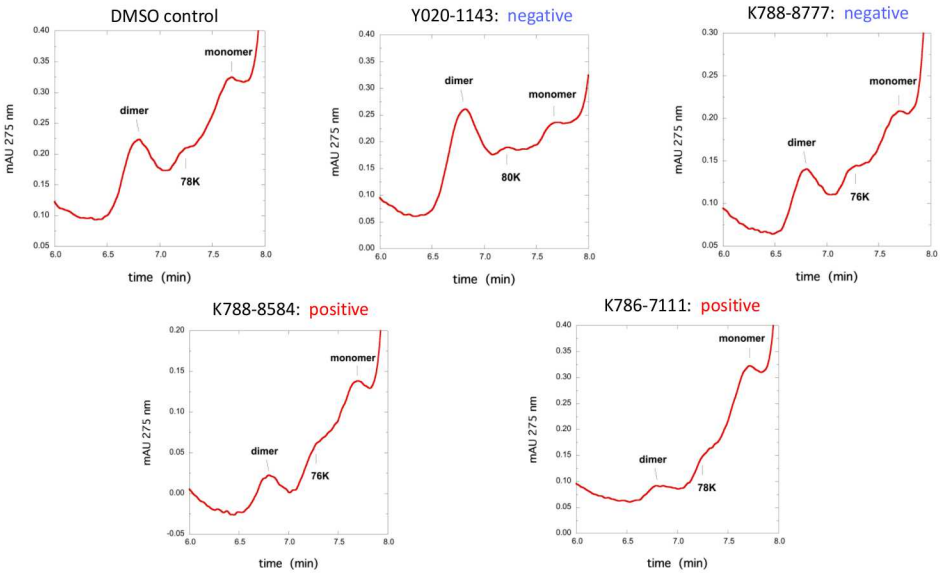

Figure 8 Sample results from dimerization assay based on SEC.

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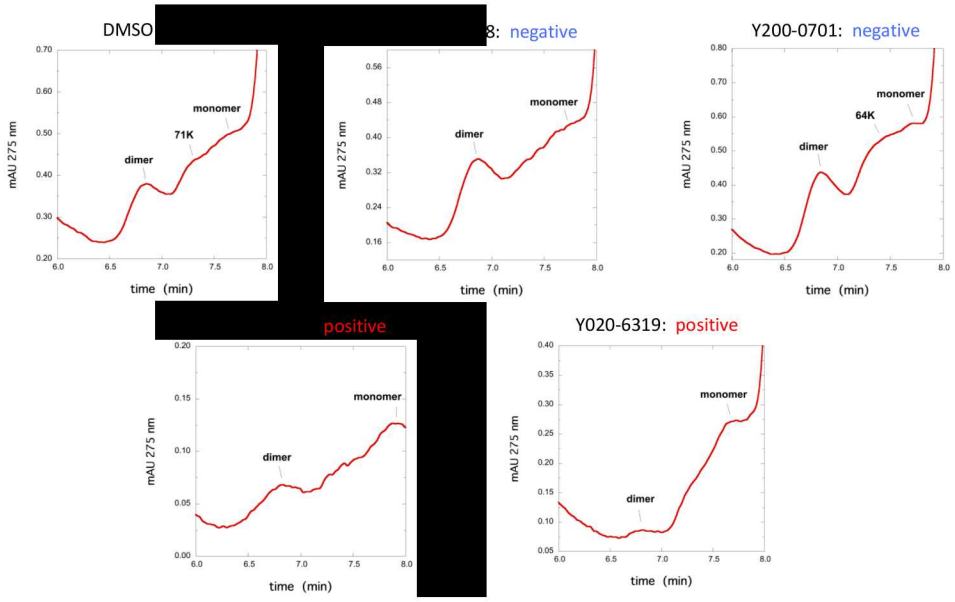

Figure 9. Sample results from dimerization assay based on SEC.

DISCUSSION:

In this work we combined in-silico and experimental screening to generate lead compounds for inhibiting the dimerization of bacterial BC. In the in-silico work we combined calculations of the positions and energetics of water molecules associated with *E. coli* BC with docking of a large number of compounds to arrive a list of 89 compounds to be tested. We expressed and partially purified this protein, verifying that it was active with a published activity test, and we developed a new assay for identifying monomer and dimer forms. At the time of this writing we have completed initial testing for 10 of the 89 compounds. Testing of the remaining 79 compounds is in progress. The fact that 2 of the 10 compounds tested show strong inhibition of dimerization is encouraging.

During the course of this project a few difficulties arose. Some confusion resulted upon comparing the results of denaturing gels with the SEC chromatograms. While the first two fractions that eluted from the Ni-NTA column showed a 50,000 g/mol band as the dominant protein in denaturing gels (see Figure 6), in the SEC chromatograms with UV detection at 275 nm running with high salt buffer (where BC is a monomer) the 50K peak was a small minority component. Time did not allow us to fully resolve the discrepancy between these two analyses and identify the nature of the stronger peaks detected by UV absorption at 275 nm. One hypothesis is that it may be due to nucleic acid that was not removed in the preparation and purification of the protein. Nevertheless, given the fact that the ratio of monomer to dimer decreased strongly when running low salt buffer compared with high salt buffer (see Figure 2), we proceeded to assay the compounds.

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The SEC assay for dimerization inhibition would be improved if BC were isolated as a pure protein. This requires an additional purification using a size exclusion chromatography column. Unfortunately this could not be accomplished within the time and funding available. We intend to finish testing the suite of 89 compounds with the current aliquots and then repeat the assay using more highly purified BC for the compounds that showed a positive result in the first round for testing.

It would be helpful to determine if BC can convert repeatedly between monomer and dimer by varying salt concentration. One concern regarding the drug-ability of bacterial BC is that it is a tighter dimeric complex than for human BC, for which dimerization inhibitors have proven successful. While additional work has shown that bacterial BC has a high turnover rate and so a dimerization inhibitor could be effective by blocking the formation of BC dimeric complexes before they form, (Humbard, Surkov et al. 2013) the efficacy would likely be greater if an inhibitor would also break up existing BC dimeric complexes. Therefore, follow-on studies will be conducted to determine if existing BC dimeric complexes can be dissociated by the inhibitor compounds.

It would also be helpful to determine the timescales required for compound binding and for the monomer-to-dimer conversion more precisely. Varying the incubation time of the inhibitor with the BC in high salt buffer prior to buffer exchange would reveal the kinetics of the binding process. Varying the incubation time in low salt buffer after rapid buffer exchange would reveal the kinetics of the dimerization process.

Running the dimerization assay as a function of inhibitor concentration for the hits discovered in this initial screen would establish the minimum concentration for effectiveness of each compound and enable ranking of the compounds. Furthermore, it has been shown previously that BC activity is much greater for the dimer form than for the monomer form. So measuring activity in the presence and absence of the dimerization inhibitors would provide an orthogonal assay to support the conclusion of dimerization by SEC. These assays will be performed in follow-on work.

Follow-on work will also include a proposal to NIH to include structural studies of the inhibitor by crystallography in collaboration with the Waldrop group. The Waldrop group has been involved in structure determination for BC for three decades. Structural data will enable another round of modeling and prediction for chemical modification of the existing lead compounds.

ANTICIPATED OUTCOMES AND IMPACTS:

The results of this project have great potential impact. A generalized two-drug per target strategy that drastically reduces or eliminates resistance in bacterial populations would avert a growing global health crisis and also provide DoD with a strategy for highly effective countermeasures against known weaponizable bacteria.

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The proposed work supports White House and government agency missions to develop medical countermeasures against highly pathogenic and multidrug-resistant bacteria (DOD, DOE, DHS, NIH, CDC). The proposed work directly addresses SNLs mission area Reducing Global Chemical and Biological Dangers, supporting the objective of acquiring expertise needed to counter the effects of natural or intentional biological events. Specifically, it addresses the request for innovative proposals to address the emerging threat of AR.

We intend to develop the best inhibitor compounds identified from our dimerization assay as lead compounds. Following our initial screen we will perform a second screen with the hit compounds, using more highly purified protein and also establish the lower concentration limit for effective inhibition. We will also perform activity assay in the presence and absence of the compounds as an orthogonal test of dimerization.

An R21 proposal to find a dimerization inhibitor for bacterial biotin carboxylase was previously submitted to NIH. The reviews requested proof-of-principle data to demonstrate that dimerization of bacterial biotin carboxylase can be inhibited. We intend to use the lead compounds identified in this Exploratory Express LDRD to support a response and a revised R21 proposal.

A proposal to DTRA was also previously submitted on this topic. In that proposal the stated goal was to find a second inhibitor for bacterial ACC by three different approaches, looking for: a dimerization inhibitor for BC, allosteric inhibitors of BC, and an active site inhibitor of BC. The reviews stated that the goals were too expansive, and that each topic would represent a major development endeavor. The review also cited lack of preliminary data to show that the intervention approaches on the ACC complex could be achieved. We intend to use the lead compounds identified in this Exploratory Express LDRD to support a response and a revised proposal that focuses on developing a dimerization inhibitor of bacterial BC.

DHHS (BARDA), has also issued a recent call to combat AR infections and we will consider this funding source as our work progresses.

Each successful BC inhibitor found will result in highly valuable IP. We intend to file a technical advance on the best lead compounds from this study.

Achaogen is a company that specializes in beating import and efflux in gram-negative bacteria (G-). They are working to develop ATP site inhibitors for BC of G-, especially focusing on penetration through G- membranes. The PI has contacted Achaogen and received a letter of intent to collaborate to bring a combination therapy against bacterial ACC to market.

Following work with *E. coli* BC we intend to expand the dual inhibitor strategy for acetyl CoA carboxylase (ACC) to target the gram-negative bacteria *Y. pestis* and *F. tularensis*, starting with attenuated *Y. pestis* and *F. tularensis* strains (*Y. pestis* KIM10 and *F. novicidia*). This will support

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Chemical and Biological Defense Program goals of DTRA by generating countermeasures to treat drug resistant infections by DoD bacterial threat pathogens.

A particular challenge for therapeutics against G- is the ability to penetrate the outer membrane and accumulate to levels sufficient for enzyme inhibition. Recently, correlations have been established between chemical and structural aspects of antibiotics and their accumulation within G- bacteria.(Richter, Drown et al. 2017) The aspects that correlate most strongly with accumulation in G- are the presence of an amine, low globularity, high rigidity, and amphiphilicity. Using these principles, a compound that was effective against only gram-negative bacteria (G+) was transformed into a compound that accumulates and kills G-. Motivated by this successful example, we will use these principles to synthesize variants of lead compounds and test for accumulation within and killing of G-. Furthermore, finding two inhibitors that both act on BC (active site inhibitor and dimerization inhibitor) will improve the chances that a covalently-linked dual inhibitor strategy will be successful. Linked inhibitors will avoid the potential problem of different pharmacokinetic and pharmacodynamics properties of the two drugs, and will also be more likely to avoid efflux in G-.(Silvers, Robertson et al. 2014) While the concept of covalently-linked dual inhibitors is well established,(Morphy and Rankovic 2006) the use of two linked drugs to target a single enzyme is rare.(Morphy and Rankovic 2006, Pokrovskaya and Baasov 2010) Avoiding efflux through pumps is another important challenge. This risk is mitigated by the possibility of covalently linking two inhibitors, as linked molecules should be highly resistant to efflux pumps.(Silvers, Robertson et al. 2014)

Since dimerization inhibitors do not compete against a substrate molecule (ATP) they have the advantage that ultrahigh affinity binding is not required. Active site and dimerization inhibitors should have broad spectrum activity as these structural aspects should be highly conserved.

CONCLUSION:

In this work we combined in-silico and experimental screening to generated lead compounds for inhibiting the dimerization of bacterial BC. In the in-silico work we combined calculations of the positions and energetics of water molecules associated with *E. coli* BC with docking of a large number of compounds to arrive a list of 89 compounds to be tested. We expressed and partially purified this protein, verifying that it was active with a published activity test, and we developed a new assay for identifying monomer and dimer forms. Finally at the time of this writing we have completed initial testing for 10 of the 89 compounds. Testing of the remaining 79 compounds is in progress. The fact that 2 of the 10 compounds tested show strong inhibition of dimerization is very encouraging.

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