THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CHARACTERIZATION AND TARGET IDENTIFCATION OF HYDRAZINE CARBONOTHIOLS, A CLASS OF *TRANS*-TRANSLATION INHIBITORS

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SPRING 2018

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

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ABSTRACT

Antibiotic resistance is a growing global epidemic with many common bacteria acquiring mutations to be multi-drug resistant. In order to combat this problem, novel antibiotics have to be developed. One promising target is *trans*-translation, which is a mechanism used by bacteria to rescue ribosomes stalled during translation. Inhibitors of *trans*-translation produce a build-up of stalled ribosomes that are unable to translate mRNA, resulting in reduced production of proteins. This is toxic to bacterial cells and leads to cell death. One class of inhibitors identified by the Keiler lab is the hydrazine carbonothiols. These compounds are shown to have broad-spectrum activity at low concetrations and have minimal toxicity in macrophages. These characteristics make them ideal candidates for further drug development. However, the mechanism of action of this class of drugs was still unknown. In order to determine the target, a chemical biology approach was utilized. This method utilizes a probe analogous to the structure of the hydrazine carbonothiols that can be photo-affinity labeled to the target of interest in the cell and can then be identified by click conjugation to a secondary fluorescent probe. Mass spectrometry analysis of the fluorescent band identified the target of these compounds to be ribosomal protein L7/L12. Initial validation studies provide minimal support for this target; thus, such experiments are critically important for future studies. By confirming the target of this class of compounds, the hydrazine carbonothiols can move onto the next stage of antibiotic development in order to curb the number of antibiotic resistant bacteria.

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ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Dr. Keiler for accepting me into his lab my sophomore year at Penn State. Having been in his lab and BMB 488 class with Dr. Ades the past two and a half years, I was able to learn a great deal of critical-thinking skills about research, especially regarding antibiotic resistance. I also want to thank my honors advisor Dr. Gilmour for answering all the questions I had about my thesis. I attribute much of my success in lab to Dr. John Alumasa who has been an outstanding mentor. He was not only able to help me figure out what aspects of my experiments to change when they did not work right, but challenged me to think critically about the results I found. His constant chuckling at almost anything I say or do in lab is a staple I always look forward to and I could never thank him enough for everything he has done for me. My friends and fellow undergraduates in both the Keiler and Ades lab: Sam, Taryn, Corey, Mia, Mary Hannah, Alexa, and Paige have also been extremely supportive by helping me through my struggles both in and out of lab for which I am extremely grateful. Finally, I would like to thank my mom and dad for always being supportive and helping me to reach my goals.

INTRODUCTION

Antibiotic Resistance is a Growing Threat

Multidrug resistant bacteria are becoming more prevalent throughout the world today. The number of untreatable bacterial infections has increased due to the emergence of these antibiotic-resistant bacterial pathogens. This exponential increase is primarily caused by over-prescription of antibiotics by healthcare providers and is also exacerbated by the misuse of antibiotics given to livestock (1, 2). As most antibiotics identified during the age of antibiotic development were derived from natural products, there were some bacteria that were inherently resistant to some of these antibiotics. The continued use of these same antibiotics in addition to bacterial mechanisms of conjugation to share plasmids containing antibiotic resistance genes has made multidrug resistance a global issue (1, 3). There have been an increasing number of antibiotic-resistant infections, especially in poorer countries without regulations and resources. For example, 88% of *S. aureus* infections in Nigeria are methicillin resistant (2).

With the World Health Organization suggesting we will soon enter a post-antibiotic era, it is essential to produce new antibiotics. Some scientists are even suggesting that 20 new classes of antibiotics have to be developed in order to support the current path of medicine (4). Thus, it is of the utmost importance to develop new broad-spectrum antibiotics with novel targets in lesser known metabolic pathways that are not currently on the market (4). One such pathway that may contain such targets is *trans*-translation.

trans-Translation is a Pathway with Potential for Antibiotic Development

The central dogma of molecular biology of DNA to RNA to protein first involves transcription of DNA into mRNA followed by translation of the mRNA into a functional polypeptide sequence. In bacterial cells, a lack of quality control measures can lead to imprecision in many processes, such as transcription, which can result in the production of mRNA without a stop codon (5). When these non-stop mRNA transcripts are then translated, the ribosome cannot terminate translation and free itself from the mRNA strand (6). This is a problem for bacterial cells as a buildup of non-stop ribosomes is toxic to the cell and reduces protein synthesis capabilities (6). Thus, in order to prevent cell death, the bacteria will employ *trans*-translation, a mechanism that rescues these non-stop ribosomes. The *trans*-translation pathway involves two main components: tmRNA and SmpB, which are encoded by the genes *ssrA* and *smpB* respectively (6, 7). The tmRNA component has qualities of both tRNA and mRNA. EF-Tu is able to recognize tmRNA as if it was a typical tRNA molecule as it is charged with an alanine residue (7, 8). Thus, the tmRNA and SmpB will form a complex recognized by EF-Tu to load the complex into the A site of the ribosome. The ribosome will resume translation on the mRNA portion of tmRNA, which contains a stop codon and a sequence encoding amino acids that tag the polypeptide chain for degradation (6, 9, 10). Release factors can then hydrolyze the peptidyl-tmRNA bond, releasing the ribosome from the mRNA. The tagged nascent peptide and non-stop mRNA released are then degraded by proteases such as ClpXP and Lon and by nucleases respectively (7, 11). This degradation process by proteases is notable since it removes a potentially harmful peptide from the cell (11).

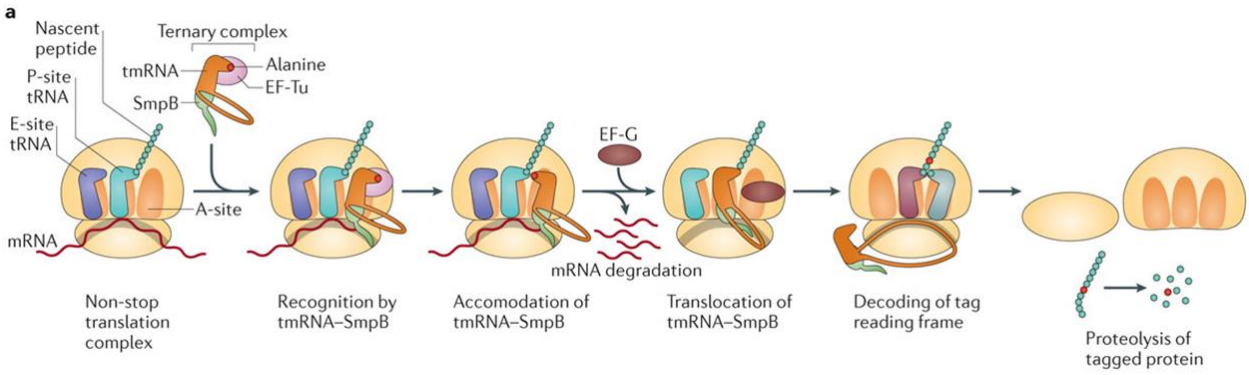


Figure 1. Mechanism of *trans*-translation pathway. During *trans*-translation the tmRNA-SmpB complex is inserted by EF-Tu into the A site of the ribosome and translation is resumed on the mRNA-like portion of tmRNA. The peptidyl-tmRNA bond is hydrolyzed by release factors releasing the ribosome from the nonstop mRNA template. The nascent peptide and mRNA are degraded by proteases and nucleases. Source (6).

Alternative ribosome rescue pathways have been discovered in those species in which *trans*-translation is not essential. These backup pathways utilize proteins ArfA and ArfB. The ArfA mechanism was originally discovered in *E. coli* and involves the ArfA protein recognizing the empty mRNA channel caused by the non-stop ribosome and recruiting release factor 2 to the ribosome to hydrolyze the peptidyl-tRNA (6, 7, 12). In contrast, protein ArfB acts through the insertion of the protein into the empty A site of the ribosome and the direct hydrolysis of the peptidyl-tRNA through a GGQ motif it contains (12). Neither of these alternative ribosome rescue mechanisms tags the dysfunctional peptide for degradation nor uses nucleases to degrade the non-stop mRNA transcript.

The *trans*-translation pathway is a promising target for antibiotic development as genes for this pathway have been found in almost all bacteria and is essential in a number of pathogenic strains of bacteria (6). Deletions of the genes *ssrA* and *smpB* have proven to be lethal in many species that are reliant upon *trans*-translation (6, 7, 11). Further, cells that have deletion of either tmRNA or SmpB have specific phenotypes associated with stress responses, such as slower growth rates, and can reduce the virulence of pathogens (7, 11).

While it has been shown that there is an analogous ribosome rescue pathway present in mitochondria of human cells (13), it has been demonstrated that inhibitors of *trans*-translation do not affect the mitochondria at concentrations where they are able to kill the bacteria (14). Additionally, cells that utilize an alternative ribosome rescue pathway such as ArfA and ArfB have also been shown to be affected by inhibitors to the *trans*-translation pathway. This makes *trans*-translation a promising pathway from which to derive novel antibiotics.

Inhibitors of *trans*-Translation were Identified through a High-Throughput Screen

In order to identify small molecule inhibitors to the *trans*-translation pathway, a high-throughput screen was utilized as these facilitate the analysis of thousands of components that exist in chemical libraries. For this reporter assay, the luciferase gene from firefly DNA that is responsible for its luminescence was used. The luciferase DNA was designed to have a transcriptional terminator placed before its stop codon so that when transcribed, it would produce an mRNA with no stop codon (9). Translation of this mRNA transcript would produce a luciferase protein in the polypeptide channel attached to a non-stop ribosome complex (Figure 2).

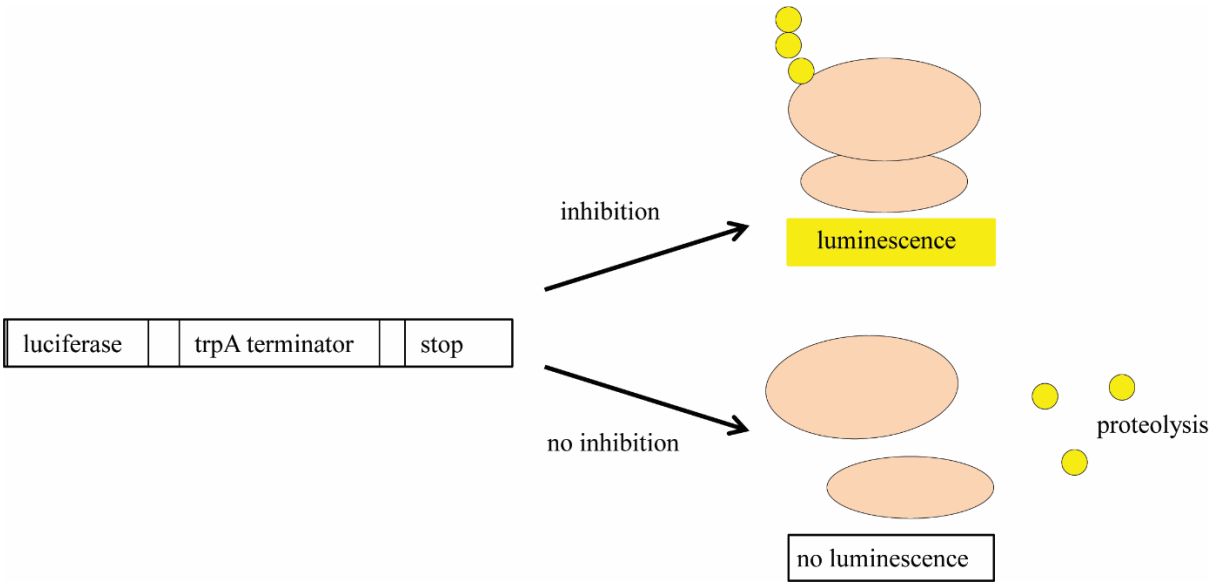


Figure 2. Luciferase Reporter Gene used in the High-Throughput Screen. The luciferase gene is placed before the trpA transcriptional terminator. *trans*-Translation is active when it tags and degrades the luciferase protein so that there is no luminescence. It is inhibited when the protein is luminescent, indicating it was not tagged and degraded by the active pathway.

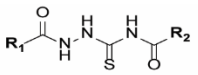
In normal cells with active *trans*-translation, the tmRNA and SmpB complex will enter the A site of the ribosome, enable the recycling of the ribosome, and release the mRNA transcript and tagged luciferase polypeptide. The tagged polypeptide would then be degraded by proteases in the cell so that there is no luminescence reading. This would also be the case if a small molecule tested does not inhibit the *trans*-translation mechanism. However, if small molecules are able to inhibit the *trans*-translation pathway, then the ribosome would not be able to be rescued and the luciferase protein would not be tagged. Thus, there would be luminescence of the peptide (Figure 2).

Of the 663,000 small molecule compounds screened to inhibit the *trans*-translation pathway, 178 compounds were found to have higher than average luminescence readings (9). Of those, only 46 compounds were determined to inhibit *trans*-translation and were used in subsequent studies. Many of these small molecules have already been shown to have therapeutic

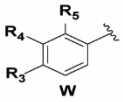
value against pathogens like *M. tuberculosis* and *B. anthracis,* further demonstrating the potential of *trans*-translation inhibition (10, 15).

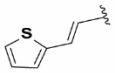
Hydrazine carbonothiol Compounds Inhibit *trans*-Translation

Of the remaining compounds identified as inhibiting the *trans*-translation pathway in the high-throughput screen, 21 were found to have a backbone structure similar to a hydrazine carbonothiol and were subsequently studied further (Figure 3).



substituents R1/R2



W X Y Z

Compound ID	HTS activity*	R1	R2	R3	R4	R5
KKL-62	6.9	W	Y	H	H	H
KKL-63	9.4	W	CH_3	Ph	H	H
KKL-64	1.5	W	CH_3	OCH_3	H	H
KKL-65	10.4	W	CH_3	CH_3	Br	H
KKL-86	4.1	W	W	$^{\$}CH_3$	H	H
KKL-136	11.9	W	CH_3	OC_3H_7	H	H
KKL-137	9.9	W	CH_3	OC_4H_9	H	H
KKL-138	2.5	W	C_2H_5	H	H	H
KKL-139	6.0	W	C_2H_5	Br	H	H
KKL-140	5.1	W	C_2H_5	OC_3H_7	H	H
KKL-141	7.1	W	C_2H_5	OC_4H_9	H	H
KKL-142	3.5	W	C_2H_5	OCH_2-Ph	H	H
KKL-143	3.0	C_4H_9	C_3H_7	-	-	-
KKL-147	1.1	W	Z	H	H	H
KKL-148	4.1	W	X	CH_3	H	H
KKL-149	2.0	W	X	OCH_3	H	H
KKL-150	2.1	W	W	$^{\$}OCH_3$	H	H
KKL-151	1.8	W	W	$^{\#}OCH_3$	H	H
KKL-152	2.0	X	Z	-	-	-
KKL-153	2.4	X	W	OCH_3	H	H

* HTS activity is the fold change in the luminescence signal relative to controls.
$ substituent only in R1 (R2 = H): # substituent in both R1 & R2

Figure 3. Hydrazine carbonothiols identified in the high-throughput screen. The chemical structure of the hydrazine carbonothiol compound is listed next to the reported increase in fluorescence compared to the control seen in the luciferase-based high throughput screen. The placement and structure of the R groups are shown.

The fold-change in luminescence discovered in the original screening process was varied and used to determine which hydrazine carbonothiol compounds would be synthesized for further analysis. A previous student in the lab was able to synthesize compounds KKL-63 and 64 identified in the high throughput screen in addition to analog KKL-588 from precursor molecules (16). Additional analogs of these compounds have been synthesized to determine the overall activity of the hydrazine carbonothiol compounds in various strains of bacteria as well as to identify the molecular target of this class of compounds. These analogs were designed to have the same backbone structure, but different functional groups than the original compounds synthesized to see how these structural modifications changed their activity (Figure 4).

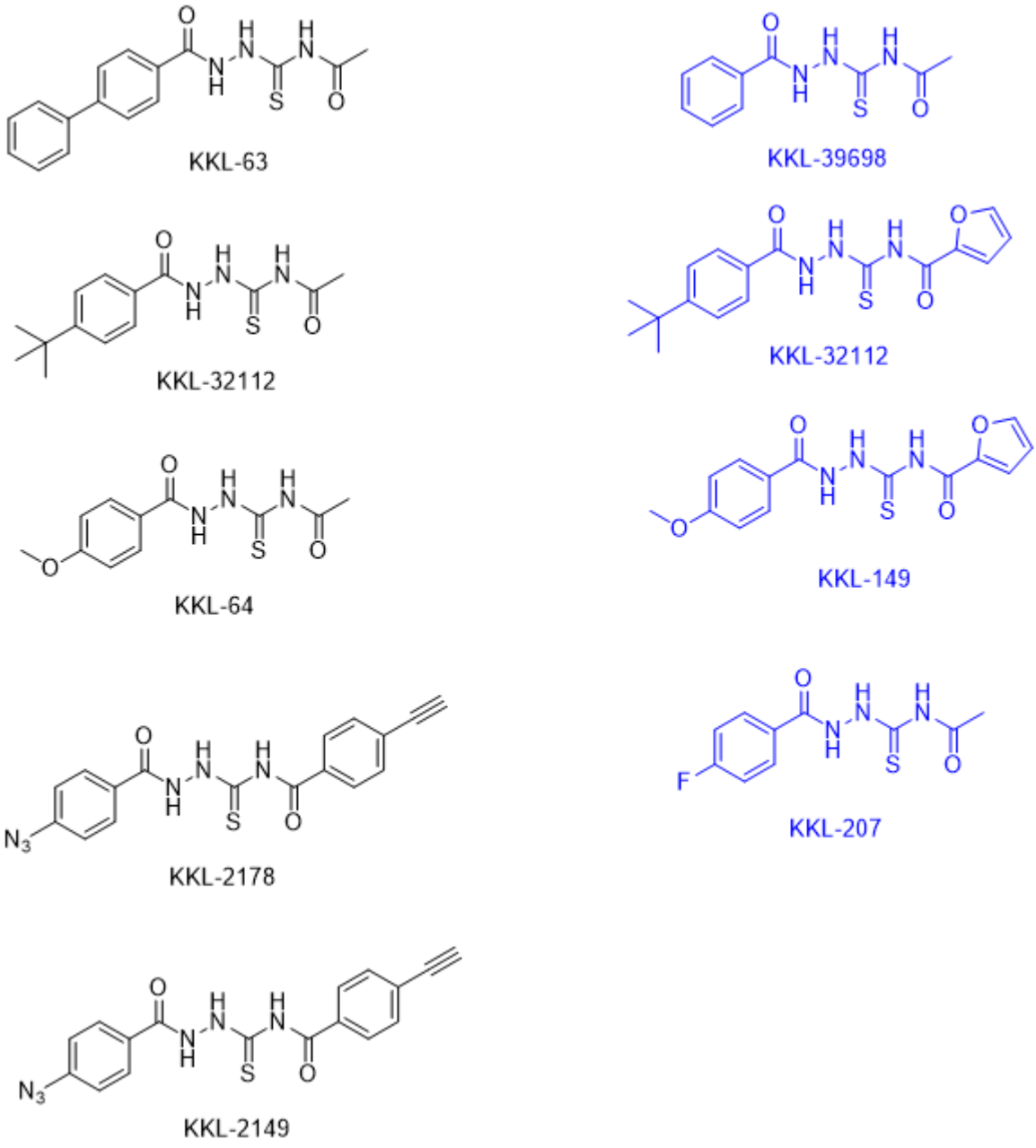


Figure 4. Structures of hydrazine carbonothiol analogs. The chemical structure of the hydrazine carbonothiol compounds used in this thesis. The compounds have the same backbone structure with various R groups added to either end of the backbone.

Chemical Biology Target Identification Approach for Hydrazine carbonothiol Compounds

Target identification strategies employed in research include predicting potential targets using docking and sampling methods to determine where a small molecule might be able to bind in a larger structure. Target discovery of specific drugs has also been achieved by comparing sequences of proteins across databases in order to determine similarities between an unknown protein and similar proteins with known targets (17).

Another common approach to target identification is through the use of resistant mutants. In this case, bacteria are grown in the presence of a high concentration of the drug of interest. Colonies that grow at these high concentrations of inhibitor are further tested to check for and confirm resistance. The resistant strain is then sequenced in order to determine what mutations arose to confer resistance to the inhibitor when compared to the original strain used in the experiment. This approach has successfully been used by researchers to identify the target of a class of compounds called the indoleamides by growing *M. tuberculosis* cells in a media with 8x the typical amount of drug needed to kill them (18). However, it is often difficult to obtain resistant mutants for a given compound. For instance, resistant mutants would not be able to be obtained if the mutations needed to eliminate inhibitor activity resulted in bacterial death. Further, multiple mutations might be necessary to build resistance to the inhibitor and it would not likely be possible for a bacterium to acquire the multiple random mutations required (10). Thus far, the resistant mutant approach has not been achievable for the *trans*-translation inhibitors (9). Consequently, a different path has to be taken to identify the target of these compounds.

For this thesis, a chemical biology approach was utilized. This approach involves the use of a probe that mimics the compounds of interest. The probe also contains an affinity and/or

fluorescent tag that can be used to isolate and identify the inhibitor (5). As the bulky fluorescent and affinity groups may interfere with the natural placement of the inhibitor in the cell, the chemical biology method may not give an accurate determination of the target (5). To fix this imprecision, a small probe mimicking the structure of the hydrazine carbonothiol compounds is combined with a secondary probe with fluorescent and affinity groups through a covalent linkage (5). This is done through the use of a newer trend identified in the past decade called click chemistry.

Click chemistry is characterized by utilizing reactants that do not interfere with native biochemical reactions and processes in cells and that are stable and nontoxic (19). One common click chemistry approach involves copper-catalyzed reactions between azides and terminal alkynes (20). The process used in this thesis involves copper catalyzed Azide-Alkyne Huisgen Cycloaddition (AAHC) and photo-affinity labeling and is adapted from the protocol of Alumasa et al. (5). Specifically, a probe from the hydrazine carbonothiol class is modified to have an azide and alkyne functional group. The azide can undergo photo-affinity labeling to link it to its target and the alkyne moiety undergoes a click conjugation reaction to link to an azide group on a secondary probe containing an affinity and/or fluorescent tag. This is beneficial as it eliminates the issues of a large probe common to the chemical biology technique while maintaining the integration of an affinity tag and fluorescence group to identify the inhibitor on a gel. Further, the use of this approach is helpful as it can be used in whole cell bacteria and is not limited to in-vitro studies (5).

This chemical biology approach to target identification has already been used with other inhibitors of the *trans*-translation pathway in the 1,3,4-oxadiazole benzamides class that were originally discovered in the high-throughput screen (10). Thus, there is precedence for the use of

this technique in this thesis to identify the target of the hydrazine carbonothiol compounds. Further, characterization of the inhibitors will be completed in order to gain insight into their therapeutic value.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli ΔtolC, *B. anthracis* Sterne, DH5α, and BL21 (DE3) cells were cultured at 37℃ in nutritionally rich lysogeny broth (LB) growth medium. BL21 (DE3) p*rplL* and BL21 (DE3) pET28 p*rplL* were cultured at 37℃ in LB growth medium with chloramphenicol (20 mg/mL) for assays and were grown in nutritionally rich terrific broth (TB) media with chloramphenicol (20 mg/mL) at 37°C for bulk culture IPTG induction. WT SB75 mCherry trpAt and *ΔssrA* SB75 mCherry trpAt strains were cultured in EZ-rich media (1X MOPS Mixture, 1.32 mM K_2HPO_4, 1X ACGU, 1X EZ Supplement, 0.2% Glucose, sterile H_2O) with ampicillin at 100 mM at 37°C. Methicillin-resistant *S. aureus* and *S. boydii* were cultured at 37℃ in Mueller-Hinton growth medium. *H. influenzae* was cultured in Brain-heart infusion media supplemented with NAD^+ and hematin at 37℃.

Table 1. List of Strains, Plasmids, and Oligonucleotides

Strains	Plasmid	Strain Description	Origin
MG1655 Δ*tolC*	N/A	*tolC*::kan	Keio collection, National Bioresource Project
B. anthracis Sterne (34F2)	pOX1	Non-pathogenic strain of *Bacillus anthracis* that lacks the capsule	Alumasa et al. (15)
DH5α	N/A	Cloning strain of *E. coli*	Keiler lab
SAM001	p*rplL*	BL21 (DE3) strain for overexpressing ribosomal protein L12 with T7 promoter	This study

SAM002	p*rplL*	BL21 (DE3) strain for overexpressing ribosomal protein L12 with T5 promoter	This study
LC1131 (SB75)	ptrcm-Cherry-trpAT-PPZ	Screening strain for *trans*-translation inhibitors	Keiler Lab
LC1132 (SB75)	ptrcm-Cherry-trpAT-PPZ	Control strain lacking the *ssrA* gene	Keiler Lab
Methicillin Resistant *Staphylococcus aureus*	N/A	USA 300	ATCC
Shigella boydii	N/A	N/A	ATCC
Haemophilus influenzae	N/A	ATCC 49247	ATCC
Plasmid		**Plasmid Description**	
p*rplL*		Plasmid expressing the *E. coli* ribosomal protein L12	
ptrcm-Cherry-trpAT-PPZ		Plasmid expressing mCherry protein that has a transcription terminator to create a nonstop mRNA	
Oligonucleotides		**Sequence**	
L12-FWD		5'-AATGTACCATGGACTATGTCTA TCACTAAAGATCAAATCATTG-3'	
L12-REV		5'-AATGTACTCGAGTCATTATTT AACTTCAACTTCAGCGCCAG-3'	

Growth Inhibition Assays

Minimum Inhibitory Concentration Assays

Bacterial strains being tested were grown overnight in their respective media and diluted to OD_{600} 0.002 in growth culture. KKL-63, 64, 147, 209, 588, 2149, 2178, 32112 and 39698 were added in duplicate or triplicate at concentrations from 20-200 μM. Diluted bacterial strains were added to the top wells in order to serially dilute the compound down the plate. The plates were then incubated at 37℃ overnight. The MIC was the minimum concentration of drug that

was able to visibly inhibit growth of the cells in a given well. For nonpathogenic strains the OD_{600} of the wells was then measured. The average of the duplicate or triplicate wells was taken and normalized to the control wells that contained bacteria, but no inhibitor to give the percentage growth in that well. A sigmoidal curve was made to compare the concentration of the drug in a given well to the percentage growth in that well in order to determine the IC_{50} or the concentration needed to inhibit 50% of growth for the compounds.

Growth Curves

B. anthracis was grown overnight and back-diluted to OD_{600} 0.2 in LB media containing propidium iodide (20 μM). Cells were transferred to culture tubes containing hydrazine carbonothiol inhibitor at half-MIC, MIC, and 2x MIC values. Zero-time points for growth at OD_{600} and fluorescence values of propidium iodide at an excitation of 493 nm and emission of 636 nm were measured using the SpectraMax i3 (Molecular Devices). Cultures were grown for 7 hours at 37°C and time points were recorded for growth and fluorescence every hour. Propidium iodide fluorescence values were normalized to the growth of the cells in the tube and compared to the DMSO negative control, which shows minimal cell death.

mCherry Reporter Assay

A secondary reporter assay utilizing mCherry protein was used as a secondary screen to confirm inhibition of *trans*-translation by compounds previously identified in the luciferase reporter assay described in the protocol by Ramadoss et al. (10). WT and ΔssrA strains for SB75 mCherry trpAt strains express mCherry protein with a termination loop before the stop codon. These cells were grown to OD_{600} 0.2 and subsequently induced with IPTG (1 mM). The cells

were added to a 96-well plate containing diluted hydrazine carbonothiol drug (100 μM and 200 μM) and incubated overnight. The SpectraMax i3 was used to record the OD_{600} of the cells and the fluorescence of the mCherry protein using an excitation of 587 nm and emission of 610 nm. The fluorescence values were normalized to the growth in that column and visualized in a bar graph. The drug was determined to inhibit *trans*-translation if the mCherry product was not degraded by *trans*-translation machinery and had an increased normalized fluorescence value compared to the WT control strain.

Target Identification

The target identification approach utilizing photo-affinity labeling and copper-catalyzed click bioconjugation was adapted from the protocol of Alumasa et al. (15). *E. coli ΔtolC* and *B. anthracis* cells were grown to mid-log phase. KKL-2178 (20 μM) the modified hydrazine carbonothiol with an azide and alkyne moiety was added to the bacterial cultures. After growth for an additional 1 hour, the cells were centrifuged at 2716 rcf. The pellet was resuspended in chilled phosphate-buffered saline and irradiated with 100 $\mu J/cm^2$ (CL-1000 Ultraviolet Crosslinker) for 10 minutes to crosslink the azide in KKL-2178 to its molecular target in the bacterial cell. The cells were lysed by the addition of lysis buffer (100 mM $NaPO_4$, 100 mM NaCl, 0.1% sodium dodecyl sulfate), PMSF (0.5 mg/mL), lysozyme (0.2 mg/mL) and sonication. The lysate was purified by centrifugation to remove any unwanted proteins from the lysed cells. The clarified lysate was incubated with secondary probe KKL-2107 (100 μM), acetonitrile (20 μM), pre-mixed THPTA (1 mM) and $CuSO_4$ (100 μM), and NH_2NH_2. KKL-2107 contains an azide moiety, fluorescent group, and Streptavidin bio-affinity tag. This enables the azide moiety

from KKL-2107 to “click” to the alkyne moiety in KKL-2178 so they are covalently linked allowing for the molecule of interest to be linked to a fluorescent group. The “clicked” product was then precipitated with chilled acetone and acetone washes with intermittent spinning. The samples were incubated with NeutrAvidin agarose resin that was washed with binding buffer (Na_2HPO_4, SDS, BME) to remove ethanol from the resin. The NeutrAvidin resin binds to the bio-affinity tag on KKL-2107 in order to purify the sample when run through a column. The sample was analyzed by SDS-PAGE and the fluorescent band was cut out to be analyzed by mass spectrometry analysis. All probes used in this experiment were synthesized by Dr. John Alumasa.

Ribosomal Protein L7/L12 Cloning

Primers for *E. coli rplL* gene encoding L7/L12 protein were designed to include sites for NcoI and XhoI (NEB) restriction enzymes. A 50 µL PCR was performed using 5x Phusion (NEB), dNTPs, Phusion buffer (NEB), genomic *E. coli* DNA (prepared from WT *E.coli* Δ*tolC* MG1655), and the forward and reverse primers for L7/L12. Amplification of the target DNA fragment was verified by gel electrophoresis. L7/L12 amplified sequence and pET28 vector were digested with restriction enzymes NcoI and XhoI. pET28 vector and L7/L12 digests were ligated together, transformed into DH5α cells, and selected for by plating on kanamycin. The presence of the insert was verified through PCR amplification of L7/L12 and by NcoI and XhoI digestion products run on a gel. The plasmid was purified and transformed into BL21 (DE3) *ΔtolC* cells and the insert was verified and sequenced.

Target Validation

IPTG-induced Growth Curves

250 μL of BL21 (DE3) Δ*tolC* p*rplL* cells were added to 25 mL LB with chloramphenicol (20 μM). Cells were grown to OD_{600} 0.40 and 12 mL of the culture was induced with IPTG (1 mM) while the remaining 12 mL culture was un-induced. Both cultures were incubated for 40 minutes. 2 mL of each culture were transferred to tubes with hydrazine carbonothiol inhibitor (0.5x MIC, MIC, 2x MIC). Initial OD readings were taken at 600 nm and were read every hour for 3 hours.

In-vitro Click Conjugation

Solution A and solution B from NEB kit E6800s, DHFR stop or nonstop template (synthesized by Taryn Ryan), KKL-2178 (20 μM), and water were combined in RNase free environment. The reaction was incubated at 37°C for 2 hours. The reaction was then crosslinked by irradiation with 100 $\mu J/cm^2$ (CL-1000 Ultraviolet Crosslinker) for 10 minutes. The crosslinked sample was combined with KKL-2099 (100 μM), acetonitrile (20 μM), pre-mixed THPTA (1 mM) and $CuSO_4$ (100 uM), and NH_2NH_2. KKL-2099 contains an azide group to "click" with KKL-2178 and a fluorescence group for easy identification on a protein gel. The sample was incubated on a rocker for 1 hour and run on a 15% SDS-PAGE gel. Fluorescence was visualized under UV light.

In-vitro Translation Inhibition Assay

Inhibition of translation by the hydrazine carbonothiol compounds was tested using NEB kit E6800s. Solution A, solution B, control template with a DHFR gene, hydrazine carbonothiol

inhibitor (100 μM), water, and ^{35}S labeled methionine were combined in RNase free environment. The reaction was incubated at 37℃ for ~1 hour. Chilled acetone was added to terminate the reaction and the sample was incubated on ice for ~30 minutes. The sample was centrifuged at 16873 rcf for 10 minutes to pellet and was re-suspended in 1X SDS loading buffer. Samples were incubated at 95℃ for 5 minutes and run on a 15% PAGE gel.

Cytotoxicity Assay

Cytotoxicity assays for the hydrazine carbonothiol compounds were performed with macrophage cells and with KKL-63, 64, and 588. 96-well tissue culture plates were seeded with 1.5 x 10^5 cells/mL in 100 μL of media overnight at 37℃ with 5% CO_2. Media was removed and cells were incubated at 37℃ and 5% CO_2 with fresh media containing 10 μL of ultrapure water, chemical compounds (0.5x MIC, MIC, 2x MIC), or nothing in triplicate. 10 μL of lysis buffer was added to the wells without compound or water. The cells were incubated for 45 minutes. Propidium iodide (20 μM) was added to the wells and incubated at room temperature for a half hour. The plate was read at 493 nm and 636 nm. The amount of fluorescence from propidium iodide was indicative of the toxicity of the compound.

In-silico modeling.

Dock to free ribosomal protein L7/L12

This protocol was adapted from Alumasa et al. (10). The docking studies were performed using the AutoDock Vina program. The Open Babel module was used to determine the minimal energy conformation of KKL-63 and the program PyMOL (Schrödinger) was used to model the

binding of KKL-63 to the L7/L12 ribosomal protein using the Lamarckian genetic algorithm. The crystal structure of L7/L12 with PDB ID 1RQU was used for these docking studies. The program generated 10 binding structures of which the highest docking scores and lowest root-mean square deviation where RMSD=0 were used to determine the ideal binding conformation of KKL-63 with L7/L12 ribosomal protein.

Supporting Information

Calculation of logP

LogP values for the hydrazine carbonothiols were calculated using the program found at http://www.molinspiration.com/cgi-bin/properties.

Band intensity determination

The intensity of the band in the radioactive translation inhibition assay was determined using the program image j (NIH).

Chemical synthesis of analogs & characterization

Chemical synthesis of KKL-64, KKL-588, and KKL-63 was completed as described in the honors thesis of G. H. Babunovic, 2015 (PSU). Additional functional groups were added to existing hydrazine carbonothiol scaffolds to create analogs. KKL-588 was re-synthesized according to this protocol. Verification of the synthesis was shown by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopy.

RESULTS

trans-Translation Inhibition tested by mCherry Reporter Assay

As stated previously, the hydrazine carbonothiol compounds were identified as inhibitors of *trans*-translation through a high-throughput screen that used firefly luciferase as a reporter gene in the assay. This is because the compounds resulted in luminescence values greater than two-fold the activation of the control reporter at 10 μM concentration in the bacteria, which qualified them as a "hit" (6). Before continuing tests with these compounds, a secondary mCherry reporter assay was conducted to further confirm that these compounds inhibit *trans*-translation. WT and *ΔssrA* SB75 *E. coli* strains expressing a mCherry mRNA transcript with a transcription terminator before its stop codon were used. The translation of this mRNA would result in a mCherry protein stuck in the polypeptide channel of a non-stop ribosome. Thus, if the *trans*-translation machinery is active it would degrade the mCherry and no fluorescence would be observed, while an inhibitor to the pathway would result in a fluorescence reading as the ribosome could not be released and the protein would not be tagged or degraded by *trans*-translation machinery.

Results show that all strains had similar growth even in the presence of the inhibitor (Figure 5A). Compared to WT controls that have active tmRNA to complete *trans*-translation, the Δ*ssrA* sample and samples of WT with inhibitor present show an increased fluorescence of mCherry protein (Figure 5B). Further, after normalizing the fluorescence in each sample to the growth of bacteria in each sample, the hydrazine carbonothiol compounds still show a significant fold difference in fluorescence compared to the WT (Figure 5C). This indicates that they inhibit the *trans*-translation mechanism.

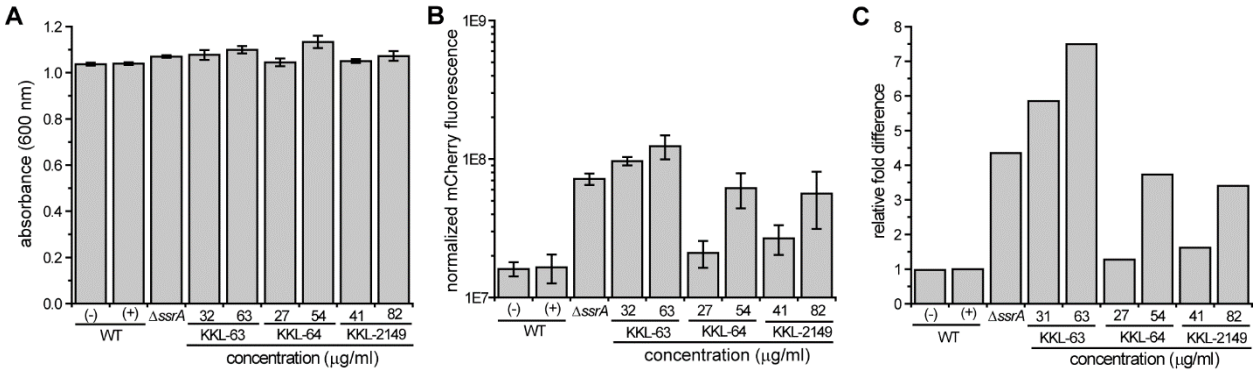


Figure 5. mCherry Reporter Assay indicates inhibition of *trans*-translation. (A) Growth of the bacterial samples was recorded at OD_{600}. Mean values of the samples in triplicate were calculated and error bars represent the standard deviation of each sample. (B) Fluorescence of the bacterial samples with excitation of 587 nm and emission of 610 nm was recorded. Error bars represent the standard deviation of each sample after calculation of the mean from the triplicate wells. (C) The fold difference was calculated by dividing fluorescence values by growth absorbance and normalizing to the WT controls at a fold difference of 1. Samples above 1 show an increased fluorescence due to inhibition of *trans*-translation.

Activity of Hydrazine carbonothiols in Bacterial Pathogens

After confirmation of *trans*-translation inhibition, the activity of the hydrazine carbonothiols in various Gram-positive and Gram-negative bacterial strains was determined. Minimum inhibitor concentration (MIC) assays were conducted in order to ascertain the least amount of drug needed to inhibit visible growth of the bacterial strain being tested. Absorbance values at OD_{600} gave precise growth of the bacteria in the presence of each drug and enabled for the determination of the IC_{50} of the compounds, or the concentration needed to inhibit 50% of growth. The hydrazine carbonothiol compounds were found to be active at low concentrations, especially in certain pathogenic strains of bacteria like *B. anthracis* and Methicillin-resistant *S. aureus* (MRSA) (Table 2).

Table 2. MIC and IC_{50} of Hydrazine carbonothiols in pathogenic strains of bacteria

Compound	Minimum Inhibitory Concentration (IC_{50}**) (μg/mL)					
	E. coli ΔtolC	***Bacillus anthracis***	***MRSA USA300****	***Shigella boydii****	***H. influenza****	***M. smegmatis****
KKL-63	0.5 (0.13)	0.1 (0.5)	3.9	3.9	1.0	7.8
KKL-64	13 (4.6)	13 (9.9)	13	13	6.7	20
KKL-149	8	12	n.d.	n.d.	n.d.	n.d.
KKL-207	26 (5.6)	26 (6.3)	n.d.	n.d.	n.d.	n.d.
KKL-588	0.9 (0.5)	1.8 (0.6)	2.8	14	2.8	14
KKL-2149	2.3 (0.3)	1.3 (0.6)	n.d.	n.d.	n.d.	n.d.
KKL-2178	2.3 (0.5)	2.3 (0.9)	n.d.	n.d.	n.d.	n.d.
KKL-32112	69	69	n.d.	n.d.	n.d.	n.d.
KKL-39698	69	69	n.d.	n.d.	n.d.	n.d.

[$]n.d. indicates MICs were not determined
*Values for pathogenic MICs were previously determined in the honors thesis of G. H. Babunovic (16).
**IC_{50} values are not determined for pathogenic strains

More effective compounds are likely better able to enter the bacterial cell, thus analysis of the relationship between the activity of the compound and its structure can aid in future optimization of the hydrazine carbonothiols. Thus, the compounds were analyzed to determine their octanal to water proportion. This was then compared to the activity of the compounds to try and ascertain a relationship between its MIC and hydrophobicity. Analysis suggests that compounds with higher logP values tend to have a lower MIC value (Figure 6).

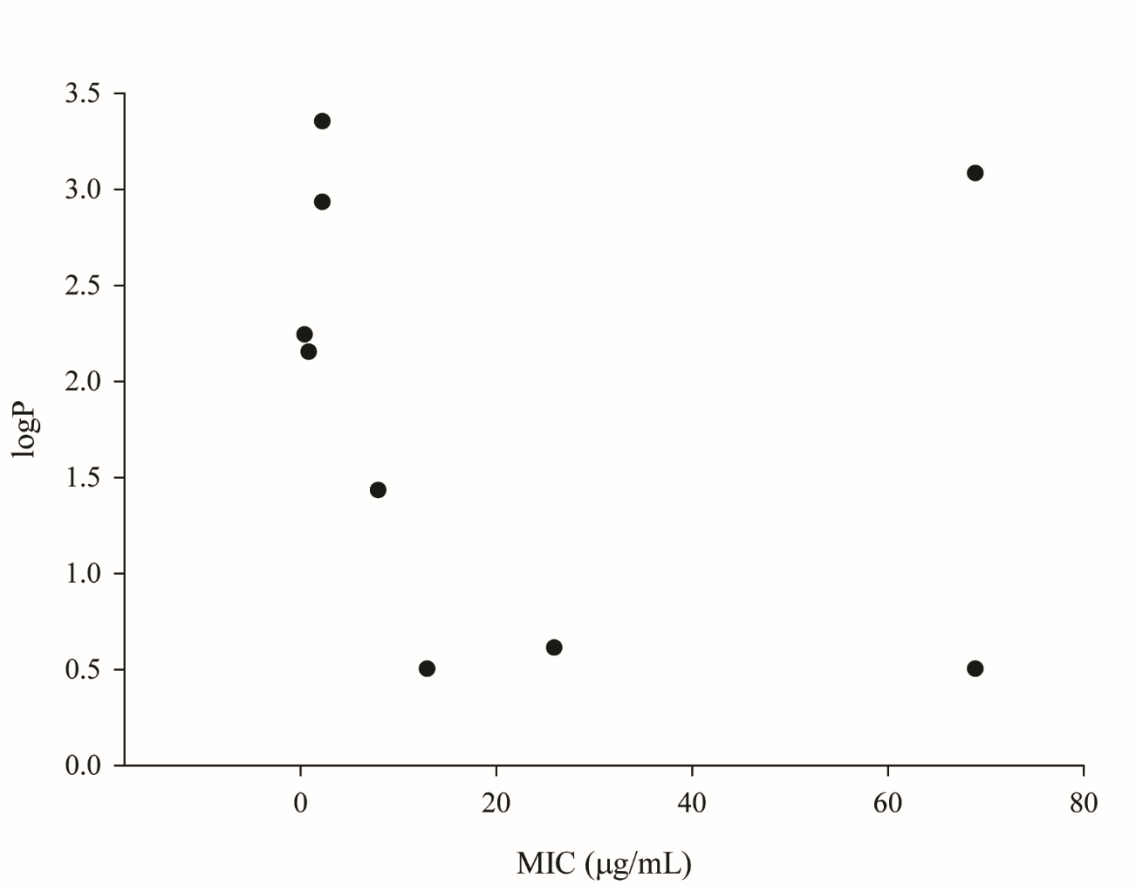


Figure 6. Relationship between logP and MIC of hydrazine carbonothiols. The lipophilicity (logP) of the compound was determined by calculating its octanal to water proportion. This value was then graphed compared to the activity the compound showed against *E. coli* Δ*tolC* bacteria. Compounds with a low MIC, such as KKL-63 (logP = 2.24) and KKL-588 (logP = 2.15), tended to have a higher logP value compared to compounds like KKL-64 (logP = 0.5).

Chemical Properties of Hydrazine carbonothiols

After determining that the compounds were active in pathogens of interest, growth curve assays were conducted in order to ascertain whether the hydrazine carbonothiols were bacteriostatic or bactericidal compounds. *B. anthracis* was grown for 7 hours without inhibitor or with the addition of a hydrazine carbonothiol compound. Time-points were taken every hour in order to determine the growth of *B. anthracis* in the presence of the compound (Figure 7).

Propidium iodide (PI) was also added to the growth medium in order to ascertain the number of dead cells. PI is a compound that is able to intercalate between the bases of DNA of cells and can fluoresce once it is bound to the DNA. However, it can only enter a dead cell that does not have an intact plasma membrane as its structure prevents entrance through the phospholipid bilayer (15). Thus, an increase in fluorescence of PI indicates more cell death.

Results show that when *B. anthracis* is grown in presence of a drug below its MIC it initially will have stunted growth, but will recover to grow properly; however, a drug concentration at or above its MIC is able to inhibit the growth of the *B. anthracis* cells (Figure 7A). Compared to the control with DMSO (the liquid the compounds are dissolved in), a low concentration of KKL-63 does not prevent growth as the absorbance value increases in a similar pattern to the DMSO sample. However, at higher concentrations the growth of the bacteria is stunted as evidenced by the lack of growth above OD_{600} 0.15 (Figure 7A). In order to ascertain if the compounds are killing the bacteria in addition to inhibiting their growth, the fluorescence output by PI was normalized to the growth of the cells in that sample. The relative fold-difference in fluorescence units for the DMSO control was determined to be 1 as DMSO is known to not kill the bacteria at low concentrations. Thus, when comparing the relative fluorescence output of the hydrazine carbonothiol compounds to the control, lower concentrations of KKL-588 and KKL-2149 do not show a large increase in fluorescence. However, at higher concentrations, all three compounds have a pronounced increase in fluorescence, indicating the compounds are bactericial (Figure 7B-D).

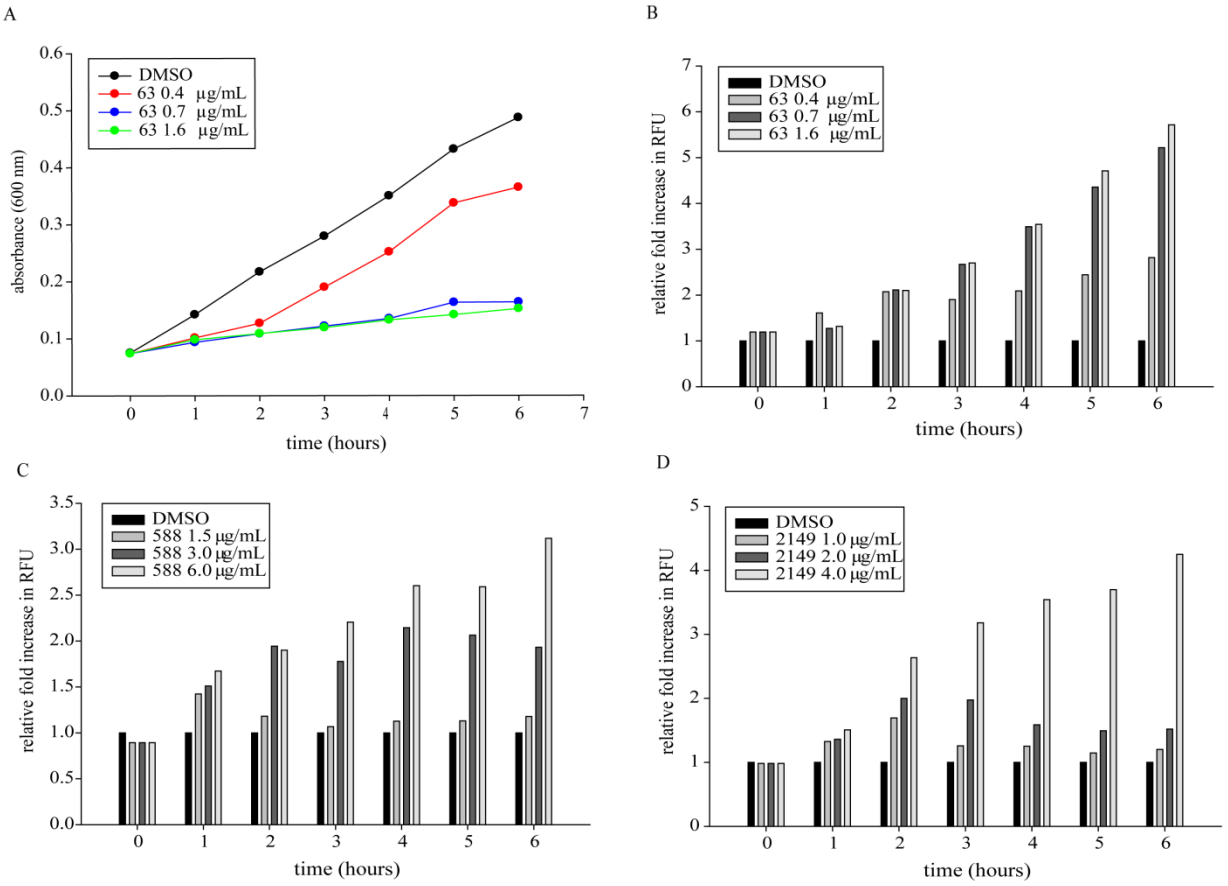


Figure 7. Growth Curves of KKL-588 in *B. anthracis* show compounds are bactericidal. (A) KKL-63 was chosen as a representative sample of the hydrazine carbonothiol class when making this figure for clarity of results. Growth of B. anthracis in the presence of KKL-63 measured every hour by recording the OD600. The fluorescence in samples given by propidium iodide excitation at 493 nm and emission at 636 nm was read. The fluorescence values were divided by the growth OD600 and were then normalized to the sample with DMSO and no inhibitor which has a fold-increase of 1. Any fold-increase above 1 is indicative of death of the cells. (B) Relative fold-increase in relative fluorescence units (RFU) of KKL-63. (C) Relative fold-increase in RFU of KKL-588. (D) Relative fold-increase in RFU of KKL-2149.

Cytotoxicity assays were then conducted in order to ascertain the therapeutic value of the hydrazine carbonothiols. For this assay, macrophages were added to a tissue-culture plate and the compounds were added to the growth medium. After incubation, propidium iodide was used as a marker for macrophages that were killed by the hydrazine carbonothiol compounds. Positive controls using the addition of a lysis reagent show maximal toxicity to the macrophages (Figure 8A, E). A negative control was also used to approximate the expected amount of spontaneous

cell death not related to toxicity of the compounds. Bright field and fluorescence microscopy images of the macrophage cells were captured for those treated with KKL-63, KKL-588, and KKL-2149 at 100 μM (Figure 8B-D and 8F-H). While some macrophages treated with the compounds are lysed, compared to the positive control the ratio of lysed cells in the experimental samples is significantly less. This is supported by a calculation of the percentage of macrophages lysed when the fluorescence of propidium iodide in each sample was normalized to the positive control. For all samples, the maximum percentage of macrophages lysed was under 20% (Figure 8I).

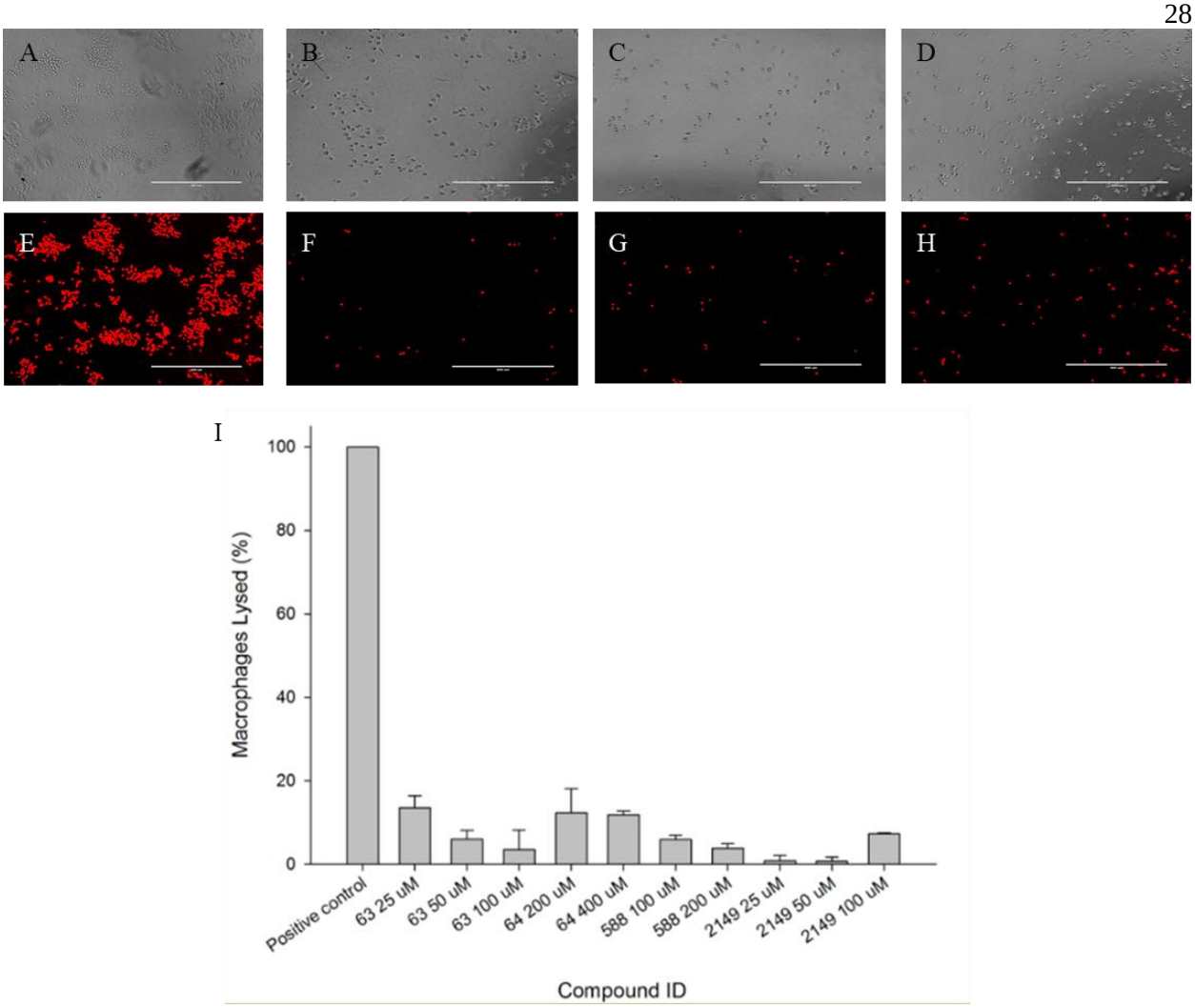


Figure 8. The hydrazine carbonothiols are not toxic to macrophages. (A, E) Bright field and fluorescence image of lysis-treated macrophages for the positive control. (B, F) KKL-63 treated macrophages at 100 μM in bright field and fluorescence. (C, G) Bright field and fluorescence view of macrophages treated with 100 μM KKL-588. (D, H) KKL-2149 treated macrophages at 100 μM in bright field and fluorescent images. (I) The fluorescence of propidium iodide in each sample was recorded and normalized to the positive control to ascertain the percentage of macrophages lysed in each sample. Error bars represent the average standard deviation in the two biological replicates with triplicate wells.

Target Identification of Hydrazine carbonothiols

After establishment of the hydrazine carbonothiols as active compounds in a range of bacterial pathogens with minimal toxicity in macrophages, they seem to be ideal drug candidates. However, it is important to discover the target of the compounds within the *trans*-translation

pathway for further development. Thus, a chemical biology approach adapted from the protocol of Alumasa et al. (5) involving a combination of photo-affinity labeling, click conjugation, and bio-affinity purification was utilized for target identification. For this click chemistry approach, a hydrazide analog that incorporates an azide and alkyne moiety, called KKL-2178, was designed and synthesized (by Dr. John Alumasa) (Figure 9).

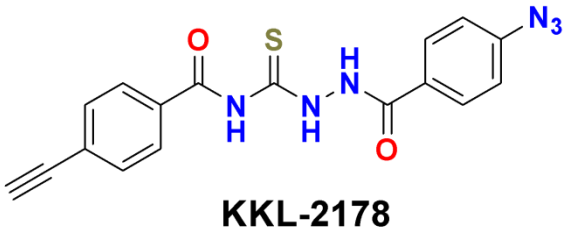


Figure 9. Chemical probe for the Hydrazine carbonothiol compounds used for target ID. The probe for the hydrazine carbonothiol compounds has an analogous structure to the parent compounds with additional alkyne and azide functional groups for photo-affinity labeling and click conjugation.

KKL-2178 was incubated with *E. coli* and *B. anthracis* cells that have reached mid-log growth (OD_{600} 0.6-0.9). Then cells were exposed to UV light to activate the azide functional group in KKL-2178 so the activated radical could react and covalently link to the molecule it is bound to in the bacterial cell, which is presumably the target of the hydrazide compounds. This photo-affinity labeling is thus selective and irreversible (5). Subsequent lysis of the cells and incubation of the lysate with a secondary probe KKL-2107 (Alumasa et a. (10)) containing an azide resulted in the click conjugation of the alkyne in primary probe KKL-2178 to the azide in secondary probe KKL-2107. This secondary probe further contained a biotin label so that the clicked lysate could be affinity purified and contained a fluorescent tag in order to identify the protein of interest on a gel (Figure 10A). The use of KKL-2178 in this process resulted in a fluorescent band between 10-15 kDa identified by SDS-PAGE (Figure 10B). Previous studies with oxadiazole *trans*-translation inhibitors have shown that use of the parental compound (without the alkyne and azide moieties) in this chemical biology approach does not result in any

fluorescence on the gel (10). Thus, KKL-2107 does not have any nonspecific effects within the assay that could affect results.

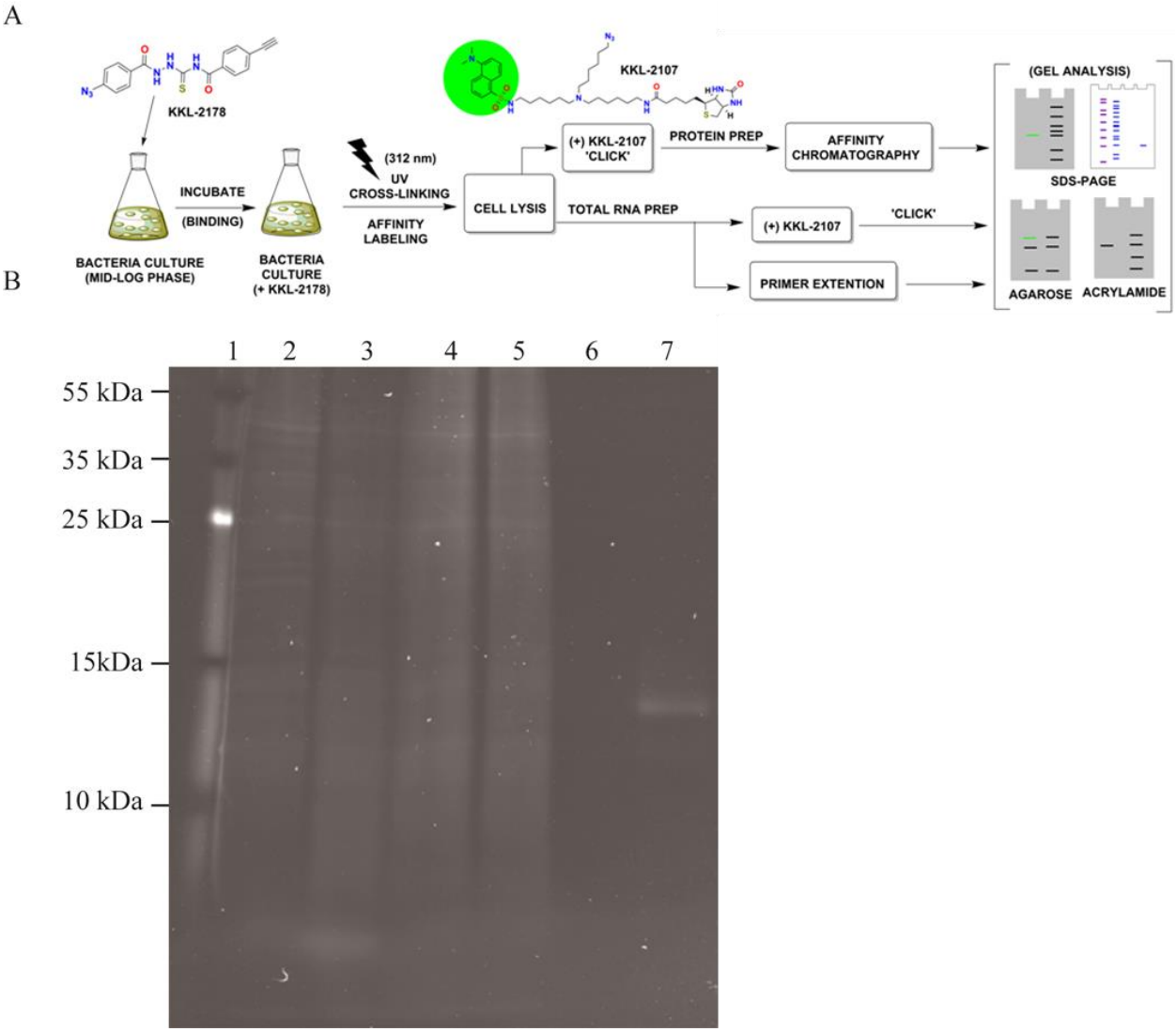


Figure 10. Chemical Biology Approach to Target Identification. (A) The scheme for the chemical biology approach is adapted from (10) and involved photo-affinity labeling, click chemistry, and affinity chromatography in order to identify the target of the compounds. (B) SDS-PAGE analysis shows KKL-2178 binds to a small protein between 10-15 kDa. Lane 1: protein ladder. Lane 2: crosslinked lysate. Lane 3: clicked lysate. Lane 4: acetone precipitated lysate. Lane 5: flow through. Lane 6: wash. Lane 7: elution fraction with fluorescent band of crosslinked protein. The fluorescent band identified as the protein binding to KKL-2178 was excised from the gel and digested with trypsin. Mass spectrometry analysis of the gel sample shows L7/l12 as the primary protein present.

After cutting out and digesting the fluorescent band, it was sent off for mass spectrometry analysis. I found that the protein the hydrazine carbono-thiol probe was binding to was L7/L12 in

the large subunit of the ribosome (Figure 10), implicating this protein as the target of the class of compounds.

Validation of L7/L12 as Hydrazine carbonothiol Target

In order to validate L7/L12 as the target of the hydrazine carbonothiol compounds, the gene was first cloned into *E. coli* in order to overexpress and purify the protein. Primers were designed for the L7/L12 gene on the *E. coli* genome and were used to amplify that region by PCR. The PCR product was then digested and ligated with pET28a vector. The recombinant plasmid was initially transformed into competent DH5α and subsequently BL21 (DE3) *E. coli* cells. The insert was verified by sequencing (Figure 11).

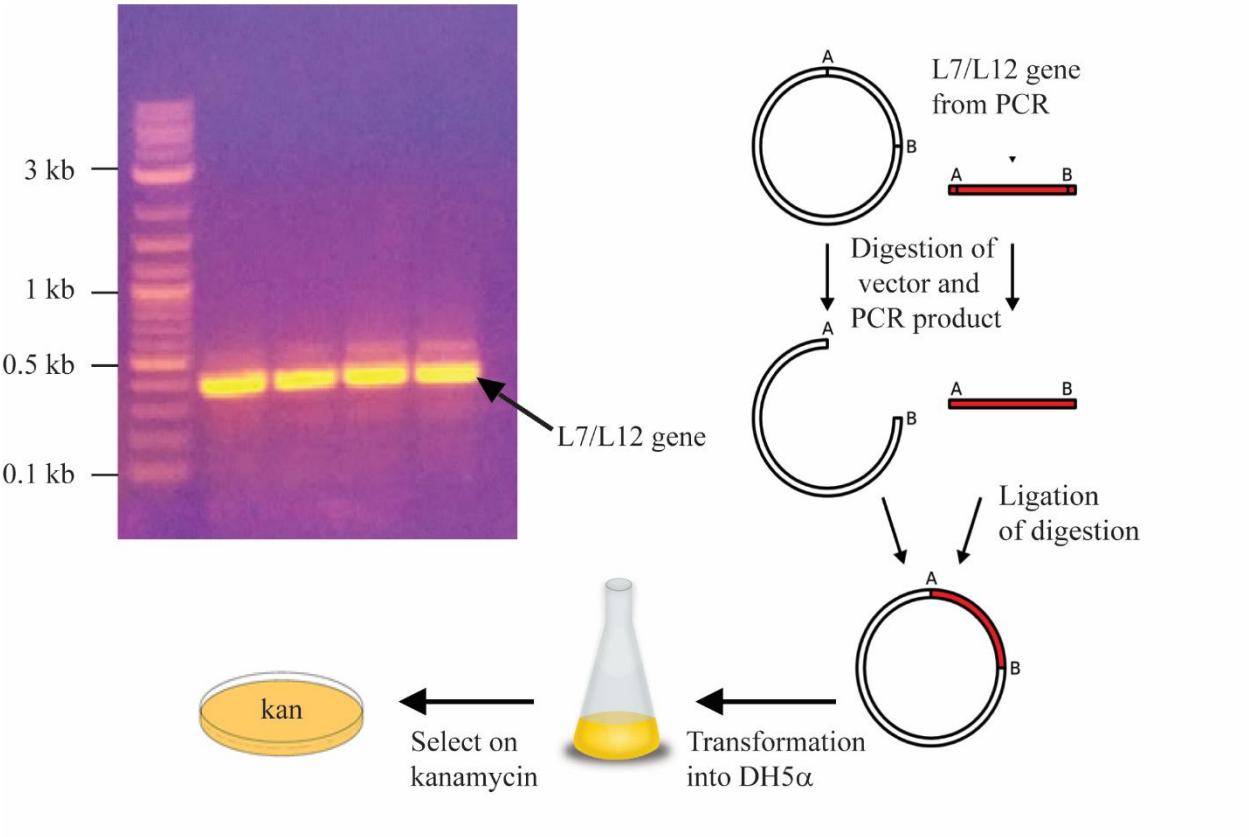


Figure 11. Scheme of L7/L12 Molecular Cloning. *E. coli* DNA was put into a PCR with primers designed to amplify the L7/L12 region. The PCR product and pET28 vector were subsequently digested with restriction enzymes and ligated together to form a recombinant plasmid. The plasmid was transformed into competent DH5α cells and selected for by the kanamycin cassette in the pET28a vector. The plasmid was then transformed into BL21 (DE3) *E. coli* cells.

This BL21 (DE3) *E. coli* strain with the *rplL* gene on a plasmid able to be induced with IPTG was initially used in a growth curve assay intended to validate L7/L12 as the target of the hydrazine carbonothiols. The induction of the bacteria to overexpress L7/L12 in the presence of the inhibitors was predicted to recover growth of the bacteria as the excess of L7/L12 free protein would bind to the KKL compound. This would then enable the ribosomal-bound L7/L12 protein to not be targeted by the compounds and allow for the continued growth of the bacteria as normal. However, the T7 promoter in the *E. coli* strain used was found to arrest growth of the bacteria upon starting the experiment as it was too efficient and used up the cell's resources.

Thus, a strain of *E. coli* that also overexpressed the *rplL* gene encoding for L7/L12 protein was placed on a plasmid with a T5 promoter, which allowed for proper growth of the bacteria. After conducting the experiment, there was no growth recovery in this new strain that was induced with IPTG as was expected. While the uninduced and induced controls grew similarly, the induced samples with compounds grew slightly worse than the corresponding un-induced samples in the multiple trials conducted (Figure 12A). In order to ensure that the correct samples were actually induced with IPTG, they were run on an SDS-PAGE gel. The gel reveals overexpression of L7/L12 as lanes 5-7 have pronounced dark bands present in the 12 kDa range of the gel that are not present in the un-induced sample lanes (Figure 12B). This ensures that the reason for no growth recovery was not related to the lack of overexpression of the ribosomal protein.

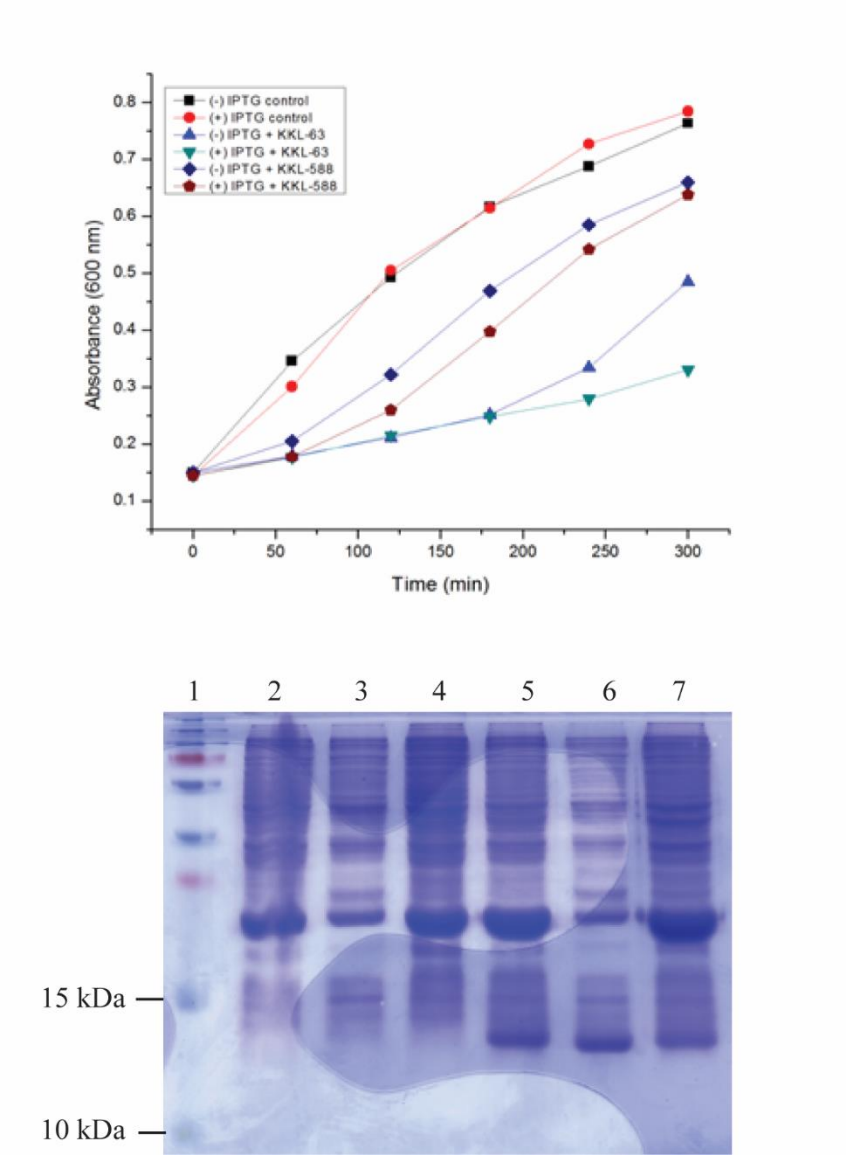


Figure 12. Overexpression of L7/L12 ribosomal protein does not recover growth of bacteria in presence of hydrazine carbonothiol compound. (A) BL21 (DE3) E. coli rplL strain with a T5 promoter was grown in the presence of hydrazine carbonothiol compound and induced with IPTG. Growth was recorded every hour by measuring the OD600 of the samples. (B) SDS-PAGE analysis of the un-induced and induced samples shows proper overexpression of L7/L12 protein. Lane 1: protein ladder. Lane 2: un-induced control. Lane 3: un-induced KKL-63. Lane 4: un-induced KKL-588. Lane 5: induced control. Lane 6: induced KKL-63. Lane 7: induced KKL-588.

In-silico docking modeling was used to predict where the compounds could be binding within the L7/L12 protein. With this, a computer program was able to predict the possible binding sites of a low-energy structure of KKL-63 within a crystal structure of L7/L12 dimer.

This allows for a prediction of where the compound is actually binding in-vivo. Using this program, the area of L7/L12 within KKL-63 was docking was predicted to be located in the N-terminal domains of the L7/L12 protein (Figure 13A). Further analysis of the binding using surface modeling shows that KKL-63 is likely binding to a pocket specifically within the N-terminal domains of the protein (Figure 13B).

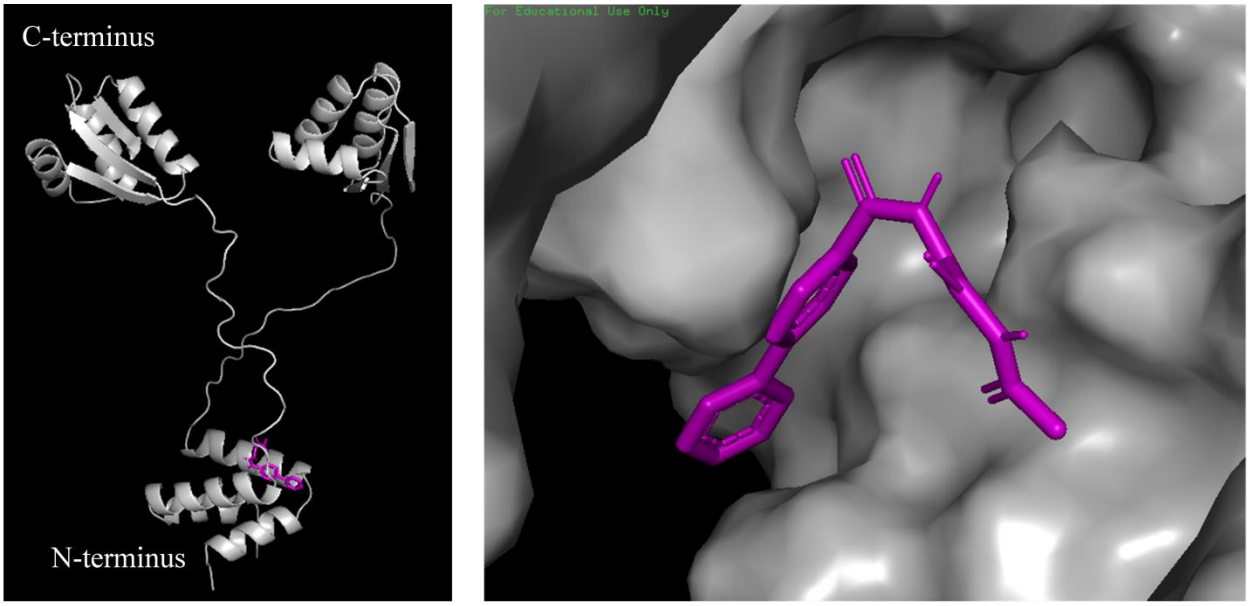


Figure 13. In-silico docking model of KKL-63 binding to ribosomal dimer L7/L12. (A) KKL-63 is predicted to bind to the N-terminal domains of the L7/L12 dimer. (B) KKL-63 is predicted to specifically bind in a small pocket within the N-terminal domains of the protein.

Translation Inhibition Testing for Hydrazine carbonothiols

With the target of the hydrazine carbonothiols predicted to be ribosomal protein L7/L12, a translation assay was done in order to test whether or not the compounds inhibit normal translation in addition to *trans*-translation. NEB-kit containing in-vitro translation components and a DHFR DNA template were incubated in the presence of select hydrazine carbonothiol compounds that range from not being active to being very active. During this time, DHFR

protein would be produced if the compounds did not inhibit the translation mechanism, while no protein would be produced if the inhibitors prevented translation. In order to monitor the assay, ^{35}S-labeled methionine was used as the radioactivity can be monitored to show translation of the DHFR protein from the mRNA template. Gel results show that no protein was produced for the negative control chloramphenicol, which is known to inhibit translation (Figure 14A). However, protein was produced for the positive control without inhibitor and for the samples with hydrazine carbonothiol compounds, showing the compounds do not inhibit translation (Figure 14A). Additionally, analysis of the intensity of the protein band indicates that minimal DHFR was produced in the negative control and that the hydrazine carbonothiol addition to the translation reaction produces equal amount of protein to the sample that did not contain any drug (Figure 14B).

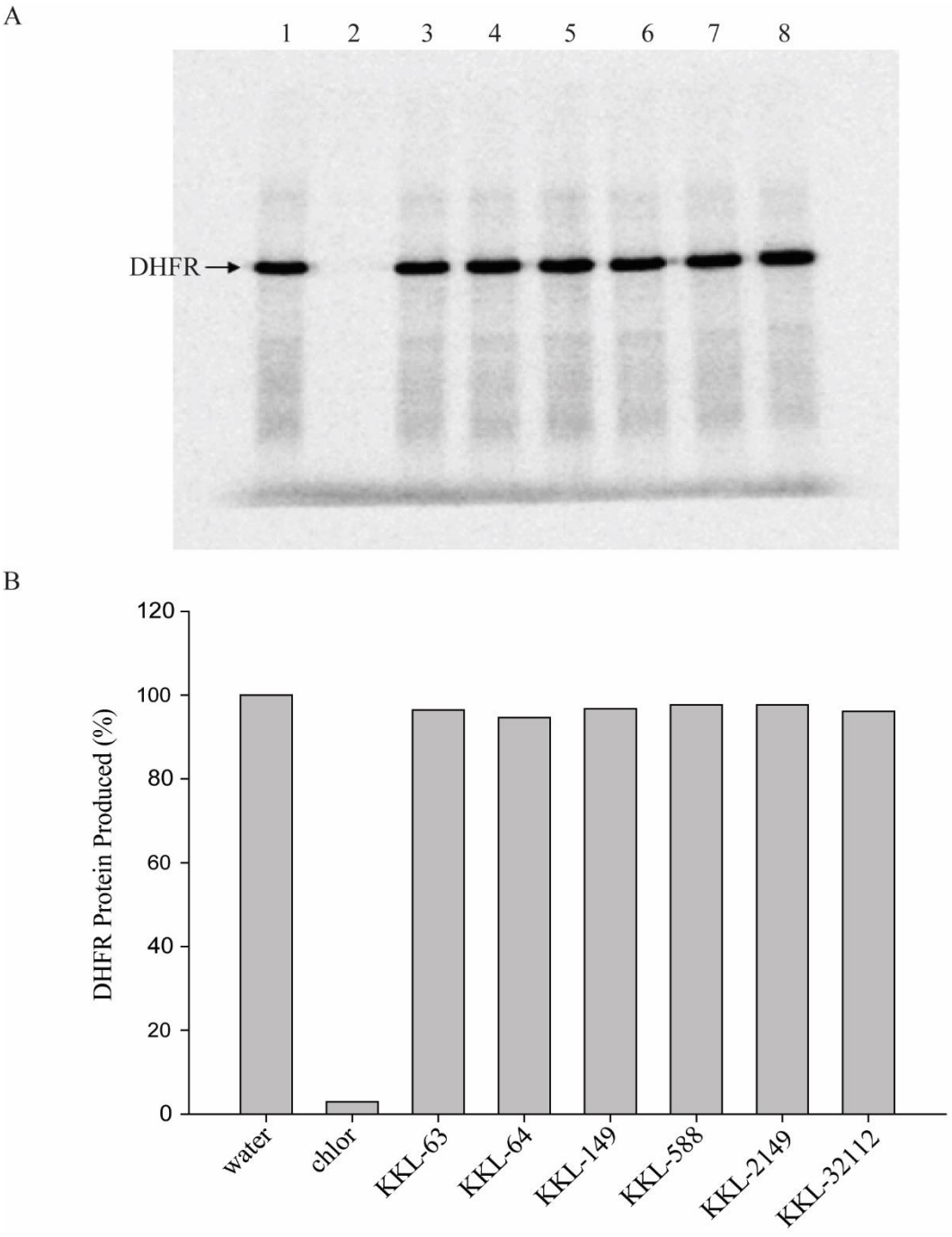


Figure 14. Translation is not inhibited by hydrazine carbonothiols. (A) Samples containing translated DHFR protein with 35S-labeled methionine were run on a 15% SDS-PAGE gel. The gel was dried and exposed on a phosphor screen. Lane 1: positive control that does not contain drug. Lane 2: chloramphenicol (chlor) treated sample, known to inhibit translation. Lanes 3-8: KKL-63, 64, 149, 588, 2149, and 32112 treated samples at 100 µM. (B) The band intensity in the image of the gel was analyzed by image j program and the values were normalized to the positive control sample showing DHFR production in the presence of no drug. A bar graph was constructed showing the percentage of DHFR protein produced for each of the samples.

DISCUSSION

The hydrazine carbonothiols are a class of compounds identified in a high throughput screen as *trans*-translation inhibitors. Initial analysis of the compounds show that they will likely have good therapeutic value as the MIC and IC_{50} values for the compounds in different bacterial pathogens show broad-spectrum activity with a small concentration of drug needed. Further, the low cytotoxicity of the hydrazine carbonothiol compounds when tested in macrophages shows they will likely have minimal toxicity within the human body. In order to further determine the value of these compounds, they should be tested in a mouse model in order to analyze toxicity of the compounds and evaluate the amount of drug needed to clear a bacterial infection.

However, when analyzing the MIC data for the assortment of compounds synthesized, there are noticeable differences in the activity of the compounds in pathogens. For instance, KKL-64 has a much higher MIC than KKL-63. When comparing the logP values determined for these compounds, KKL-63 had a high value at 2.24 while KKL-64 had a lower value of 0.5. Further, it was shown that these differences in logP correlate to the MIC of the compound, with more hydrophobic compounds being more active in-vivo. This is likely due to an increased number of hydrophobic groups facilitating the entry of those compounds into the bacterial cell; thus, a smaller concentration of drug has to be used compared to a compound with lower membrane permeability. This is directly correlated to the initial hits on the high-throughput screen, as the compounds that had a better MIC value were found to have higher screening values. Thus, for future drug design using the luciferase-based reporter assay, compounds that have a higher value on the HTS should have more effective antibacterial activity.

The chemical biology approach taken to identify the target of the hydrazine carbonothiols revealed the crosslinked protein to be L7/L12. Ribosomal protein L7/L12 is composed of two

dimers wherein each dimer contains two C-terminal domains and an N-terminal domain that are connected via a flexible hinge region (21). The C-terminal dimers of the protein are not associated with the ribosome; rather they bind factors during translation such as EF-Tu (22). Thus, these C-terminal domains are also responsible for the association of the tmRNA-SmpB complex into the ribosome during the *trans*-translation pathway (23, 24). The N-terminal domains of L7/L12 allow for the dimers to associate with each other and with the L10 domain in the 50S subunit of the ribosome (23, 24). The in-silico docking studies conducted with a crystal structure of a single free L7/L12 dimer predict that KKL-63 is able to bind in a pocket of the N-terminal domain of the ribosomal protein.

The lack of growth recovery of the *E. coli* bacteria tested in the growth curve experiment might suggest that the hydrazine carbonothiol compounds are not binding to free L7/L12 protein. Unpublished results with a triazole-based *trans*-translation inhibitor in the Keiler lab that also targets ribosomal protein L7/L12 showed a recovery of growth in the samples induced with IPTG. This indicates that those compounds were able to bind to the free overexpressed protein and that there was enough protein produced in the cell that the drug was no longer able to bind the ribosomal L7/L12, allowing *trans*-translation to resume as normal. This lack of recovery by the hydrazine carbonothiol compounds suggests that they are active when binding L7/L12 that is on the ribosome, not free floating in the bacterial cell. This can be ascertained in the future by purification of the L7/L12 protein, which can be used in an isothermal titration calorimetry (ITC) experiment that determines the binding of the hydrazine carbonothiol compounds to the protein.

Additionally, validation studies were attempted for these compounds. An in-vitro click experiment was done wherein translation machinery was incubated with a DHFR template without a stop codon to create a non-stop ribosome complex. KKL-2178 was added to the

sample so that the azide moiety could presumably crosslink to the L7/L12 ribosomal protein in the nonstop ribosome. The sample was then click conjugated to KKL-2099, a modified secondary probe that only contains an azide and fluorescent tag (no affinity tag). No visible band was able to be seen on the 15% SDS-PAGE gel, which was likely due to the small-scale of the experiment making it so not enough of the crosslinked, fluorescent sample was run on the gel. Thus, this experiment should be tested again with a larger scale in order to validate the target of the hydrazine carbonothiols as ribosomal protein L7/L12.

One notable observation in the data is that the mCherry and luciferase-based reporter screens show that the *trans*-translation pathway is inhibited by the hydrazine carbonothiol compounds, while the translation assay shows that there is no inhibition of this mechanism by the same compounds. This is significant considering the target of the compounds is the L7/L12 protein in the 50S subunit of the ribosome, which is required for ribosome activity. These results indicate that there is likely some structural change in the ribosome during the *trans*-translation pathway that enables the drug to bind in a pocket that is not available during the normal translation process. These results would also support why the compounds are not able to bind to the free L7/L12 protein in the cell as it might not be in the correct conformation that it is found in when *trans*-translation is active.

Previous investigators in the Keiler lab have posited that the binding of the *trans*-translation inhibitors to their target in the ribosome limit the flexibility of the ribosome such that it can still perform normal functions during translation, but cannot accommodate that limited flexibility during *trans*-translation (10). This could likely be the case for the hydrazine carbonothiols as tRNA that enters the ribosome during translation is significantly smaller than the tmRNA that must enter the A site during *trans*-translation. As such, a limited flexibility in

the ribosome may not allow for tmRNA to fit. However, as there is no crystallized structure of L7/L12 protein in a ribosome in a nonstop complex, these changes in flexibility cannot be confirmed to be the cause for the differences observed between translation and *trans*-translation for these compounds.

In summary, the hydrazine carbonothiol compounds are promising for future antibiotic development. These compounds have been found to have minimal toxicity to macrophages while maintaining effectiveness against bacterial pathogens. Future development of the drugs should focus on more hydrophobic structures as these have higher activity to maintain the same level of bacterial cell death. Further, the target of these compounds being in the *trans*-translation pathway provides for a novel target in the world of drug development for which bacteria are not inherently resistant. The target specifically being the L7/L12 ribosomal protein only when the ribosome is in a non-stop complex also enables for these inhibitors to only be effective during *trans*-translation and not normal translation. Thus, they would likely have no impact on human cells' translation ability. The broad-spectrum activity of these compounds against a variety of gram-positive and gram-negative pathogens further supports why the hydrazine carbonothiol compounds should be looked upon as promising for development of a novel class of antibiotics.

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APPENDIX

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Licensed Content Publisher	Springer Nature
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Licensed Content Title	Mechanisms of ribosome rescue in bacteria
Licensed Content Author	Kenneth C. Keiler
Licensed Content Date	Apr 13, 2015
Licensed Content Volume	13
Licensed Content Issue	5
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EDUCATION

Pennsylvania State University, University Park, PA
Bachelor of Science in Biochemistry and Molecular Biology 5/2018
Concentration: Molecular and Cell Biology
Minor in Psychology
Honors in Biochemistry and Molecular Biology

THESIS

Titled "Characterization and Target Identification of Hydrazine carbonothiols, a class of *trans*-Translation Inhibitors"
Thesis Supervisor: Kenneth Keiler

SCHOLARSHIPS/AWARDS

Jacqueline Hemming Whitfield Student Research Endowment Fall 2015-Spring 2016
Edward B. Nelson Undergraduate Research Award Spring 2016

LABORATORY EXPERIENCE

BMB Research Lab, Dr. Kenneth Keiler **Spring 2016-Fall 2017**
The Keiler lab uses the *trans*-translation pathway to identify potential targets for the development of new antibiotics to curb antibiotic-resistant bacteria.

This research project's goal is to identify the target of the hydrazine carbono-thiol class of compounds inhibiting the *trans*-translation mechanism of action and to validate the discovered target by in-vivo, in-vitro, and in-silico assays. Preliminary results are being gathered for publication.

POSTER PRESENTATIONS

Undergraduate Research Exhibition **Spring 2017**

Presented poster titled "Identification and Validation of the Target of Hydrazine carbonothiols"

Undergraduate Research Exhibition **Spring 2016**

Presented poster titled "Investigation of Conditions that Limit Growth in *Caulobacter crescentus* ArfB Mutant"

VOLUNTEER AND SHADOWING ACTIVITIES

Mt. Nittany Medical Center **Spring 2017-Fall 2017**

Emergency Room Volunteer (28 hours)

Responsible for transportation of patients to CT and Ultrasound, communicating with families, and assisting medical staff

Temple University Family Medicine **Summer 2017**

Shadowed David O'Gurek, M.D. (20 hours) in an urban outpatient clinic in North Philadelphia. Observed how he interacted with the underserved population in the area and runs a suboxone clinic for heroin addicts to help them lead more productive lives

Hershey Primary Care Scholars Program **May-June 2017**

Shadowed Physicians at the Harrisburg Practice of Hershey Medical Center

Learned about the various fields within primary care from physicians in those fields. I also was able to shadow physicians at the outpatient Harrisburg clinic and inpatient setting at the Penn State Children's Hospital in Hershey

Plymouth Meeting Family Medicine **Spring 2017**

Shadowed Hermine Stein, D.O. (20 hours) in a suburban outpatient clinic. Observed how she was interacting with her patients as if life-long friends and asked them personal details to make them more comfortable and establish relationships.

Abington Memorial Hospital **Summer 2015**

Hospital Elderly Life Program (HELP)

Responsible for communicating with elderly 65+ patients who typically had dementia or other mental incapacities to make their hospital stay more comfortable and communicate with them when no family or visitors were present

EXTRACURRICULAR ACTIVITIES

Penn State University Vole Ballet Club **Fall 2015-Fall 2017**

Perform in recitals held each semester with pieces choreographed by other students

Fundraising for THON

R&R THON Security Leader **Fall 2017-Spring 2018**

Responsible for the safety of all dancers, students, and THON families THON weekend by resolving situations with volunteers in the stands, directing fellow committee members, and being a leader in the absence of a THON Captain

R&R THON Committee Member **Fall 2016-Spring 2017**

Responsible for the safety of all dancers, students, and THON families THON weekend and ensuring volunteers could go to the floor/event level to see dancers

Check-In Specialist responsible for entering families and volunteers into the digital line to get onto the floor during THON and also answering any questions asked by spectators and families