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THE *TRANS*-TRANSLATION INHIBITION IN *MYCOBACTERIUM SMEGMATIS*

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ABSTRACT

Antibiotic resistance is a growing problem worldwide and has been spreading at an alarming rate over the past few decades. We are in dire need of new antimicrobial compounds that target novel pathways in bacteria. The focus of this project is the *trans*-translation pathway because it is required for growth and virulence in most pathogenic bacteria. More importantly, this ribosome-rescuing mechanism is only found in bacterial cells; therefore, pathogenesis can be prevented without harming eukaryotic cells. In order to investigate the inhibition of this pathway, series of experiments were performed on *Mycobacterium smegmatis. M. smegmatis* was chosen because it is non-pathogenic, grows quickly, and is a great model for *M. tuberculosis*. To identify potential antibiotics, a compound library at Novartis was screened for compounds that inhibited the *trans*-translation in *E. coli*. MIC and MBC assays were run on 27 of the screened molecules. Results showed that some of these molecules had bactericidal activities with very low MIC (≤ 10 μM). One of these compounds, E5, was tested using fluorescent reporter assays. The results suggested that E5 inhibits the *trans*-translation pathway by hindering proteolysis step of *trans*-translation. Currently, assays are being developed to identify a target of another inhibitor, F2.

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ACKNOWLEDGEMENTS

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INTRODUCTION

Ever since the discovery of the first natural antibiotic, penicillin, by Alexander Fleming in 1928, the use of antibiotics has become universal all around the world (1). It seemed that the war against infectious diseases was won without a doubt. However, this victory did not last long because resistance started to accumulate. The resistance appeared in different forms including increased or decreased efflux, enzymatic inactivation, target modification, bypass, repair or amplification, sequestration, intracellular localization or biofilm formation (2).

Tuberculosis (TB) is one of the diseases for which resistance has accumulated considerably over the years. In developed countries, TB is not a serious problem because cheap and effective treatments are available. Also, due to TB vaccinations, it is very rare to find outbreaks of TB cases in developed countries. However, tuberculosis is still one of the leading causes of disease and deaths worldwide, especially in poorer developing countries due to lack of resources and proper medical care (3). The prevalence of resistant TB, both MDR and XDR-TB, is increasing due to the expanded yet inappropriately controlled use of antibiotics (4, 5). Unfortunately, there has not been a new discovery of antibiotics for TB for the last 50 years (6). We are in a dire need of finding a new antibiotic class. In order to overcome the accumulating resistance, a new target has to be identified that has not been extensively exploited by the

current antibiotics. Making modifications on existing scaffolds will not be enough (7).

One of the pathways that has not been exploited is the *trans*-translation pathway in bacteria. Using this novel target prevents cross-resistance emerging between existing and new drugs. This pathway is responsible for ribosome-rescue when translation is stalled. Bacterial translation does not require any specific signals from the 3' end of the mRNA. Therefore, the bacterial ribosomes often initiate translation despite mRNA errors. A stop codon is necessary for the peptide release from the ribosome. If stop codons are missing from the mRNA, the ribosomes that initiated the translation are stalled at the 3' end. As more ribosomes are stalled over time, proper protein synthesis cannot occur; therefore, the cell becomes sick. The *trans*-translation pathway is essential for cell survival and virulence (8, 9).

Trans-translation can rescue stalled ribosomes from the non-stop mRNA translation via a key element called tmRNA. Transfer-messenger RNA (tmRNA) is a bacterial RNA molecule with both tRNA and mRNA properties. There are three main steps in the pathway: association of key components, tagging of the defective peptides, and proteolysis of tagged peptides (9). First, the bifunctional tmRNA molecule forms a ribonucleoprotein complex with several different components to bind to the stalled ribosome. SmpB, a small protein that binds to the tRNA portion of tmRNA, initially comes into the complex and associates with tmRNA (8). Then, the tmRNA molecule is charged with alanine (10, 11, 12) and interacts with GTP-bound EF-Tu, which is responsible for binding of tmRNA to

the stalled ribosome (13). When the ribonucleoprotein complex recognizes nonstop translation, the tRNA portion of the tmRNA enters the A site of the ribosome (14). At this point, the second main step of the *trans*-translation pathway takes place. The nascent polypeptide is transferred to tmRNA. The mRNA portion of the tmRNA, which encodes a specific peptide tag, is then used as a reading frame to resume translation while the truncated or erroneous mRNA is released and degraded. Translation continues until the ribosome encounters stop codon and is released (9, 15). In the third and final step of the pathway, the defective peptide is transported to the ClpXP protease and degraded (9, 16). Proper removal of the defective peptides is an essential part of the *trans*-translation pathway because accumulation of these peptides could inhibit growth and metabolic reactions in bacteria (Figure 1).

Targeting the *trans*-translation pathway has several advantages. First of all, this unique ribosome-rescue mechanism is only present in bacteria (9). If a new drug can successfully inhibit the *trans*-translation pathway, it will be able to kill the bacteria without hurting humans. Evidence from *Neisseria gonorrhoae*, *Shigella flexneri,* and *Yersinia pseudotuberculosis* suggest that *trans*-translation is essential for bacterial growth and virulence (9, 17). Inhibition of this pathway can cause growth defects and even death in bacteria.

Developing a model system for TB is an important part of drug discovery. Using *Mycobacterium tuberculosis* is the best way to test possible candidates; however, it is often dangerous to work with the bacteria and requires BSL-3 facilities. Also, it takes 2 weeks to grow up a saturated culture of *M. tuberculosis*.

Instead of using dangerous and inefficient *M. tuberculosis*, a bacterium from the same genus called *Mycobacterium smegmatis* was used in this set of experiments. *M. smegmatis* is a non-pathogenic bacterium that shares many traits with the *M. tuberculosis* such as bacilli growth and biofilm production. One of the most practical benefits of *M. smegmatis* is that it only takes 48 hours to grow a saturated culture. *M. smegmatis* is safe, fast-growing, and closely related to *M. tuberculosis*; therefore, it is an excellent model organism for initial drug discovery experiments (18).

Previously, high throughput screening was performed on 650,000 small molecules from a Novartis chemical library to find chemicals that inhibit the *trans*-translation pathway. In order to achieve this, an *E.coli* luciferase reporter system with a trpA terminator was utilized to ensure the use of the *trans*-translation pathway. In this particular system, luminescence is produced only if a compound inhibits one of the steps in *trans*-translation pathway. Out of 650,000 compounds, 51 showed possible inhibition of *trans*-translation in *E. coli* reporter system (Figure 2). In order to evaluate the efficacy of these inhibitors in a new model system, MIC and MBC assays were performed in *M. smegmatis.* After effective compounds were identified, the molecular targets of individual compounds were examined. First, special strains of M. smegmatis with *mCherry* reporters were used to assay proteolysis of tagged proteins. The second approach involved purification of EF-Tu protein and an in vitro binding assay using fluorescence polarization. If an inhibitor competitively binds to the EF-Tu protein, the amount of fluorescence will be less than the free protein. This would imply that the molecule

inhibits the initial association of key components step of the *trans*-translation pathway by competitively binding to the EF-Tu. Identifying the molecular targets of these inhibitors is important because modifications can be made to improve specificity as well as efficacy of these drugs.

MATERIALS AND METHODS

Bacterial strains

For MIC and MBC assays, *M. smegmatis* strain MC2 155 was grown at 37°C in lysogeny broth. To each *M. smegmatis* culture, Tween-80 was added at 0.1% the total volume to avoid clumping. All overnight cultures were grown for 48 hours in a 37°C roller drum.

The *mCherry* reporter strains (LEC240, LEC282, and LEC283) were grown at 37°C in 7H9 medium with ADC enrichment for fluorescent reporter assays and growth curve assays. The *mCherry* reporter strains of bacteria were generously donated from Dr. Cox at UCSF. Each strain of bacteria contained a plasmid with a kanamycin resistance gene. A final concentration of 20 μg/mL kanamycin was used to select for the plasmid-containing bacteria. Middlebrook 7H9 broth was made following the instruction provided by Dr. Cox. After sterilization, ADC enrichment was added at 10% the total volume. Overnight cultures were set up with 0.05% Tween-80 and 20 μg/mL kanamycin. The remaining medium was kept at 4°C and covered in foil in order to avoid light exposure. Overnight cultures were grown for 48 hours just like the wile-type *M. smegmatis* strain MC2 155.

Small Molecule Inhibitors

From the previous luciferase reporter assays, small molecule inhibitors with *trans*-translation inhibition activity were purchased from Life Chemicals, Inc.

Compounds were diluted in 100% DMSO to the final concentration of 10 mM. The inhibitors were stored at -80°C.

MIC Assays

Minimum inhibitory concentration (MIC) assays were performed using the CLSI guidelines for determining antimicrobial activity of the compounds. Overnight cultures of *M. smegmatis* were grown in lysogeny broth. Cultures were diluted to a final concentration of OD_{600} = 0.001 in 96-well microtiter plates and inhibitors were added initially at 100 µM and serially diluted 2-fold. Untreated, DMSO, and tetracycline controls were also run on each trial. Distilled water was applied to the small area between the wells in order to prevent the cultures from drying out. Any speck or cloudiness in the well was considered positive for growth. After 48 hours of incubation in 37°C, the last well that showed no bacterial growth was determined as the MIC.

MBC Assays

For minimum bactericidal concentration (MBC) assays, 10 µL from wells containing the MIC, 2X MIC, and 4X MIC of each inhibitor was diluted 10-fold in fresh LB broth in new 96-well microtiter plates. Six 10-fold serial dilutions were made and 10 µL from each well was spotted on LB plates, and grown for 48 hours at 37°C. Similar to the MIC assay, untreated, DMSO, and tetracycline were used as controls. If the inhibitor resulted in 1000-fold reduction in cfu/mL from the

original inoculum, it is determined as bactericidal. Otherwise, the inhibitor is classified as bacteriostatic.

Growth Curves

Overnight cultures of *mCherry* reporter strains (LEC240, LEC282, and LEC283) were diluted to OD_{600} = 0.05 into 7H9 medium with ADC enrichment. The medium was pre-warmed for 10 minutes with kanamycin (20µg/mL), 20% Tween, and E5 inhibitor (2 µM only for LEC282 experimental group) for 10 minutes before inoculation. OD_{600} of the cultures was measured every 2-3 hours and the values were plotted versus time. The doubling time of each strain was calculated using best-fit curves.

Fluorescent Reporter Assay

The *mCherry* reporter strains LEC240, LEC282, and LEC283 were grown overnight, diluted to OD_{600} = 0.05, and grown for 24 hours in a shaking water bath at 37°C. Kanamycin (20µg/mL), 20% Tween, DMSO and E5 inhibitor (2 µM only for LEC282 experimental group) were added to 7H9 medium with ADC enrichment and pre-warmed for 10 minutes before the inoculation. Cells were observed on agarose pads every 2-3 hours using a Nikon Eclipse 90i microscope, 60X TIRF N. A. 1. 4 objective, with a Nikon CoolSNAP HQ CCD camera. The fluorescence values were determined via Hamamatsu Photonics program.

Fluorimeter Readings

During the fluorescent reporter assay, 1.0 mL of untreated LEC282 was spun down to harvest cells. The OD600 of LEC282 determined amount of other cultures. This was done to ensure that each culture had approximately the same number of cells. After discarding the supernatant, cell pellets were stored at -80°C for two weeks before analysis.

The pellets were thawed and resuspended in fresh 7H9 broth after two weeks. In order to release the fluorescent protein, the samples were lysed with chloroform. The fluorescence of each lysate was measured using a fluorimeter. For the analysis, 50 µL aliquots of samples were loaded into 396-well black bottom plates in duplicates. Samples were excited at 587 nm and the emission at 610 nm was measured.

In order to directly compare the amount of fluorescent protein over total protein, the total amount of protein was determined using a Bradford Assay. Standard curves were created using known concentrations of BSA. Bio-Rad Protein assay agent was mixed with the protein samples and put in a 96-well plate. Using the equation calculated from the BSA standard curve, sample absorption at 595 nm was converted to the amount of proteins.

Amplification and Isolation of *M. smegmatis tuf* gene

For in vitro binding assays, purified EF-Tu protein is needed. The first step of this purification was the amplification of the *M. smegmatis tuf* gene. Using the NCBI nucleotides database, the *tuf* gene sequence was obtained. Primers were

designed using the obtained sequence. The sequence data was entered into NEBcutter V2.0 website. From the 0 cut section, NdeI and HindIII were chosen for restriction enzymes. The PCR was done with Phusion Polymerase and 5X Phusion GC buffer in following conditions: 30 sec at 98°C, (10 sec at 98°C, 30 sec at 58°C, 1.5 minutes at 72°C) x 29, and 10 minutes at 72°C. Primer sequences were as follows:

Forward: 5’-ACACATATGGTGGCGAAGGCGAAGTTCGAGCGGA-3’

Reverse: 5’-AGAAAGCTTTCACTTGATGATCTTGGTGACGCGG-3’

RESULTS

A7, D3, D7, E2, and E5 compounds had bactericidal effect with low MIC

A total of 27 inhibitors were tested using both MIC and MBC assays. The range of inhibitory concentrations was from 0.78 to 100 μM (Table 1). As mentioned in the materials and methods section above, three types of controls were used for each set of analysis: untreated, DMSO, and tetracycline. The untreated cells were healthy at all times without any signs of growth inhibition. All inhibitors were dissolved in DMSO; therefore, DMSO was used as a control to exclude the possibility that DMSO is causing the inhibitory effect rather than the inhibitors themselves. From the data, it is obvious that DMSO had no effect on the growth of *M. smegmatis* and can be assumed that any observed inhibitory effects are in fact due to the inhibitory molecules. Tetracycline is a known inhibitor of *M. smegmatis* and was used to compare the efficacy of the candidate molecules. As expected, the MIC of tetracycline was very low (0.01 μM). Out of all tested inhibitors, 20 molecules showed some kind of inhibitory effects on the bacteria. Seven compounds showed no inhibitory effect on *M. smegmatis* (A6, B1, B3, B6, C4, F7, and G6). MIC values of all inhibitors are summarized into a table (Table 1). To help visualize the best inhibitor molecules, the data was made into a bar graph (Figure 3).

From the 20 molecules that showed inhibitory effects, A7, D3, D7, E2, and E5 showed bactericidal activity with very low MIC (<10 μM) and were determined

as the best inhibitors. These compounds were bactericidal at the MIC or slightly above their MICs. The MIC values of these best inhibitors were 1.7, 1.2, 3.4, 1.7, and 5.9 μM respectively. E1, E4, and G4 exhibited very low MIC; their MICs were 7.4, 6.3, and 6.3 μM respectively. However, E1 and G4 were bactericidal only at 4X MIC while E4 was bacteriostatic. B4, F1, and F2 also exhibited submillimolar MIC values; their MIC values were 17.8, 15.6, and 31.3 respectively. Similar to the five best inhibitors, these compounds were bactericidal at MIC or slightly above their MICs.

Structures of the best inhibitors were examined to see if there are any similarities in the functional groups. With an exception of E2, all inhibitors shared three aromatic ring structures with amide bond in the middle. However, in terms of the functional groups that are attached to the aromatic rings, there was no obvious similarity. Also, the aromatic structures were specific features to the best inhibitors. Most of the compounds from the Novartis chemical library contained similar forms of aromatic structures (Figure 4).

Fluorescent reporter assay revealed that E5 inhibits ClpXP proteolysis step

It was hypothesized that E5 inhibits the activity of proteolysis step of *trans*-translation. In order to test this hypoethsis, strains of *M. smegmatis* with *mCherry* reporters for proteolysis were used. The *mCherry* reporter strains contain plasmids that encode fluorescent mCherry proteins that are constitutively expressed. There are three types of the special strains: LEC240, LEC282, and LEC283. LEC240 is the wild-type *mCherry* that always expresses the fluorescent

proteins. LEC282 is tmRNA-tagged that appears dark under the microscope. When the mCherry protein is synthesized in LEC282, tmRNA-tag is recognized by ClpXP protease and the whole peptide is degraded; therefore, the cells appear dark. LEC283 is a tag-variant strain that has the tmRNA-tag; however, the last three amino acid sequences are modified from LAA to ASV. This modification stabilizes the peptide and the protease can no longer recognize and degrade it. Therefore, LEC283 also appears bright under the microscope (Figure 5).

If an inhibitor were to inhibit proteolysis, the cells would become bright despite the presence of tmRNA-tag. In order to measure the effect of E5 without killing the majority of cells, growth curves were plotted to determine the appropriate concentration of inhibitor (Figure 6). Initially, this experiment was conducted in lysogeny broth (LB) medium; however, the *mCherry* reporter strains exhibited abnormally excessive clumping and irregular growth more so than the wild type *M. smegmatis* strain. Due to these abnormalities, accurate absorbance values could not be measured. Upon switching the medium from LB to the Middlebrook 7H9 broth, excessive clumping disappeared. From this point afterward, all assays involving *mCherry* reporter strains were performed in Middlebrook 7H9 medium.

Previously, the MIC of E5 was measured to be 5.9 µM. Initially, 1 µM of E5 was added to the medium; however, the fluorescence values were not significantly different from the control group. Subsequently, the final concentration was doubled to 2 µM and the amount of fluorescence drastically

increased. Despite the changes in the amount of fluorescence, the doubling time of LEC282 + DMSO (4.58 hours) and LEC + E5 (4.8 hours) were not drastically different. The concentration of the inhibitor was set to 2 μM for the rest of the experiment (Table 2).

Addition of E5 to LEC282 caused an increase in fluorescence compared to the DMSO-treated LEC282 controls (Figure 7). At all time points, LEC240 and LEC283 controls were expressing maximum fluorescence of 255. Both untreated LEC282 and DMSO-treated LEC282 expressed little to no fluorescence compared to aforementioned two strains and E5-treated group. It was safe to assume that DMSO was not responsible for inhibition of ClpXP proteolysis and the autofluorescence was not hindering the outcomes of the experiment.

After 3 hours of E5 treatment, treated cells started to express fluorescence more vigorously than the untreated and DMSO-treated LEC282 cells. Around 8 hours after the inoculation, the inhibitor treated cells were fluorescing at an intensity of 228 (arbitrary) while the LEC240 and LEC283 controls had intensity of 255. After 24 hours of incubation, the amount of fluorescence declined for the E5 treated group. The experiment was performed multiple times and it revealed that the largest amount of fluorescence was observed after 7 to 8 hours of incubation. The fluorescence reporter assay provides promising evidence that the mCherry proteins are not degraded by ClpXP due to the effect of E5; therefore, it is likely that E5 is indeed inhibiting the proteolysis of the *trans*-translation pathway.

Fluorimeter reading was not conclusive

The fluorescent reporter assay using microscopy gave promising data about E5. In hopes of better quantifying the level of fluorescence, fluorimeter readings were taken for each sample. As mentioned in the materials and methods, 1 mL aliquots of the samples were taken at each time point and stored at -80°C. The cell pellets were resuspended with the Middlebrook 7H9 medium and lysed with chloroform. Unfortunately, fluorescence values were very inconsistent (data not shown) and did not match the results from the microscopy.

Experiments were done multiple times; however, the outcomes were the same. No consistent fluorescence values could be measured using fluorimeter. Quite often, the amount of fluorescence for all groups was about the same; however, it was obviously not true as seen in the microscopy data. After discussing the issue with laboratory members and Dr. Keiler, it was speculated that the lysis buffer might be causing the inconsistency. The fluorimeter approach was no longer pursued because better lysis buffer could not be found and no conclusion was made from these results.

PCR amplification of *M. smegmatis tuf* gene was successful using new primers. Vector ligation, transformation, and protein purification are in progress.

F2 itself was not one of the five best inhibitors from the MIC assays. However, when Dr. John Alumasa made modifications on this compound and created Dansyl-F2, it showed 4-fold increased effectiveness against bacterial

growth. In attempts to find a molecular target of the F2 compoud, Dr. Alumasa incubated biotinylated-F2 with *B. anthracis* cell lysates. After running the lysates through columns, he discovered that a protein called EF-Tu is the binding target of F2 inhibitor (J. Alumasa, unpublished results). As mentioned before, EF-Tu is one of the essential components of the *trans*-translation pathway. If such outcomes could be replicated in other species of bacteria, it could be concluded that EF-Tu is a universal binding target of the F2 inhibitor. To answer this question, *M. smegmatis* EF-Tu has to be made and purified. *M. smegmatis tuf* gene is responsible for the synthesis of EF-Tu protein in the bacterium. Therefore, amplification and isolation of this gene was set as a priority.

The genomic DNA of *M. smegmatis* was isolated by following Gentra System, Inc. Gram-Negative Bacteria Protocol. Initially, attempts were made to amplify the *tuf* gene using forward and reverse primers with following sequences:

Forward (NheI): ACAGCTAGCGTGGCGAAGGCGAAGTTCGAGCGGA

Reverse (HindIII): AGAAAGCTTTCACTTGATGATCTTGGTGACGCGG

Using these primers, *tuf* gene seemed to be amplified and the subsequent cloning into pGEM-T Easy vector was successful. However, when diagnostic double-digest was performed to confirm the presence of the insert, release of the gene was not observed. After many failed attempts, sequencing data revealed that the *tuf* gene was amplified; however, the NheI restriction site of the forward primer sequence disappeared for unknown reason. Fortunately, a HindIII restriction site was still intact. Three more colonies with similar phenomenon

were cultured and the plasmids were sent for sequencing. All three data showed that the NheI restriction sites were missing (data not shown).

A new primer was designed with an NdeI restriction site. The sequences of the primer are in the materials and methods section. One of the problems with the old primer was that amplification of the gene from the genomic DNA was very difficult. Yield was often very low and subsequent amplification was required. However, with the new primer, direct amplification from the genomic DNA resulted in efficient amplification and yield was high enough that gel purification was successfully performed on the amplified PCR product.

DISCUSSION

The results shown above definitely provide sufficiently evidence that targeting *trans*-translation for novel antibiotic discovery is a promising approach. Out of 27 inhibitors tested, 20 of them showed some form of inhibitory effects. Surprisingly, all 20 inhibitors had MICs of 100 µM or less. The five best inhibitors A7, D3, D7, E2, and E5 exhibited MIC below 10 µM. In comparison to the *trans*-translation inhibitor of TB currently in the market, pyrazinamide (MIC > 0.8 mM) (19), the candidate molecules tested are much more effective at killing the bacteria. The fact that these small molecules showed great antimicrobial activities suggest that targeting this ribosome-rescuing mechanism is encouraging and should be investigated in further detail. Targets of individual candidate molecules must be specified and the inhibitors must be tailored so that specificity and efficacy improve.

From the fluorescent reporter assay, E5 inhibitor showed very promising data for novel antibiotic discovery. MIC and MBC results show that it is bactericidal on *M. smegmatis* with low MIC values. Not only that, the fluorescent reporter assay suggested that the target of E5 is the proteolysis step of the *trans*-translation. Ben Chambers conducted the same study with A7 and D7; however, these particular inhibitors did not inhibit ClpXP proteolysis step of *trans*-translation (Ben Chambers, unpublished results). TB Alliance also tested E5 for inhibition of TB growth and toxicity. In normal culture setting, the MIC of E5 was

25.0 µg/mL and 48.2 µg/mL in low oxygen settings. For pyrazinamide (first-line antibiotic for TB), the MIC was >128 ug/ml.

While the outcomes were promising, there was a limitation to the fluorescent reporter assays. The fluorescence count was done via a microscopy program. Cells were captured and individual cell's fluorescence counts were measured and averaged. The problem is that it is impossible to scan and measure the entire sample. Also, after 8 hours of incubation, cells started to clump up. When cells were clumped up in a bundle, the amount of fluorescence seemed to increase as a whole. Therefore, it was difficult to measure fluorescence of individual cells (Figure 8). In order to overcome the limitations of the microscopy, fluorimeter readings were used. However, due to problems with lysis buffer and inconsistent data presentation, this kind of approach to identify inhibitor target was no longer used. This suggests that different kinds of approach may be better for identifying the target of E5. One of the suggestions was the use of pulse-chase analysis to evaluate the effect of E5 on protein ability. However, this study could not completed due to problems with contaminated reagents.

In light of the recent discovery that Dr. John Alumasa has made, the focus of the research has shifted to F2 compound and EF-Tu. He found that F2 binds to EF-Tu in *B. anthracis*. Therefore, the purpose of this research is to find out if F2 has the same molecular target in *M. smegmatis*. If that is the case, we can correlate the finding to tuberculosis because *M. smegmatis* and *M. tuberculosis* share genetic similarities. While the new primer seems to amplify the desired

gene very well from the genomic DNA, the cloning of the insert into pGEM-T Easy vector caused problems for a while. Originally, the insert was expected to be sub-cloned into pGEM-T Easy vector and subsequently cloned into pET28b overexpression vector. This way, EF-Tu protein could be made in mass amount after transforming the gene into *E. coli BL21*. Therefore, both cloning into pGEM-T Easy vector and direct digestion of the PCR product are being used to make EF-Tu protein.

Antibiotic resistance is accumulating at an alarming rate. Bacteria are adapting to the antibiotic challenges while we are running out of options to clear the infection. However, the data suggests that inhibition of the *trans*-translation brings hopes to antibiotic discovery. Research to identify the molecular targets of the best inhibitors must be continued and understood in more detail. Only then, modifications can be made to improve the effectiveness of the inhibitors. Understanding the modes of actions of these molecules and the *trans*-translation pathway would be a step closer to finding novel antibiotics to kill *M. tuberculosis.*

FIGURES

Figure 1. The *trans*-translation pathway. Three main steps of the *trans*-translation pathway are illustrated. Possible inhibitors of each step are evaluated in this particular set of experiments. Source: Keiler, KC. “Biology of *trans*-translation.” *Annual Reviews of Microbiology.* 2008. 62:133-51 (9).

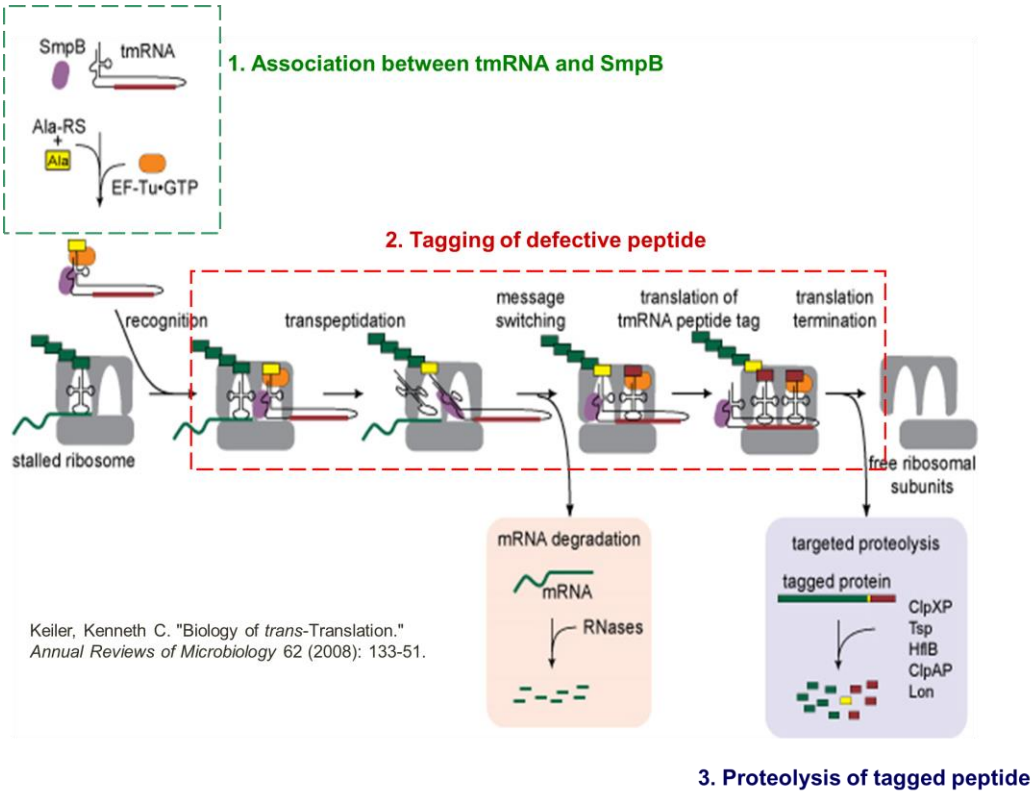


Figure 2. Screening of *trans*-translation inhibitors in *E. coli* luciferase reporter system. The luciferase reporter system with trpA terminator forces the ribosome to be stalled and ensures the use of the *trans*-translation. The tagging by tmRNA is followed by the degradation of tagged peptide by ClpXP, eliminating luminescence. If either tagging or the proteolysis step of the pathway is inhibited, then the luciferase peptide is no longer subjected to degradation and the cells appear bright. Source: Ben Chambers and Hoon Chang. *Inhibition of the trans-Translation Pathway in Mycobacterium smegmatis.* 2011. The Pennsylvania State Univerity, University Park.

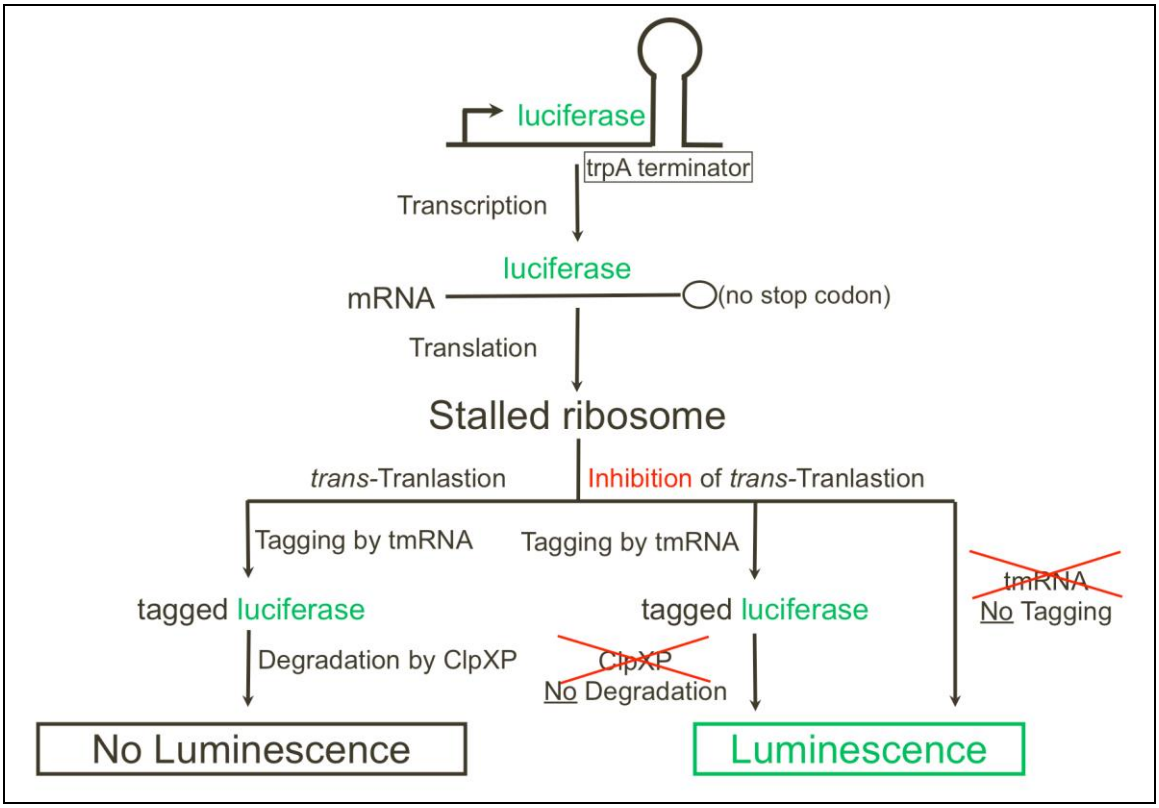


Figure 3. Average MIC values of inhibitors. MIC values of all inhibitors are plotted below. Black bars represent ineffective molecules. There was no inhibitory effect up to 100 μM for the seven molecules (A6, B1, B3, C4, F7, and G6). Small red bars indicate the best five inhibitors (A7, D3, D7, E2, and E5), with bactericidal effects at MIC or slightly above their MICs.

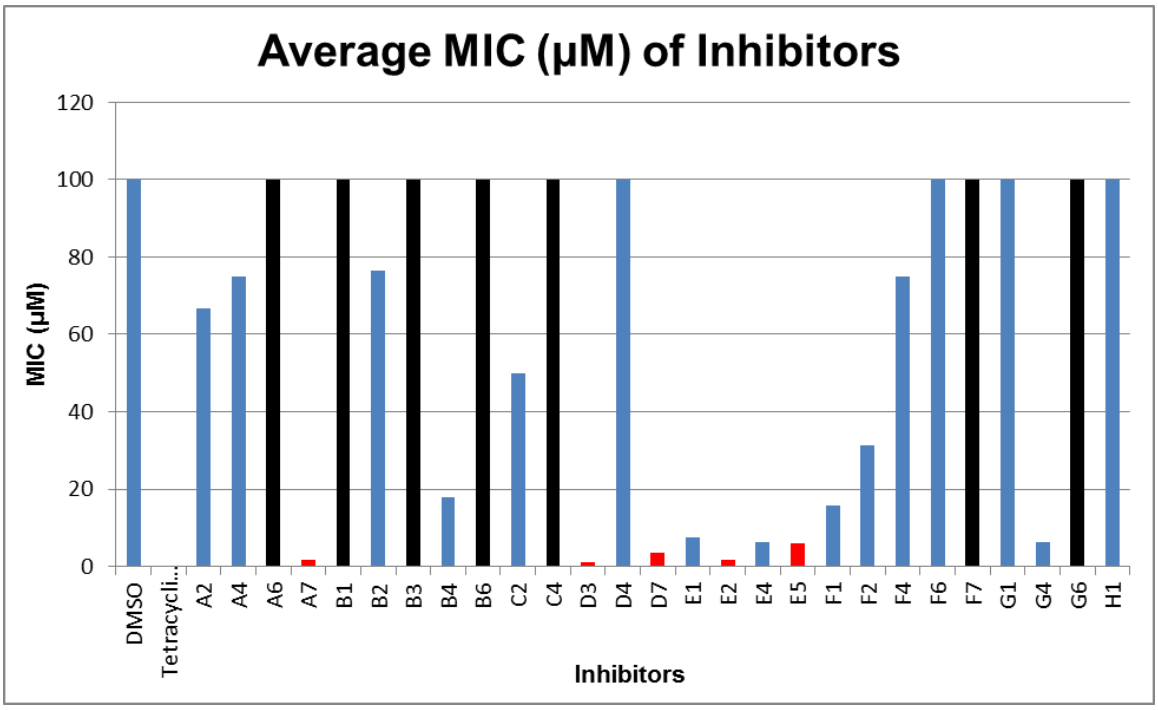


Figure 4. Structures of the best inhibitors. Similar aromatic structures are present with an exception of E2 molecule. However, there are no definite patterns to the functional groups that are attached to the aromatic rings.

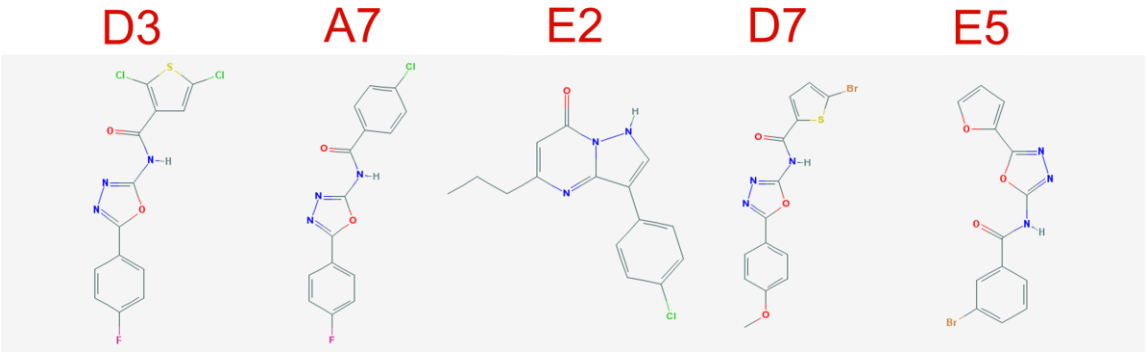


Figure 5. Fluorescent reporter assay design. LEC282 is a special strain of *M. smegmatis* called *mCherry* reporter strain, which contains a plasmid DNA that encodes mCherry fluorescent protein. In this particular case, the tmRNA tag is built into the plasmid. Under normal circumstances, the tmRNA tag targets the protein for degradation by protease ClpXP, giving no fluorescence. However, if an inhibitor hinders the ClpXP proteolysis, the protein avoids degradation and produces fluorescence. Source: Ben Chambers and Hoon Chang. *Determining the Targets of trans-Translation Inhibitors in Mycobacterium smegmatis.* 2012. The Pennsylvania State University, University Park.

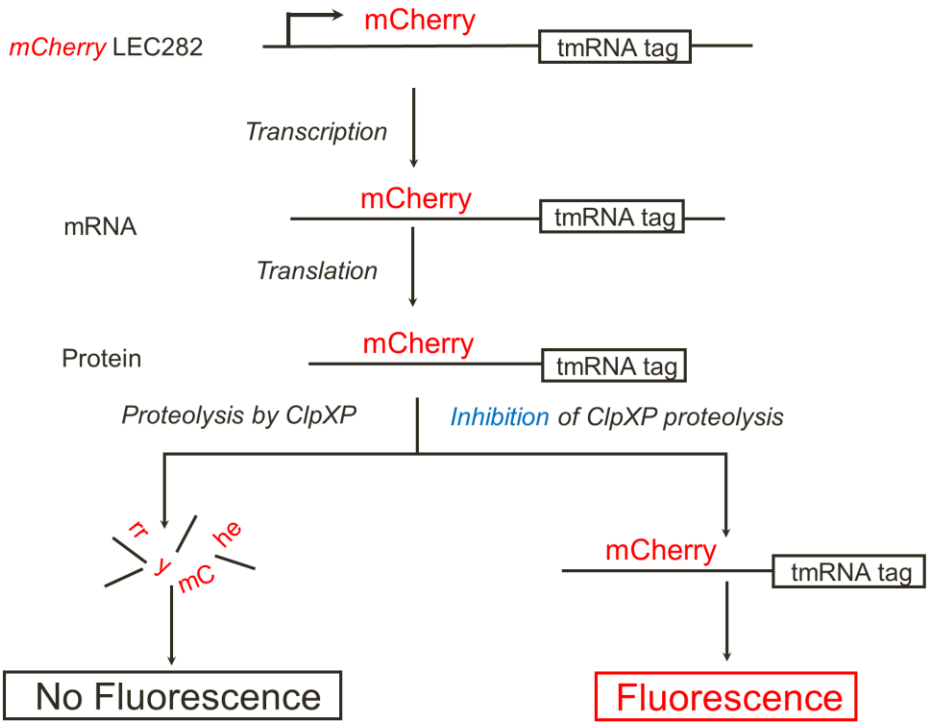


Figure 6. Growth curves of *mCherry* reporter strains. Overnight cultures of *mCherry* reporter strains were back diluted to OD_{600} = 0.05. At 0, 3, 6, 8, 10, 12, and 24 hour time points, 1 mL of each sample was collected and the absorbance values were measured. The OD_{600} absorbance values were plotted into a growth curve shown below.

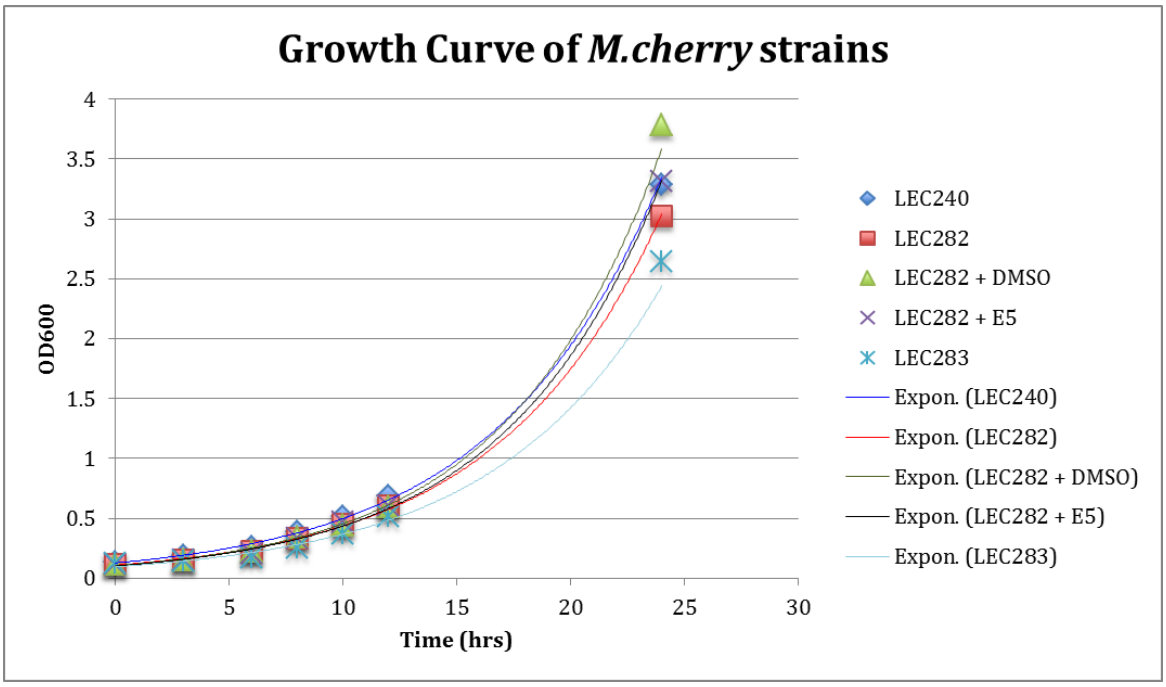


Figure 7. Inhibition of proteolysis in vivo. The amount of fluorescence measured by the microscopy is plotted below. Through all time points, both LEC240 and LEC283 are expressing maximum fluorescence of 255. Untreated and DMSO-treated LEC282 show little to no fluorescence. After 3 hours of incubation, E5-treated LEC282 shows significantly more fluorescence. After 8 hours, the cells are most fluorescent.

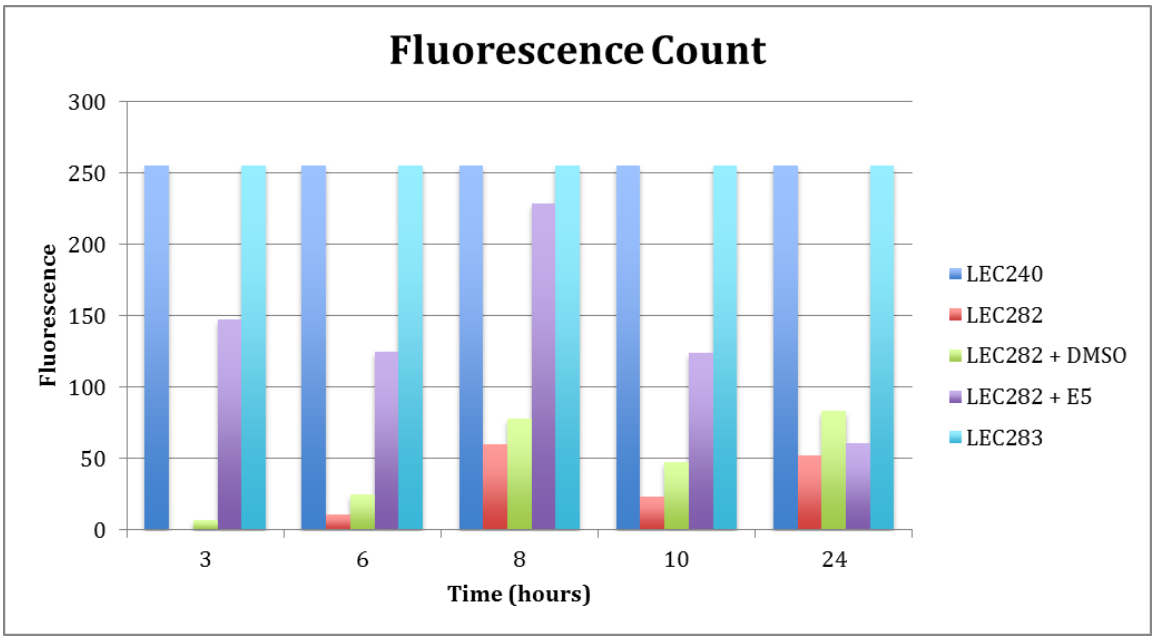
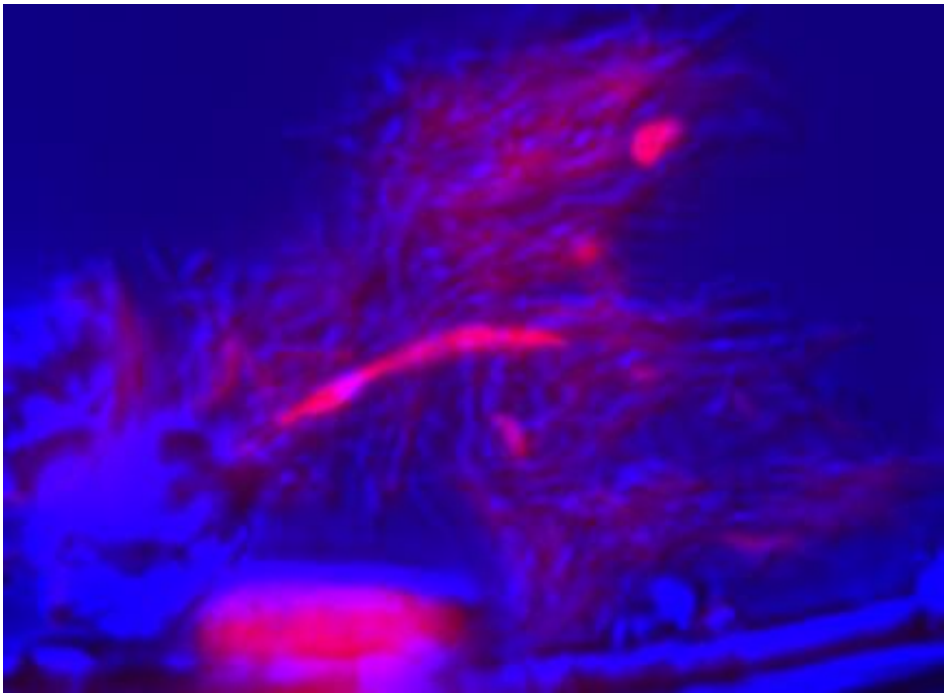


Figure 8. Micrographs of E5-treated cells after 8 hours of incubation. E5-treated group of LEC282 seem to clump up after 8 hours of incubation. It is difficult to measure the fluorescence level of individual cells.



TABLES

Table 1. MIC and MBC results summary. Inhibitors with bactericidal effect with low MIC values are considered more effective candidate drug. The best five inhibitors are highlighted in yellow. IE means that the inhibitor was ineffective and thus had no inhibition on bacterial growth. For inhibitors that received IE, MBC was not performed; therefore, MBC determination was not applicable or N/A. Static inhibitors were able to inhibit growth in MIC assays; however, when the inhibitors were removed or bacteria were inoculated in fresh medium, the bacteria started growing back again.

Inhibitor	MIC (μM)	Cidal or Static	Inhibitor	MIC (μM)	Cidal or Static
DMSO	IE	N/A	D7	3.4	cidal
Tetracycline	0.01	0.01	E1	7.4	cidal at 4x MIC
A2	66.7	static	E2	1.7	cidal
A4	75	cidal	E4	6.3	static
A6	IE	N/A	E5	5.9	cidal
A7	1.7	cidal	F1	15.6	cidal
B1	IE	N/A	F2	31.3	cidal
B2	76.6	76.6	F4	75	cidal
B3	IE	N/A	F6	100	cidal
B4	17.8	cidal	F7	IE	N/A
B6	IE	N/A	G1	100	cidal
C2	50	cidal	G4	6.3	cidal at 4x MIC
C4	IE	N/A	G6	IE	N/A
D3	1.2	cidal	H1	100	cidal
D4	100	static			

Table 2. Doubling time of *mCherry* reporter strains. Based on the growth curves shown in Figure 6, doubling times of *mCherry* reporter strains including those of the control and E5-treated group were calculated. The ranges of doubling time were from 4.5 to 5.2 hours. Compared to the wild-type *M. smegmatis*, the *mCherry* reporter strains had similar doubling time.

Strains	Doubling Time (hours)
LEC240	5.15
LEC282	5.03
LEC282 + DMSO	4.58
LEC282 + E5	4.8
LEC283	4.95
WT *M. smegmatis*	4 to 5

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