SMALL MOLECULE INHIBITORS OF THE TRAN-TRANSLATION PATHWAY HAVE ANTIBIOTIC ACTIVITY AGAINST MYCOBACTERIUM SMEGMATIS

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ABSTRACT

Antibiotic resistance has been spreading at an alarming rate over the past few decades. Tuberculosis (TB), the most prevalent infectious disease in the world, is becoming even tougher to treat now that many of the drugs used to fight it are becoming more and more ineffective. It is necessary to begin developing new antimicrobial compounds that target novel pathways in bacteria so that TB can be better treated in the future. One such novel pathway is the trans-translation pathway which is responsible for rescuing stalled ribosomes. It is required for growth and virulence in many pathogenic bacteria but is not found in eukaryotic cells. This provides selective toxicity and a favorable approach to fighting pathogens. In an attempt to find new drugs to treat TB, this study used *Mycobacterium smegmatis* as a model for *Mycobacterium tuberculosis*, the bacterium that causes TB, because *M. smegmatis* is non-pathogenic and grows quickly making it much easier to work with. Small molecules previously shown to inhibit the trans-translation pathway in *E. coli* were tested in *M. smegmatis* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays to determine how effective each one is at preventing growth of the bacteria. After the best inhibitors (A7, D3, D7, E2, and E5) were identified, UV mutagenesis and fluorescence assays were used to search for the target and mechanism of action for each inhibitor. The fluorescence assays using mCherry-expressing strains of *M. smegmatis* suggested that inhibitor E5 is likely inhibiting the proteolysis step of the trans-translation pathway while inhibitors A7 and D7 are inhibiting a step before proteolysis. Future work will concentrate on improving the fluorescence assays and running binding assays with possible target proteins to continue the search for the specific targets of each of the best inhibitors.
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Tuberculosis (TB) is one of the world's most dangerous and common infectious diseases. One-third of the world's population tests positive for exposure to *Mycobacterium tuberculosis* (the bacterium that causes TB) and there are over 8 million new cases of active TB each year. If left untreated, the mortality rate of TB is close to 50% (25). The recent developments of multi-drug-resistant TB and extensively drug-resistant TB are further adding to the danger that this disease poses. Currently, patients who are diagnosed with active TB disease within the US are administered the common drug regimen that has been used for decades. Consisting of four antibiotics (ethambutol, isoniazid, pyrazinamide, and rifampicin), this treatment program is effective but takes at least six months to completely cure the patient. Six months of treatment is a very long time for patients to stick to the daily plan of taking their antibiotics and receiving regular medical checkups (10).

Making this treatment program even tougher are the intense side effects that most patients experience. The side effects associated with anti-TB drugs can include intense nausea, vomiting, loss of appetite, and dark urine. The occurrence of these side effects often prompts patients to cut back on their drug consumption or stop taking the drugs completely (17). Another discouraging factor that many patients face is the sheer amount of pills they must take. The treatment of even the most curable form of active TB disease requires taking four pills every night, one of which is very large in size and therefore tough to swallow. When patients stop taking their TB treatment, symptoms can
return and they can become actively infectious again. Stopping the intake of even a single drug can allow the infecting bacteria to become resistant and for the treatment to fail. When the bacteria become resistant to one or more of the anti-TB drugs, treatment becomes much tougher and requires several new second-line antibiotics (4).

The irresponsible self-treatment exhibited by TB patients over the past few decades has resulted in the recent increase in the incidence of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) TB cases. In the 2011 WHO Global Tuberculosis Control report, it was estimated that there are currently over 650,000 prevalent cases of MDR-TB worldwide (25). MDR-TB is defined as TB that is resistant to rifampicin and isoniazid, the key first-line anti-TB drugs. XDR-TB is resistant to rifampicin, isoniazid, quinolones, and at least one of the second-line injectable drugs. Without treatment, the mortality rates of MDR-TB and XDR-TB are over 80%. Even with treatment, the mortality rates still approach 50% (4). These incredibly lethal strains of *M. tuberculosis* can spread to other people just as easily as the normal strain. The inhalation of fewer than ten aerosolized bacteria is capable of causing infection (25).

In order to prevent a terrible epidemic in the coming decades, new treatments for TB need to be found. The current drug regimen that has been used for decades is losing its effectiveness. Resistance to these drugs is increasing at an alarming rate. Finding newer and better treatments is key in defeating TB in the future. New drugs that are easier to administer and cure patients in less time would cut down on the number of
patients that give up on treatment and help contribute to the development of drug-resistant strains of TB.

One possible approach is to develop new drugs that target novel pathways in bacteria. This way, cross resistance between existing drugs and new drugs will not be a problem. One novel target that is being explored is the *trans-*translation pathway. It is responsible for rescuing ribosomes that stall during translation (13). When an mRNA transcript is damaged or truncated and missing a stop codon, translation stalls when the ribosome reaches the end of the transcript. Without a stop codon, the proper release factors cannot be recruited to finish translation and release the ribosome. This means that the ribosome is stuck to the damaged transcript and unable to move on and begin the translation of other transcripts in the cell. As more ribosomes become stalled over time, the cell loses its ability to effectively produce enough proteins to keep the cell healthy. Recovery from stress is severely limited when the pathway is inhibited (13,14). Therefore, having the *trans-*translation pathway is very important to bacteria.

An explanation of how the *trans-*translation pathway works is shown below in Figure 1. The main effector molecule involved in the pathway is called tmRNA. Its name comes from the fact that it possesses the characteristics of both tRNA and mRNA. The pathway can be divided into three main steps: binding of the key components, tagging of the defective peptide, and proteolysis of the defective peptide (13). In the first step, several molecules must associate into a complex which is able to bind to the stalled ribosome. The key association occurs between tmRNA and SmpB. SmpB is a small
protein that binds to the tRNA-like domain of tmRNA so that the complex can bind to the ribosome (1,8,9,12,22,26). The tmRNA molecule is then charged with an alanine residue and becomes associated with GTP-bound EF-Tu which allows the tmRNA to bind the ribosome (2,20). Using its tRNA-like domain, the tmRNA enters the A site of the stalled ribosome (15,24). The second main step of the pathway then occurs and works by the regular mechanism of translation. After the tmRNA binds to the ribosome and the peptide strand is transferred to the tmRNA alanyl residue, the truncated mRNA is released and degraded (19,27). The mRNA-like domain of tmRNA provides a sequence that can be read by the ribosome and used to complete translation of the peptide (13). This mRNA-like domain of tmRNA encodes a specific peptide tag that is attached to the end of the defective peptide (15). The function of the 8-17 amino acid long tag (varies among species) is to target the defective peptide for proteolysis (6,7,15). In step three of this pathway, the defective peptide is transported to the protease where it binds and is then degraded (13). The most commonly used protease in this pathway is ClpXP (5).

Proteolysis of the defective peptides is an important step in the pathway because without it, the cell would fill up with defective peptides that could get in the way and cause negative side effects such as inhibiting growth or other intracellular processes.

The main benefit of targeting the trans-translation pathway is that it is found in all bacteria but not in eukaryotes (13). This means that any drug developed to target this pathway would have good selective toxicity already built in. The risk of negative reactions with the human body would be very low. In bacteria however, this pathway is very important to proper growth and development. For example, it has been shown that
tmRNA is absolutely required for growth and virulence in *Neisseria gonorrhoeae* and *Shigella flexneri* (13). Also, the pathogenesis of *Yersinia pseudotuberculosis* and *Salmonella enterica* requires the presence and activity of the trans-translation pathway (11,16). When the pathway is not working correctly, it is common to see growth defects in bacteria when they experience a rapid change in their gene expression profiles (14).

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**Figure 1. The trans-translation pathway.** The pathway used to rescue stalled ribosomes in bacteria is composed of three main steps: association of the key components, tagging of the defective peptide, and proteolysis of the tagged peptide by proteases. Source: Keiler, KC. “Biology of trans-Translation.” Annual Reviews of Microbiology. 2008. 62:133-51 (13).
In trying to develop new drugs to treat TB, it is very difficult to work directly with *Mycobacterium tuberculosis*. It is very dangerous and requires special BSL-3 facilities to work with in the lab. Another drawback is the two weeks that are required to grow up a culture of the bacteria that can be used in in vivo assays. Instead of working with the risky and time-consuming *M. tuberculosis*, it is much more practical to begin testing in a model system. One of the more common model systems for *M. tuberculosis* is *Mycobacterium smegmatis*, a non-pathogenic species of the genus Mycobacteria (23). *M. smegmatis* has a very similar shape to *M. tuberculosis*. Both grow as long bacilli that are capable of filamentous growth. Clumping is very common in *M. smegmatis* cultures and can cause some minor problems in assays. The main benefit of *M. smegmatis* is that it is very fast-growing compared to *M. tuberculosis*. Instead of two full weeks to grow up a usable culture, it takes only 48 hours. Because *M. smegmatis* is safe, fast-growing, and closely related to *M. tuberculosis*, it serves as an acceptable model in the initial testing of new drug candidates for the treatment of TB (23).

Work to develop the *trans*-translation pathway as a novel target for antimicrobial drugs has been under way since 2009. Previous screens were done in *E. coli* to determine small molecule compounds that possibly inhibit the pathway. A luciferase reporter system that incorporated a transcript missing its stop codon to guarantee use of the *trans*-translation pathway was set up in *E. coli* (see Figure 2 below). This system was used for a high-throughput screen of the small molecule library at Novartis. Out of the 650,000 small molecules in the library, a total of 51 showed possible inhibition of the *trans*-translation pathway. Because this screen was done in *E. coli*, the first step for this
study was to test each of the small molecules in *M. smegmatis* for inhibitory action. MIC and MBC assays using a wide range of concentrations were utilized to evaluate the effectiveness of each small molecule at inhibiting the pathway in *M. smegmatis*.

![Luciferase reporter system](image)

**Figure 2. The luciferase reporter system used for initial screening in *E. coli*.** The luciferase gene included a stem-loop structure for termination of transcription that resulted in a transcript without a stop codon. This guaranteed use of the trans-translation pathway. If the pathway worked correctly, the luciferase peptide was tagged and degraded giving dark cells. However, if the pathway was inhibited at either the tagging or proteolysis steps, the luciferase peptide remained intact and the cells were bright.

Once the best inhibitors in *M. smegmatis* had been identified, the goal was to determine the target and mode of action for each one. Multiple approaches were utilized to find these answers. First, UV mutagenesis was used in an attempt to generate strains of *M. smegmatis* that were resistant to the promising inhibitor D7. After resistant mutants were
isolated and verified, the \textit{smpB} and \textit{ssrA} genes were sequenced and compared to the wild-type strain to look for the mutations that resulted in resistance. The second approach was a fluorescence assay that incorporated strains of \textit{M. smegmatis} that were carrying the gene for the fluorescent mCherry protein on a plasmid. This assay concentrated specifically on whether or not the proteolysis step was being blocked by the inhibitors. Both of these methods were used to gain a general understanding of the mechanism utilized by each of the main inhibitors so that further down the road, more specific binding assays could be carried out. The reason for trying to determine the targets and mechanisms of action for the drugs is so that alterations can be made to make the drugs more specific and more effective at lower concentrations.
MATERIALS AND METHODS

Strains and Growth Conditions

All MIC and MBC testing was done using *M. smegmatis* strain MC2 155. The wild-type *M. smegmatis* was grown in tubes of lysogeny broth (LB) at 37°C. To each *M. smegmatis* culture in LB, Tween-80 was added at 0.1% the total volume to avoid clumping of the bacteria during growth. Overnight cultures were grown for 48 h in a 37°C roller drum.

The mCherry-plasmid strains of *M. smegmatis* were provided as a gift from Dr. Jeffrey Cox at the University of California, San Francisco. The plasmid vector used to carry the mCherry gene also had a gene for kanamycin resistance. Therefore, bacteria containing the plasmid were marked by resistance to kanamycin. All the overnights for the mCherry-plasmid carrying strains were grown in tubes of 7H9 broth in a 37°C roller drum. During the fluorescence assays, they were grown in flasks of 7H9 in a 37°C shaking water bath. Middlebrook 7H9 broth is used specifically for the growth of Mycobacteria species. The 7H9 broth was made following the protocol provided by Dr. Jeffrey Cox along with the mCherry strains. After sterilization of the 7H9 broth medium, albumin-dextrose-catalase (ADC) enrichment was added at 10% the total volume. Upon setting up cultures of the mCherry strains, 0.05% Tween-80 was added to the 7H9 broth. Kanamycin was also added at a final concentration of 20 μg/mL to select for the plasmid-carrying bacteria. The 7H9 broth was kept at 4°C and covered in foil before use to keep it fresh. Overnights were grown for 48 h just like the wild-type strain.
Small Molecule Inhibitors

The small molecule inhibitors were identified in collaboration with Novartis using their small molecule library. They were selected based upon their ability to inhibit the trans-translation pathway in \textit{E. coli}. The small molecules that showed activity were then purchased from Life Chemicals, Inc. Each of the compounds was dissolved in dimethyl sulfoxide (DMSO). They were stored in 10 mM stocks at -20°C.

MIC Assay

Minimum inhibitory concentration (MIC) assays were used to determine the effectiveness of each of the small molecules in inhibiting the growth of \textit{M. smegmatis}. The assays were carried out in 96-well round bottom plates that were UV-sterilized for 30 min before use. Overnight cultures of the \textit{M. smegmatis} were back-diluted to an \( \text{OD}_{600} \) of 0.001 directly before exposure to the inhibitor. Aliquots of 50 \( \mu \text{L} \) of the back-diluted culture were then distributed to each well. Starting with the top row, the small molecules were applied using serial two-fold dilutions starting at 100 \( \mu \text{M} \) in the first well and ending at 0.78 \( \mu \text{M} \) in the eighth and final row. Untreated, DMSO, and tetracycline controls were also run on each plate. Distilled water was added to the small spaces in between wells on the plate to provide a humid environment for the bacteria during incubation. After mixing each well, the plate was covered and incubated 48 h at 37°C. The wells were observed by eye for any sign of growth after two days. The MIC was recorded as the lowest concentration where no bacterial growth was visible.
MBC Assay

Following the MIC assays, minimum bactericidal concentration (MBC) assays were also run to determine if the small molecules were bactericidal or bacteriostatic at their respective MICs. The MBC assays were carried out by titrating the bacteria on LB agar plates to determine the number of colony forming units (CFU) per mL. Bacteria from the MIC well and the two wells above that (higher concentrations) were further diluted in fresh LB broth in a new UV-sterilized 96-well round bottom plate. Six serial ten-fold dilutions were carried out and then 10 μL of each of the six dilutions ($10^{-1}$ to $10^{-6}$) were plated as separate spots on the LB plates. The MBC assay was performed on the untreated, DMSO, and tetracycline controls for comparison to the small molecule inhibitors each time. The plates were incubated at 37°C for 48 h. After incubation, the number of colonies were counted and the dilution factors were used to calculate CFU/mL. Any concentration of small molecule inhibitor that reduced the number of CFU/mL by 3.0 log units compared to the untreated control was classified as bactericidal. Any concentration that reduced the growth by less than 3.0 log units was classified as bacteriostatic.

UV Mutagenesis

Before treating the *M. smegmatis* with UV radiation to induce mutations, a UV kill curve was constructed by treating a culture of *M. smegmatis* at maximum energy in a UVP CL-1000 Ultraviolet Crosslinker for 20 sec at a time and growing up the culture on LB plates to determine the CFU/mL at each time point. From this standard curve, the time that resulted in the death of 99.9% of the bacteria was chosen as the time that the
cultures would be treated with UV radiation. The optimum UV treatment time was found to be 2-4 min.

Overnight cultures were back-diluted to an OD$_{600}$ of 0.001 and allowed to grow in a 37°C roller drum for 4 h. After incubation, the cultures were poured into a large plastic petri dishes that had been UV-sterilized for 30 min. The petri dishes were then placed in the UV cross-linker without their lids and treated with UV radiation for 2-4 min. The UV-treated cultures were then used to carry out MIC assays using one of the most effective small molecules inhibitors, inhibitor D7. Repeated two-fold dilutions were used again starting at 100 μM in the top well of each column. Bacteria that were not UV-treated were used as a control in these MIC assays. Another control used was UV-treated bacteria that were not exposed to the inhibitor. After incubating at 37°C for 48 h, any growth observed in wells that had a concentration above D7’s average MIC of 3.4 μM was considered to be a possible D7-resistant mutant. The bacteria from these wells were used to inoculate overnight LB cultures and run repeat MIC assays to confirm that the mutated strain was indeed resistant to D7.

**Sequencing the smpB and ssrA Genes of the D7-Resistant Mutant**

The smpB and ssrA genes of D7-resistant mutant 4A were sequenced and compared to the wild-type *M. smegmatis* genome. The genomic DNA of mutant 4A was isolated using the Small-Scale Lysis Technique (3). A PCR using Deep Vent DNA polymerase was then used to amplify the smpB and ssrA genes. The PCR products were separated on a gel for analysis and then purified using the QIAquick Gel Extraction Kit produced
by Qiagen. The PCR products were A-tailed using Taq DNA polymerase and then ligated into the pGEM-T Easy Vector using the pGEM-T Easy Vector System I by Promega. The ligation was allowed to run overnight at 4°C. The ligation product was then transformed into chemically competent DH5α E. coli cells and spread onto LB-ampicillin plates containing 0.1 mg/mL ampicillin. Before inoculating the plates with the transformed cells, they were covered with 25 μL of 1 M IPTG and 40 μL of 30 mg/mL X-Galactose in DMF and incubated at 37°C for 30 min to allow the DMF to evaporate. The plates were then incubated overnight at 37°C. White colonies were then picked and re-streaked on fresh LB-ampicillin plates to confirm they were white and thus possessed an insert within the vector. Isolated colonies were then grown up in overnight cultures. The plasmid was isolated from the bacteria using the QIAprep Spin Miniprep Kit produced by Qiagen. An analytical digestion was then performed using the restriction enzymes Fast-Digest SpeI and Fast-Digest NcoI. After a gel was run to show that the insert was the correct length, the plasmid was sent for sequencing at the Huck Institutes of the Life Sciences at the Pennsylvania State University.

**Fluorescence Assays**

Strains of *M. smegmatis* containing plasmids carrying genes for the red fluorescent protein mCherry were used for these assays. Overnight cultures were grown in 7H9 media containing 0.05% Tween-80 and 20 μg/mL kanamycin. The overnights were back-diluted to an OD$_{600}$ of 0.005 in 15 mL of 7H9, 37.5 μL of 20% Tween-80, and 30 μL of 10 mg/mL kanamycin in an autoclaved 125 mL glass flask. The tmRNA-tagged strain (LEC282) was then inoculated with the small molecule inhibitors at sub-MIC
levels. For inhibitors A7 and D7, 1.0 μM was used; for inhibitor E5, 2.0 μM was used.

Each time the assay was performed, multiple controls were run along with the inhibitor-treated culture. The wild-type mCherry strain (LEC240) and the tmRNA variant tag strain (LEC283) were used as positive fluorescence controls. An untreated sample of the tmRNA-tagged strain (LEC282) and a DMSO-treated sample of the tmRNA-tagged strain were used as negative fluorescence controls.

The flasks were then incubated in a 37°C shaking water bath for 24 h. Samples were taken every couple hours up to the 10 or 12 h time point. After that, the last samples were collected at around 24 h. For most runs, the time points used were 3, 5, 7, 10, and 24 h. At each time point, the OD_{600} was recorded and 5 μL of the culture was placed on a glass slide with a cover slip for observation under the fluorescent microscope. Representative pictures were taken at each time point for each sample. The red component of the fluorescence was then recorded from the image and used to compare the levels of fluorescence between the controls and the inhibitor-treated culture.

**Fluorimeter Readings**

This was done as an extension to the fluorescence assays in a preliminary attempt to better quantify the results and compare the levels of fluorescence. During the fluorescence assays at each time point, 1.0 mL of the untreated tmRNA-tagged control culture was spun down to harvest the cells. Depending on the OD_{600} readings, different amounts of each culture were also spun down. The OD_{600} reading of the untreated tmRNA-tagged control culture was used to standardize the other volumes so that
approximately the same number of cells were harvested for each sample. The supernatant was removed and the cell pellets were kept frozen at -80°C for a 2-3 weeks before they could be analyzed.

After two weeks, the cell pellets were removed from the freezer and resuspended in fresh 7H9 broth. The cells were then lysed using chloroform to release the fluorescent protein. Aliquots of each sample were then analyzed on a fluorimeter to determine the amount of fluorescent protein in solution. Two different types of fluorimeters were used in these readings. The first fluorimeter read all the samples together on a 396-well black bottom plate. For this fluorimeter, 50 μL aliquots of the samples were loaded into each well. For the second type of fluorimeter, the samples were loaded one at a time into the same quartz fluorimeter cuvette which was rinsed with distilled water and ethanol in between each reading. A total of 1.0 mL of each sample was used to take the readings on the second fluorimeter. For both fluorimeters, the samples were excited at 587 nm and the emission at 610 nm was collected. These are the excitation and emission wavelengths of the fluorescent mCherry protein.

The total amount of protein in each sample was also measured. This way, the level of fluorescence could be expressed as the amount of fluorescent protein over total protein and the values could be directly compared. The total amount of protein was determined using a Bradford Assay. The Bradford Assay used Bio-Rad Protein Assay reagent. It was carried out in a 96-well round-bottom, clear plastic plate. A set of standards was used to construct a standard curve that revealed the linear relationship equation
between absorption at 595 nm and the total amount of protein present in solution. This equation was then used to convert the values of absorption at 595 nm for the samples to total amount of protein.
RESULTS

MIC and MBC Assays

Over a period of four months, a total of 26 possible small molecule inhibitors were tested against *M. smegmatis*. The minimum inhibitory concentration (MIC) assays tested the ability of each small molecule to inhibit growth across a range of concentrations from 0.78 to 100 \( \mu \text{M} \). The complete set of average MIC and MBC values for each inhibitor is listed below in Table 1. Out of the 26 small molecules tested, 20 were capable of inhibiting growth of *M. smegmatis* at concentrations of 100 \( \mu \text{M} \) or lower.

Three controls were run during each assay. The untreated control confirmed that the bacteria were healthy and able to grow. The DMSO-treated control was to make sure that inhibition of growth was not due to DMSO, the solvent that each of the small molecules was suspended in. Because the DMSO never prevented growth of the *M. smegmatis* in any of the assays, it was safe to conclude that any inhibition of growth was due to the small molecules alone. Also, a tetracycline-treated control was run to compare the effectiveness of the small molecules with that of a current drug used to kill *M. smegmatis*. To determine the MIC of tetracycline in *M. smegmatis*, serial ten-fold dilutions were used instead of the serial two-fold dilutions used on the inhibitors. For each assay that was run, the three controls consistently gave the expected results.

When determining the MIC of the inhibitors, any speck or cloudiness in the well was considered positive for growth. The MIC was defined as the well with the lowest concentration of small molecule that had absolutely no growth. Lower MIC values
correspond to more effective inhibitors. Figure 3 below has been included to help visualize the best inhibitors. The shorter the bar is on the graph, the lower the MIC value, and thus, the more effective the inhibitor is.

**Table 1. Average MIC and MBC values of each inhibitor tested.** Each assay was repeated at least in duplicate. More effective inhibitors were tested up to six times each. Lower MIC and MBC values correspond to more effective inhibitors. The best inhibitors, or “top tier” inhibitors, are shown in bold. DMSO was used as a control because it was used to dissolve each inhibitor. Tetracycline was also included as a control and a way to compare the effectiveness of these inhibitors to current drugs. A MIC of ‘IE’ means that the inhibitor was ineffective up to 100 μM and unable to inhibit growth of the bacteria. For the MBC values, ‘N/A’ means the inhibitor was ineffective and ‘static’ means that the inhibitor was not bactericidal at concentrations up to 4xMIC.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MIC (μM)</th>
<th>MBC (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>A2</td>
<td>66.7</td>
<td>static</td>
</tr>
<tr>
<td>A4</td>
<td>75.0</td>
<td>75.0</td>
</tr>
<tr>
<td>A6</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>A7</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>B1</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>B2</td>
<td>76.6</td>
<td>76.6</td>
</tr>
<tr>
<td>B3</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>B4</td>
<td>17.8</td>
<td>18.1</td>
</tr>
<tr>
<td>B6</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>C2</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>C4</td>
<td>IE</td>
<td>N/A</td>
</tr>
<tr>
<td>D3</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>
After repeating the MIC assay multiple times on each potential inhibitor, the five inhibitors with the lowest MIC and MBC values were placed into a group referred to as the “top tier” of inhibitors. The five inhibitors that were members of the top tier were A7, D3, D7, E2, and E5. Their average MIC values were 1.7, 1.2, 3.4, 1.7, and 5.9 μM, respectively (see Table 2 below). Inhibitors D3 and E5 were bactericidal at their respective MIC values meaning that their MBC values were the same as their MIC values. Inhibitors A7, D7, and E2 were bactericidal at slightly higher concentrations with average MBC values of 2.0, 4.3, and 3.1 μM, respectively. Comparison of their chemical structures (Figure 4) reveals that there are a few common functional groups conserved among the five inhibitors. However, those functional groups are not special to the best
inhibitors as they are found in many of the small molecules that were in the Novartis library. Further testing and manipulation of the structures followed by new MIC assays is required to determine which functional groups are critical to their mechanisms of inhibition. This “top tier” was selected as the set of inhibitors that would be further explored down the road to determine their targets and mechanisms of action.

The second best group of inhibitors, or “second tier,” included the inhibitors B4, E1, E4, F1, F2, and G4. Their average MIC values were 17.8, 7.4, 6.3, 15.6, 31.3, and 6.3 μM, respectively. Inhibitors B4 and F2 had MBC values equal to their MIC values meaning they were bactericidal at their MICs. Inhibitors E1, F1, and G4 were also bactericidal, but at concentrations greater than their MICs. Despite the very low MIC value of 6.3 μM for inhibitor E4, it was shown to be bacteriostatic in the MBC assays. One problem that was observed for inhibitor B4 was that it was difficult to keep it dissolved in solution. Its insolubility issues would make it challenging to develop it into an effective drug.

Table 2. The “top tier” inhibitors. These inhibitors demonstrated the lowest average MIC and MBC values in wild-type *M. smegmatis*. These are the inhibitors that were chosen for further testing to determine their targets and mechanisms of action.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MIC (μM)</th>
<th>MBC (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>D3</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>D7</td>
<td>3.4</td>
<td>4.3</td>
</tr>
<tr>
<td>E2</td>
<td>1.7</td>
<td>3.1</td>
</tr>
<tr>
<td>E5</td>
<td>5.9</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Figure 4. Molecular structures of the most effective inhibitors. There are no common functional groups that are statistically important and no obvious relation between structure and effectiveness.

UV Mutagenesis

Initial UV kill curves showed that 99.9% of the *M. smegmatis* were killed after being exposed to UV radiation for 2-4 minutes. This exposure time was then used to create mutant strains of *M. smegmatis* that were tested for increased resistance to inhibitor D7. Over a couple weeks of UV treatment assays, six possible D7-resistant mutants were isolated. Upon retesting with a MIC assay, five of those six isolates showed MIC values of 25 μM (see Table 3). Compared to the average MIC of D7 which was 3.4 μM, these five isolates possessed MIC values only approximately 8 times higher than the wild-type *M. smegmatis*. The most resistant isolate produced by these UV mutagenesis assays was isolate 4A. MIC testing showed that D7 had an average MIC of 50 μM in this environment.
isolate, over 15 times greater than the MIC of D7 in the wild-type strain. A couple of the individual MIC assays even produced limited growth in the presence of 100 μM of D7. This amount of increase in the MIC of D7 was considered very significant and isolate 4A was thought to be the most resistant to D7.

Because isolate 4A was shown by repeated MIC assays to be the most resistant mutant, it was chosen for sequencing. Synthetic primers and Deep Vent Polymerase were used to successfully amplify the \textit{smpB} and \textit{ssrA} genes. These two genes encode SmpB and tmRNA, respectively. SmpB and tmRNA are two of the most important molecules involved within the tagging step of the \textit{trans}-translation pathway. Previous research using GFP-expressing strains of \textit{E. coli} had shown that inhibitor D7 likely inhibits the tagging step (N. Ramadoss, unpublished results). For this reason, it was suspected that one of these two molecules (SmpB or tmRNA) was the likely binding site for D7. Mutations resulting in alteration of the target would result in the resistance that was being observed in Isolate 4A.

After gel purification of the PCR product, A-tailing, ligation into pGEM-T Easy Vector, and transformation into CaCl$_2$-competent DH5α \textit{E. coli} cells, the plasmids were sequenced at Huck Institutes of the Life Sciences at the Pennsylvania State University. Analysis of the sequences showed that they were 100% identical to the gene sequences found in wild-type \textit{M. smegmatis}. With this information, it was concluded that isolate 4A’s resistance to D7 was not due to the fact that the SmpB or tmRNA molecules had been altered.
Table 3. Average MIC values of D7 in the D7-resistant mutants. The D7-resistant mutants were generated by UV mutagenesis. In wild-type *M. smegmatis*, D7 has a MIC of 3.4 μM. The isolated resistant mutants mostly showed MIC values about five times greater. Isolate 4A was the most resistant to D7, producing an average MIC value of 50 μM which was approximately ten times greater.

<table>
<thead>
<tr>
<th>D7-Resistant Mutant Isolates</th>
<th>Average MIC of D7 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>25.0</td>
</tr>
<tr>
<td>1B</td>
<td>25.0</td>
</tr>
<tr>
<td>2A</td>
<td>25.0</td>
</tr>
<tr>
<td>3A</td>
<td>25.0</td>
</tr>
<tr>
<td>4A</td>
<td>50.0</td>
</tr>
<tr>
<td>5A</td>
<td>25.0</td>
</tr>
</tbody>
</table>

**Fluorescence Assays**

These assays were used as the second method in which to search for the targets and mechanisms of action of the most effective inhibitors. Three of the five top tier inhibitors (listed in Table 2) were chosen for these assays. Inhibitors A7, D7, and E5 were selected to be run first because stocks of D3 and E2 were low and had lost activity. The usefulness of the assay would be determined before fresh D3 and E2 stocks were ordered.

The fluorescence assays using mCherry concentrated on determining if the proteolysis step of *trans*-translation was being blocked by the inhibitors. Inhibitors were tested on the strain of *M. smegmatis* that contained a plasmid carrying the tmRNA-tagged fluorescent mCherry protein. The gene had the tmRNA tag encoded at its 3’ end to target the mCherry protein for destruction by ClpXP. This peptide tag was identical to
the tag that is added by tmRNA during the trans-translation pathway. Therefore, this assay skipped the step where the ribosome stalls and requires rescuing by tmRNA. Instead, the tmRNA tag was automatically built into the sequence of the gene. If the proteolysis step was not blocked by the inhibitor, the mCherry protein would be degraded like normal by ClpXP and the cells would be dark with no fluorescence. However, if the proteolysis step is blocked, the mCherry protein will not be degraded and the cell will remain fluorescent. The LEC240 strain contains the wild-type mCherry gene without the tag. Therefore, it is not degraded and the cell is always fluorescent. The LEC283 strain contains a variant tagged version of the mCherry gene. This alternate tag is not recognized by ClpXP and thus, the fluorescent protein is not degraded and the cells remain bright. The wild-type and variant tagged strains served as positive fluorescence controls. The flowchart of this assay can be found in Figure 5.

**Figure 5. Flowchart describing the fluorescence assays.** These assays were run using the mCherry strains of *M. smegmatis*. The tmRNA tag is built into the gene in the plasmid and targets the protein for degradation by the protease ClpXP. Inhibition of ClpXP proteolysis leads to fluorescent bacteria.
The fluorescence assays were repeated multiple times for each inhibitor. At each time point, OD$_{600}$ readings were taken to ensure that each culture was growing normally. Initial assays done with simple LB broth (instead of the 7H9 broth) showed some abnormal growth patterns for the mCherry-containing *M. smegmatis* strains. The fluorescence readings for these runs were not considered good data. After switching over to the Mycobacteria-specific 7H9 broth, OD$_{600}$ readings were normal for every run carried out over the two month period. This means that all the fluorescence data collected from these runs was reliable and could be used for interpretation and forming conclusions.

Apart from the OD$_{600}$ readings taken at each time point, each culture was also observed under a fluorescent microscope. Five μL of each culture was placed on a clean glass slide and covered with a glass cover slip. The bacteria were observed under 1000x magnification and pictures deemed to be representative of the culture at large were taken. The images were later analyzed for the amount of fluorescence emitted by individual cells. In particular, the value that was used was the red component of the fluorescence since the mCherry protein emits red fluorescence at the wavelength of 610 nm. The goal was to take pictures of individual cells so that the fluorescence could be easily seen. However, this was often impossible with the higher time points because intense clumping was seen in all cultures past the 7 h time point. Clumps of bacteria interfered with fluorescence measurements and may have had an impact on the results.
For inhibitor A7, the fluorescence assay was repeated five times. All the runs produced similar results, some of which can be seen below in Figure 6. The two positive fluorescence controls, the wild-type mCherry (LEC240) and the variant tagged (LEC283) strains, both produced high levels of fluorescence as expected (shown in the upper graphs). The microscope’s maximum detection limit for fluorescence was at 255 units. Many of the time points for the positive controls were above the maximum and are displayed on the bar graph as 255 units of fluorescence but should be considered as >255 units. The two negative fluorescence controls, the untreated tmRNA-tagged and DMSO-treated tmRNA-tagged strains, both showed very low levels of fluorescence as expected. The level of fluorescence in the A7-treated culture remained similar to the negative controls throughout the assays. Micrographs shown in Part C of Figure 6 further demonstrate how the A7-treated bacteria are dark like the DMSO-treated negative control. The positive wild-type mCherry control shows obvious fluorescence while the other two are almost completely dark. These results lead to the conclusion that proteolysis is not being blocked in the bacteria treated with A7. Instead, it is likely that inhibitor A7 functions by inhibiting a step prior to the proteolysis step of the **trans**-translation pathway.
Figure 6. Results of the fluorescence assays run with inhibitor A7. The fluorescence values recorded by the fluorescent microscope on 10/28/2011 (A) and 10/31/2011 (B). Maximum detection occurs at 255 units of fluorescence so any bar reaching 255 on the graph should be considered >255. (C) Images taken with the fluorescent microscope showing that the A7-treated bacteria are dark like the DMSO-treated tmRNA-tagged negative control.
For inhibitor D7, the fluorescence assay was repeated eight times. Although the first run produced fluorescence in the D7-treated bacteria, all other runs agreed on the fact that the bacteria treated with D7 were dark with no fluorescence. A couple of the runs are shown below in Figure 7. Again, the two positive controls, the wild-type mCherry and variant tagged strains, are consistently fluorescent (upper graphs). Most of them reaching the maximum level of measurement for the scope. The two negative controls and the D7-treated tmRNA-tagged strain all possess similar levels of fluorescence at each time point. The images shown in Part C of Figure 7 also show that the D7-treated bacteria remain as dark as the negative controls. These results lead to the conclusion that D7 works by targeting a step before the proteolysis step of *trans*-translation.

For inhibitor E5, the results are drastically different than those for A7 and D7. Looking at Figure 8 below, it is clear that the bacteria treated with inhibitor E5 emitted higher levels of fluorescence than the two negative controls, the untreated tmRNA-tagged (LEC282) and DMSO-treated tmRNA-tagged (LEC282+DMSO) strains. The levels of fluorescence for the E5-treated sample were consistently 1.5-2.5 times greater than the negative controls. The pictures shown in Part C of Figure 8 also show the clear appearance of fluorescent cells in the culture treated with E5. The mCherry protein with the tag is not being degraded, resulting in the bacteria remaining fluorescent. These results give good reason to conclude that E5 is likely inhibiting the proteolysis step of the *trans*-translation pathway in *M. smegmatis*. 
**Figure 7. Results of the fluorescence assays run with inhibitor D7.** The fluorescence values recorded by the fluorescent microscope on 10/21/2011 (A) and 10/24/2011 (B). Maximum detection occurs at 255 units of fluorescence so any bar reaching 255 on the graph should be considered >255. (C) Images taken with the fluorescent microscope showing that the D7-treated bacteria are dark like the DMSO-treated tmRNA-tagged negative control.
Figure 8. Results of the fluorescence assays run with inhibitor E5. The fluorescence values recorded by the fluorescent microscope on 10/31/2011 (A) and 11/04/2011 (B). Maximum detection occurs at 255 units of fluorescence so any bar reaching 255 on the graph should be considered >255. No untreated tmRNA-tagged control was run in B because there were not enough clean, autoclaved 125 mL flasks. (C) Images taken with the fluorescent microscope showing that the E5-treated bacteria are much brighter than the DMSO-treated tmRNA-tagged negative control.
**Fluorimeter Measurements**

The fluorimeter measurements were incorporated as a preliminary attempt to better quantify the level of fluorescence in the various samples. Cells were spun down and collected at each time point for each culture and stored at -80°C until they could be analyzed. Following resuspension in fresh 7H9 broth and lysis with chloroform, the amount of fluorescent protein in each sample was measured using two different fluorimeters. The fluorescence values produced by both fluorimeters were very inconsistent (data not shown). Even the values for samples run in duplicate at the same time produced wildly different values.

Although the wild-type mCherry positive controls were clearly the most fluorescent under the scope, the fluorimeter data often presented the wild-type mCherry samples as some of the least fluorescent. The DMSO-treated tmRNA-tagged negative control cultures also presented very unexpected results. Quite often, they produced extremely high fluorescence values that clearly differed from what was observed under the fluorescence microscope. The high amount of inconsistency associated with this preliminary fluorimeter data and the fact that the results do not match what was seen under the microscope suggest that the lysis buffer may be interfering with the assay. For this reason, the fluorimeter data will not be taken into account until the problems with the lysis buffer are solved and the values are more consistent. No conclusions can be drawn from these results but it is important to continue pursuing this method to verify what was seen under the microscope during the fluorescence assays.
DISCUSSION

The above results demonstrate that inhibition of the trans-translation pathway is an effective way to prevent the growth of *M. smegmatis*. 20 out of the 26 small molecule inhibitors tested showed effectiveness at concentrations of 100 μM or less. The top tier of inhibitors included compounds A7, D3, D7, E2, and E5. The MIC values of this group were all below 6.0 μM with D3 being the best inhibitor at a MIC of 1.2 μM. To get a better indication of how effective these inhibitors are, it is best to compare them with the drugs currently used to treat TB. Isoniazid, rifampicin, and ethambutol all have MIC values one to two orders of magnitude lower than the top tier inhibitors (18). However, when compared to pyrazinamide (average MIC >800 μM) which has recently been suggested to work by targeting the trans-translation pathway, the top tier inhibitors are much more effective (21). The fact that the small molecule inhibitors work at concentrations so much lower than a current drug that targets the same pathway is significant. It means that this route of drug development for TB is promising and should be pursued further. In order to continue moving these small molecule inhibitors forward, their targets need to be identified and their mechanisms of action need to be deciphered. After this is complete, the small molecule inhibitors can be altered to increase specificity for tighter binding and better inhibition of the trans-translation pathway.

The first attempt to determine the target of one of the inhibitors was done using UV mutagenesis. After generating some D7-resistant mutants and retesting the MIC values,
the *smpB* and *ssrA* genes of the most resistant mutant (isolate 4A) were sequenced. The nucleotide sequences of the *smpB* and *ssrA* genes were identical between the resistant mutant and the wild-type *M. smegmatis*. This means that it is impossible to conclude if D7 targets either of these molecules. Resistance could be due to mutations that have general effects such as increased production of efflux pumps, decreased membrane permeability, or activation of an enzyme that degrades D7. If the resistance is due to one of these general reasons, D7 could be targeting SmpB or tmRNA but there is no way to know it. Even though D7 is still capable of binding to its target, other factors are blocking its function. Another explanation is that instead of targeting SmpB or tmRNA, D7 targets another piece of the trans-translation pathway. This alternate target may have been mutated in isolate 4A, thus not allowing inhibitor D7 to bind and function. Overall, the UV mutagenesis produced no conclusive information on what the target of inhibitor D7 is. All that was learned is that *M. smegmatis* is capable of developing resistance to this inhibitor. In the future, whole genome sequencing or the sequencing of other important genes in the tagging steps of trans-translation could be carried out on isolate 4A and the other D7-resistant mutants to determine exactly where the resistance is coming from.

Although this path of searching for the target could have been continued, multiple factors suggested that it was better to move on to a different type of assay. First of all, the initial kill curves done to determine the necessary length of UV exposure revealed that *M. smegmatis* is very resistant to UV damage. Where bacterial cultures such as *E. coli* are completely killed off after only 20 s of treatment, some of the *M. smegmatis*
remain viable even after 8 min of treatment. This may be due to the abnormally thick cell wall filled with mycolic acids that is characteristic of the Mycobacterium genus. The UV rays may not be able to penetrate the thick cell wall found in *M. smegmatis*. Another reason to abandon the UV mutagenesis method was that each set of runs was using up large volumes of the inhibitor stock. Many UV treatments produced no resistant mutants meaning that a whole plate of MIC assays was wasting inhibitor D7. The high concentrations of 25, 50, and 100 μM that were required to search for sufficiently resistant mutants required a lot of inhibitor stock to be used up. Continuing with this risky method would have been irresponsible. Moving on to the fluorescence assays which only required sub-MIC concentrations of the inhibitors was a good choice.

Overall, the fluorescence assays using the mCherry-expressing strains of *M. smegmatis* worked well. Under the fluorescence microscope, the results of dark or bright cells was clear and easily observable. The Middlebrook 7H9 broth provided great growth with consistent doubling times of around 4 h. Out of the three inhibitors that were tested in depth with this assay, inhibitor E5 showed strong indication that it was inhibiting the proteolysis step of trans-translation. The pre-tagged mCherry protein was not being degraded by ClpXP like it should have been. Inhibitor E5 could be interfering with the targeting of the protein to the ClpXP complex, the binding of the protein to ClpXP, or with the physical mechanics of ClpXP. Inhibitors A7 and D7 produced results that suggested that the proteolysis step was not being inhibiting. This means that for these two inhibitors, it is probably the tagging step that is being blocked.
The results for each of the three inhibitors all agree with previous data that was obtained recently in GFP-reporter assays done in *E. coli* (N. Ramadoss, unpublished results). After screening of the library with the luciferase-reporter system was complete, a GFP-reporter assay was performed to get an initial indication of which of the two main steps (tagging of proteolysis) of trans-translation each inhibitor affects. In *E. coli*, A7 and D7 were found to be tagging inhibitors and E5 was found to a proteolysis inhibitor. The results from the fluorescence assays support the idea that these inhibitors affect the same steps and probably have the same mechanisms of action in *M. smegmatis*. Because A7 and D7 affect the tagging steps and E5 affects the proteolysis step, there is a possibility that using combination therapy to attack both parts of the pathway could produce a synergistic effect that would result in an even higher level of inhibition of trans-translation.

Even though cultures treated with inhibitor E5 did clearly show fluorescence, there are still a couple problems to consider. The fluorescence demonstrated by E5-treated bacteria was much less than the fluorescence of the two positive controls, the wild-type mCherry and variant tagged strains. In most cases, it was only twice that of the negative controls and a mere fraction of the positive controls. If the proteolysis step of trans-translation is being inhibited, it would make sense to see more fluorescence in the cultures. But because sub-MIC concentrations are being used, it is tough to determine how much effectiveness is lost with each decrease in concentration. If the proteolysis step is only being partially inhibited because the concentration of E5 is so low, this would explain why only limited fluorescence was observed.
Another thing to consider is that during microscope observations, clumps of bacteria seemed to exhibit more fluorescence than single cells. The clumping of bacteria may have trapped extra E5 within the clump as well, leading to an increase in the relative concentration of the inhibitor. With higher concentrations, more fluorescence would have been observed. Clumps of bacteria also made it difficult to get a true reading of the fluorescence levels from the photos, especially at the later time points when clumping was much more prevalent. Trying to find a single cell in a single phase of the image to measure was difficult and there was often background fluorescence that may have artificially increased the measurement and led to skewed values. Bias may have also been accidentally included in the fluorescence readings. Because only a few pictures were taken of each time point for each culture, it was difficult to get a true overall representation of the total amount of fluorescence. All effort was made to view the entire field of cells for each slide, but it is impossible to say for sure that each sample was assigned a fair fluorescence value.

It was because of these problems that an effort was made to quantify the fluorescence in terms that could be truly trusted. The plan was to collect small samples of each culture at each time point and quantify the amount of fluorescent protein by using a fluorimeter. Dividing the amount of fluorescent protein by the amount of total protein would have made it possible to directly compare the samples and interpret whether or not treatment with the inhibitors led to an increase in fluorescence over the negative controls. Although the plan was promising, the execution was unsuccessful. It is suspected that there was a problem with the lysis procedure that was used to break
open the cells and release all the proteins. Using chloroform may have damaged the mCherry protein or altered the fluorimeter readings by causing diffraction of the light.

In the future, it will be important to return to the fluorescence assays and find a way to optimize the protocol for lysis of the cells. Fluorimeter measurements are required to confirm that inhibitor E5 is causing an increase in fluorescence compared to the negative controls. If this can be confirmed, there is a high probability that E5 functions by targeting the proteolysis step of \textit{trans}-translation. The next step could then be to run binding assays to see if E5 is binding to ClpXP directly. Overall, the search for the targets of the best inhibitors must continue. Finding their targets and determining their mechanisms of action is a crucial step in moving these small molecules down the path to becoming drugs. Once the method of inhibition is known, alterations can be made to improve the small molecules and make them more specific to their binding targets. This would lead to greater inhibition and thus a greater negative impact on the health of the bacteria. Understanding the function of these inhibitors may also make it possible to gear them better towards the killing of \textit{M. tuberculosis} and the treatment of tuberculosis, the overall goal of this research.
REFERENCES


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Education:

The Schreyer Honors College at The Pennsylvania State University, University Park, PA
   Bachelor of Science in Microbiology
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Experience:

Undergraduate Researcher in the Keiler Lab at Pennsylvania State University, Biochemistry and Molecular Biology Department, Fall 2010-current.
   Have used MIC assays, MBC assays, UV mutagenesis, DNA isolation, cloning, genome sequencing, and fluorescence assays to test the effects of pre-selected small molecules on the trans-translation pathway in Mycobacterium smegmatis. Currently trying to determine the targets and mechanisms of action of the most effective inhibitors.

Summer Intern at Benchmark Analytics, Inc. in Center Valley, PA, Summer 2010 and 2011.
   Ran microbiological tests on water, dairy, and soil samples to check for bacterial contamination.
Awards and Honors:

Schreyer Honors College Academic Excellence Scholarship (2008-2012)

• For being a member of and fulfilling the requirements of the honors program

First Place in the Life Sciences category of the PSU Undergraduate Research Exhibition (2011)

• Presented a scientific poster describing the research I have completed

Penn State Thompson Endowed Scholarship (2011-2012)

• Given by the BMB department to a senior with excellent academic standing

Penn State Charles E. and Joyce Knauss Mathues Scholarship (2010-2011)

• Given by the BMB department to a junior with excellent academic standing

Penn State Holtzinger Endowed Scholarship in Science (2010-2011)

• Given by the BMB department to a junior with excellent academic standing

The Evan Pugh Scholar Award for Seniors, Penn State (2012)

• For maintaining a cumulative GPA of at least 3.98 throughout my college career

The Evan Pugh Scholar Award for Juniors, Penn State (2011)

• For sustaining a GPA of at least 3.99 over my first five semesters

President’s Sparks Award for Sophomores, Penn State (2010)

• For earning a perfect 4.0 GPA through my first three semesters

President’s Freshman Award, Penn State (2009)

• For completing my first semester with a 4.0 GPA

Inducted into Phi Eta Sigma Honors Society (2009)

• Admission allowed to first year students with a GPA > 3.5

Dean’s List (GPA > 3.5) at Penn State University (every semester)

Activities:

Penn State Baseball Club - Division II, Member (2008-current)

• Help organize fundraising events, team practices, travel plans, and coach a team in the campus-wide intramural fall baseball league

• Won the NCBA (National Club Baseball Association) National Championship in 2011