IDENTIFICATION OF PKS13 INHIBITORS FOR ANTITUBERCULAR DRUG DISCOVERY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Identification of Pks13 Inhibitors for Antitubercular Drug Discovery

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Frontline tuberculosis drugs, i.e. Isoniazid, have become inefficient as strains of *Mycobacterium Tuberculosis* have become multi-drug resistant, creating an urgent need for novel antitubercular drugs. The key to the bacterium resistance lies within its thick and complex cell wall, where mycolic acids (MAs) are the major constituents and contribute to the permeability of the cell wall and the resistance of the organism. The last step in their biosynthetic formation is done by polyketide synthase 13, which produces the α-alkyl-β-ketoester precursors leading to MAs formation. We have identified a compound in previous studies, IMTB-28, able to bind to the Pks13 Thioesterase domain, a domain where no major research has been done before. Inhibition of Pks13 TE domain by IMTB-28 prompt structural changes in Pks13 that make it unable to function properly and stop product formation. This research identified a compound, TAMU196, within a series of IMTB-28 analogs, with great potency against the Pks13 TE domain and Mtb cells, and low toxicity against human cells. Eight commercially available compounds were also identified having 100% inhibition against the Pks13 TE domain at 10 μM concentration. These inhibitors can be used as scaffolds for structural changes in the path to becoming the next antitubercular drug and save the lives of patients with multi-drug resistant tuberculosis worldwide.

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Finally, I would like to thank my mother and brother for bringing happiness and optimism into my life.

NOMENCLATURE

Mtb	*Mycobacterium Tuberculosis*
TB	Tuberculosis
MAs	Mycolic Acids
Pks13	Polyketide Synthase 13
TE	Thioesterase
HDF	Human Dermal Fibroblast
IC_{50}	Half Inhibitory Concentration
MIC_{50}	Half Minimal Inhibitory Concentration
O.D	Optical Density
RFU	Relative Fluorescence Units

CHAPTER I

INTRODUCTION

The Need for Novel Antitubercular Drugs

Tuberculosis (TB) remains today as one of the deadliest diseases as strains of *Mycobacterium Tuberculosis* (Mtb), the causative agent of tuberculosis, have developed resistance to commonly used TB antibiotics, i.e., Isoniazid and Rifampicin [1]. Only in the year 2014, TB infected around 9.6 million people, killing 1.5 million [2]. This has placed an urgent need in the development of novel TB compounds.

The success of Mtb as a pathogen lies within its thick and complex cell wall, which is the key factor in its physiological and pathogenic aspects, and its antibiotic resistance and susceptibility [3]. Its main constituents, mycolic acids (MAs), are α-branched and β-hydroxylated long chain fatty acids that help Mtb increase resistance against chemical damage, hydration, and hydrophobic antibiotics. The MAs essentiality for the survival of Mtb, and having their biosynthetic pathway inhibited by the most efficient anti-TB drug Isoniazid as an effect [4], makes the MAs pathway a crucial source for effective target identification for novel anti-TB drugs.

Pks13 TE Domain Drug Discovery

Within the *Corynebacterineae* suborder, MAs have a common 2-alkyl 3-hydroxyl segment, found to be biosynthesized by precursors formed by polyketide synthase 13 (Pks13). This enzyme contains an acyl transferase domain, a ketosynthase domain, an acyl carrier protein domain, and a thioesterase domain (TE); and catalyzes the last step in the MAs biosynthetic pathway by synthesizing the α-alkyl-β-ketoester precursors that form the mentioned MA segment

[3]. Inhibition of Pks13 would lead to a discontinuation in the formation of MAs, which would result in the disruption of the bacterial cell wall and elimination of Mtb. In previous studies, we have identified a lead benzofuran compound, IMTB-28 (See Figure 1), that inhibits the Pks13 TE domain by blocking the release of the MA precursors by de-esterification. We have solved the inhibitor structure in complex with the TE domain, and we have used this information to synthesize IMTB-28 analogs containing the benzofuran core and differing R groups[5].

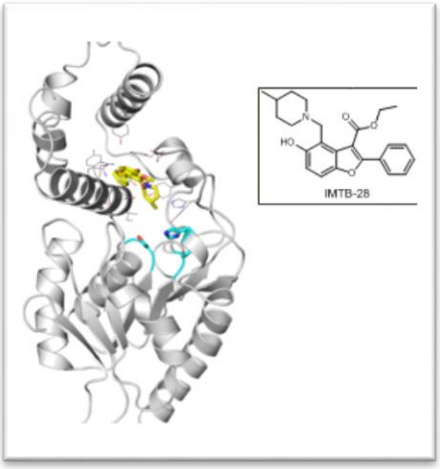


Figure 1. IMTB-28 binding to Pks13 Thioesterase Domain. The picture taken from previous studies, and depicts the binding site of the IMTB-28 lead molecule, containing a benzofuran core.

This research strives to identify potent IMTB-28 analogs through the use of *in vitro* studies to assess the inhibitory potency of these compounds against Pks13 TE domain, their killing activity against Mtb cells, and their cytotoxicity activity against human dermal fibroblast cells. This research will also strive to find active inhibitors, or hits, of the Pks13 TE domain using high throughput screening. Inhibition of Pks13 TE domain by these inhibitors will prompt structural changes in Pks13 that will make it unable to function properly and stop MAs formation. Success will result identifying potent and active Pks13 able to be used as scaffolds for further investigation in the path for developing a drug able to combat resistant strains of TB and save millions of lives.

CHAPTER II

MATERIALS AND METHODS

In Vitro Assays

Absolute IC_{50} of Compounds Against Pks13

Assay

Compound dilutions were first made in a 2-fold series (10 μM- 0.00098 μM) using dimethyl sulfoxide (DMSO, Sigma Aldriech) as solvent in a 96-well plate (VWR). The 100 μL assay was then performed using Tris buffer (0.25M, pH 7.0) , purified Pks13 TE domain (100 nM), 1 μL compound dilutions from the dilution plates, and 4-Methylumbelliferyl heptanoate (MUH, 20mM, Sigma-Aldrich). The constituents of the assay were added and mixed in this exact sequence. The assay mapping was divided into positive controls (Pks13 TE + MUH+ Compound) from rows A-D and columns 1-11, a negative control (MUH + Compound) from rows E-H and columns 1-11, and a DMSO control from column 12, were used for data analysis. The plate was then red with a microplate reader (Omega FLUOstar) at 560 nm. The %inhibition was calculated and plotted against log(concentration) using Prism (GraphPad Software) to determine IC_{50} values.

Absolute MIC of Compounds in Wild Type $Mtb^2$7000 Cells

Cell Culture

The $Mc^2$7000 Mtb cell line was commercially available. The culture was made with 10 mL 7H9 Broth using OACD (1x), Tyloxapol (50%), Pantothenic acid (50 mg/mL), Malachite green (0.25 mg/mL), and 1 mL of P1 Mtb cells (0.01 O.D). This was incubated at 37^0 C for 7 days.

Single and Multiple Carbon Source Assay

Three-fold dilutions plates were made with a $[0.5\ mM]_0$. Using the cell culture at an O.D of 0.8 at 560 nm, cells were prepared with 2 different carbon sources in 2 different media for a final O.D of 0.01 at 560 nm: one 20 mL Dextrose 7H9 Broth prep-media with a final concentration of Dextrose (0.5 %), NaCl (0.085 %), Tyloxapol (0.25 ug/mL), Pantothenic Acid (50 mg/mL), and Malachite Green (0.25 mg/mL), in the assay plate; and one 20 mL Dextrose + Acetate M9 prep-media with a final concentration of Sodium Acetate (0.25%), Dextrose (0.25 %), Magnesium Sulfate (2 nM), Calcium Chloride (125 µM), Malachite Green (0.25 mg/ mL), Tyloxapol (0.05%), Pantothenic Acid (50 mg/mL), in the assay plate. Using the dilution plate, 4 µL of compound from Row A was transferred to Row A in 2 assay plates, Row B to Row B in both assay plates, respectively. A portion of 196 µL of M9 prep-media and 7H9 prep-media was added into their respective assay plates, giving a total assay volume of 200 µL. For rows A-H, from column 2-11, the final concentrations of the compound in the assay plate ranged from 10 µM- 0.00050805 µM in a 3-fold series. Undiluted Rifampicin (10 uM) was used in column 12 as positive control, DMSO was used in column 1 as negative control, the wells at the edge in row H were used as cell-only control, and row H was used as the diluted Rifampicin control. The plates were incubated at 37^0 C for 7 days, then stained with Resazurin (0.25 mg/mL) for 2 days, and finally red with a microplate reader (Omega FLUOstar) with a fluorescence protocol at 560 nm. The data was analyzed through a %growth calculation and a dose-response was determined by plotting the %inhibition against the log(concentration) of the compound using GraphPad Prism (Graphpad Software) to determine the MIC_{50} value.

Absolute MIC_{50} of Select Compounds in IMTB-28 Resistant mutant Mtb $Mc^{2}7000$ Cells

Cell Culture and Assay

Resistant cells were obtained from previous studies. The cell culture and assays were performed using the procedure for wild type but with resistant mutant cells. The plates were stained with Resazurin after 5 days, and red with a fluorescence protocol after 2 days.

Human Dermal Fibroblast Cytotoxicity Assay

Human dermal fibroblasts (HDF) cells were purchased from ATCC (Manassas, VA). HDF cells were cultured in DMEM (Lonza) media supplemented with 10% fetal bovine serum (Lonza) and penicillin/streptomycin (Lonza). For the cytotoxicity assay, compound stocks were serially diluted in phosphate buffered saline (PBS) plus 10% DMSO. On the day of assay, HDF cells were trypsinized, counted and resuspended at a concentration of 64,000 cells/ml in media. Cells were plated, overlaid with the compound serial dilutions and incubated at 37C. After 48h, Resazurin dye was added and the assay plates cultured for another 24h. The next day the absorbance of the Resazurin was measured on a microplate reader to assess cell death. Cytotoxicity was determined as a percent of dead cells versus living.

High Throughput Screening

Assay

A compound library consisting of three 384-well plates (VWR) with a total of 924 compounds were screened against Pks13 TE for a period of 2 hours at room temperature for hit identification. The assay was done with the aid of the CyBio automated system, with a formulated protocol for a 50 µL assay containing Tris buffer (0.25M, pH 7.0), the Pks13 TE domain (100 nM), compounds from the compound library plates (10µM), and MUH substrate (20 µM), all added and mixed in this exact sequence. A positive control (Pks13 TE domain +

MUH), a negative control (MUH only), and a reference control (Pks13 TE domain + a potent IMTB-28 analog) were used. Once the assay was completed, the plates were red in a fluorescence protocol using a microplate reader (Omega FlUOstar), and the enzyme activity was analyzed through calculating 1-%activity (Pks13 + compound activity/ Pks13 activity).

CHAPTER III

RESULTS AND DISCUSSION

Potent Pks13 Inhibitors in the IMTB-28 Analogs Series

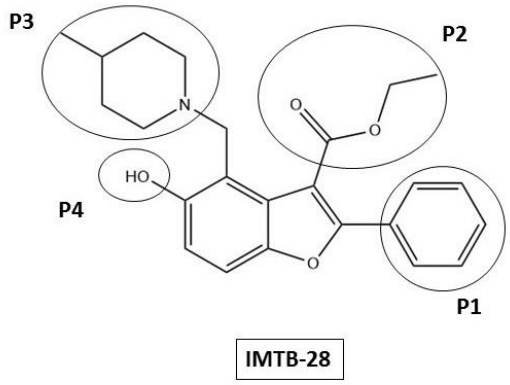
IC_{50} and MIC_{50} testing

The first step to discover potent pks13 TE domain inhibitors in the IMTB-28 analog series was to assess their inhibitory activity based on IC_{50} and simultaneously validate the Pks13 TE domain as their specific target. The same optimized assay conditions from previous laboratory work on Pks13 were used for this experiment, consisting of a final Pks13 TE domain concentration of 100 nM, and the use of MUH as a substitute substrate to mimic Pks13 TE domain substrate with a final concentration of 20 μM.

Results were obtained by a construction of a dose-response graph and evaluation of the activity of the antagonist (the IMTB-28 analog) versus the activity of the agonist (MUH), and calculating the concentration of the antagonist needed to inhibit half of the biological response formed by the agonist [6]. A small IC_{50} value indicated greater inhibitory activity by the compound as a small concentration was needed to competitively inhibit Pks13 activity and prevent MUH binding leading to product formation.

After validating Pks13 TE domain as their direct target, the most active compounds (See Figure 2) were tested in an MIC_{50} experiment against Mtb $Mc^2$7000 cells to determine their cell killing activity [7]. The results were obtained through a dose-response of the half minimal inhibitory concentration (MIC_{50}) of the compound needed to inhibit 50% of cell growth. This experiment was done in single (dextrose) and multiple (dextrose + acetate) carbon source, the

latter being the one most reflective of compound activity inside our body, to assess the effect of carbon source in compound activity.



IMTB-28 Analog	IC_{50} (μM)	MIC_{50} (μM)	
		Dextrose	Dextrose+Acetate
TAMU185	0.839	0.0299	1.124
TAMU196	1.12	0.0777	2.159
TAMU190	0.29	0.012	>10
TAMU195	0.5	0.0149	>10

Figure 2. Summary results of selected potent IMTB-28 analogs series. Structure of IMTB-28 as the structure of the compounds are confidential and cannot be disclosed on the left, and a table of results which include IC_{50} values, and MIC_{50} values w/ different amount of carbon sources on the right.

Analogs w/ Spirocyclic Amines (TAMU185 & 196)

In comparison to the other active analogs, both TAMU185 and 196 contained a spirocyclic amine as one of the P groups, and killed the cells in both single and multiple carbon source with an MIC_{50} less than 10 μM (See Figure 3). TAMU185, containing a secondary amine instead of an ester as TAMU196, showed greater activity against Pks13 and Mtb cells.

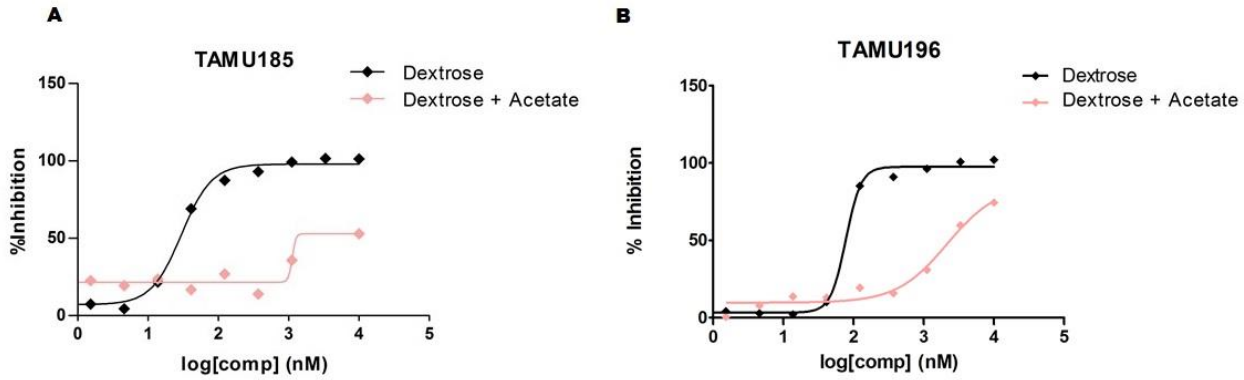


Figure 3 . Multiple carbon source dose-response graph of potent IMTB-28 analogs. (A) Graph of TAMU185. The activity of the compound decreases as the dose-response shifts from an MIC_{50} of 0.029 to 1.24 μM. (B) Graph of TAMU196. This compounds has a smother and more sigmoidal shape graph than TAMU185, and its activity decreases from 0.078 to 2.159 μM, having a larger shift decrease than TAMU185.

In the dose-response graph, a shift in the sigmoidal curve was seen in the multiple carbon source experiment leading to a higher MIC_{50} value and lower compound activity against the Mtb cell. A possible reason was that because the Mtb cells have access to not just one, but two carbon sources, more cells were reproduced and more compound was required to kill 50% of the cell population resulting in a greater MIC_{50}. This possibility, however, fails to explain why other IMTB-28 compounds do not show activity against Mtb cells in multiple carbon sources but do kill in single carbon source. TAMU190 and TAMU195 have only one P group different than TAMU185 and TAMU196, where in place of a spirocylic amine have a cyclic amine. This cyclic amine allows them to have more potency against the Pks13 TE domain, but makes them unable to kill cells in multiple carbon source MIC_{50}.

Analogs w/ Cyclic Amines (TAMU190 & 195)

These benzofuran molecules contained a cyclic amine as one of the P groups; TAMU195 contained an ester while TAMU190 contained a secondary amine. TAMU190 showed greater potency with a two-fold increase in both IC_{50} determination and MIC_{50} with dextrose-only carbon source. These analogs showed no MIC_{50} <10 μM in mixed carbon source.

Resistant Mutant MIC_{50}

Once established that these compounds were potent against Mtb cells, the next step was to do an experiment to verify the Mtb killing efficiency of the inhibitors due to specifically targeting the Pks13 TE domain within the Mtb cell. For this, an MIC_{50} assay was performed using grown IMTB-28 resistant mutant Mtb cells rather than wild type cells as in previous MIC_{50} experiments, and holding the remaining assay conditions from the previous MIC_{50} single carbon source assay constant. The results were also obtained through a dose response w/ % growth of the cells, and these MIC_{50} values were compared to the values obtained from the wild type single carbon source assay (See Figure 4). A positive outcome, where the Pks13 inhibitors fail to kill cells resistant to them, was determined by the resazurin dye reduction to resofurin resulting in an overall pink color.

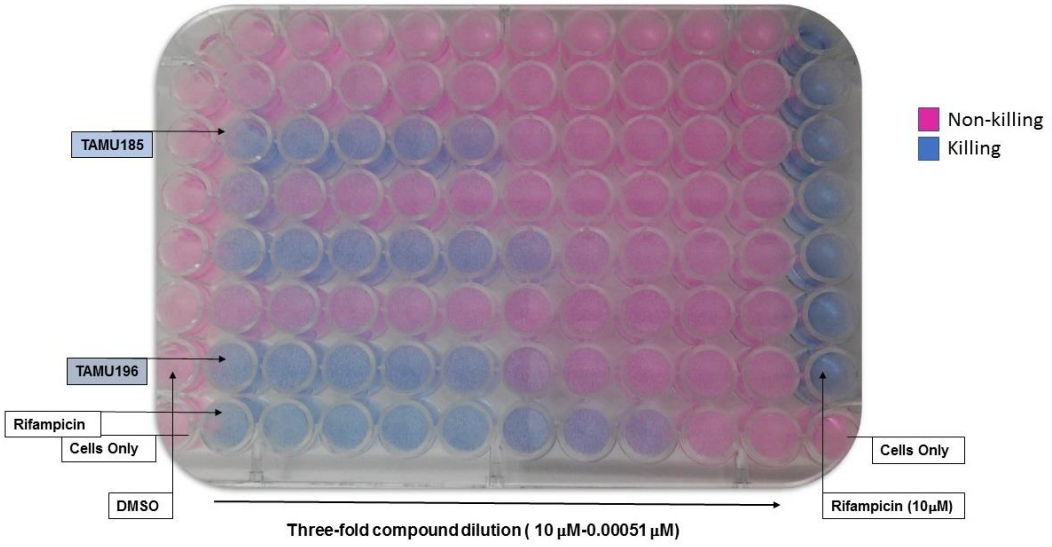


Figure 4.Compounds against resistant IMTB-28 Mtb $Mc^2$700 cells MIC_{50} plate. Coloring of the wells after Rezasurin staining for two days. Both compounds show activity against cells resistant to their activity against Pks13 TE domain.

Both TAMU185 and TAMU196 had a negative result of inhibiting the growth of cells that were resistant to their main benzofuran structure making them IMTB-28 analogs. This meant that even though these compounds killed Mtb cells and inhibited the Pks13 TE domain

directly, there was no certainty both compounds killed the cells because of inhibiting Pks13 and something else vital for cell survival, or inhibiting something else vital for cell survival and not Pks13 once inside the Mtb cell where they needed to look for the Pks13 target. However, both compounds remain potent inhibitors against Mtb and Pks13 TE domain, but further investigation will need to be done to verify the specific target of these compounds once inside the cell as they show potency against IMTB-28 resistant mutant Mtb cells (See Table 1).

Table 1. MIC50 values of compounds against wild type and resistant mutant Mtb cells*.

IMTB-28 Analog	*Wild Type Mtb Mc²7000* MIC_{50} *(μM)*	*IMTB-28 Resistant Mtb Mc²7000* MIC_{50} *(μM)*
TAMU185	0.03	0.08
TAMU196	0.07	0.05

*Both compounds showed activity against the resistant mutant cells despite resistivity of the cells against them, with similar MIC_{50} values against wild type cells. This indicated the possibility of cell killing activity of the compounds due to binding to Pks13 and another target, or cell killing due to binding to a target other than Pks13 within the cell.

HDF Cytotoxicity Testing

After determining two compounds within the IMTB-28 series, TAMU185 and TAMU196, to have great potency against the Pks13 TE domain and have killing proficiency against Mtb cells in a multiple carbon source environment, the consequent step was to verify if these compounds toxic to Mtb were also toxic to human cells and thus unable to be key compounds for tuberculosis drug discovery. A quick and simple method to test the cytotoxicity of these compounds against human cells was through an HDF cytotoxicity assay in collaboration with another laboratory member. This fibroblast-based assay uses human lung fibroblast cells sensitive to mycobacterial cytotoxicity, and are able to respond to the viability of the bacilli in a dose-response behavior[8]. After obtaining the results, the percent cell viability of the cells was

calculated and plotted against the log of the concentration of the compound to determine IC_{50}. TAMU185 and TAMU196 gave an IC_{50} of 12.5 µM and >100 µM, respectively (See Figure 5).

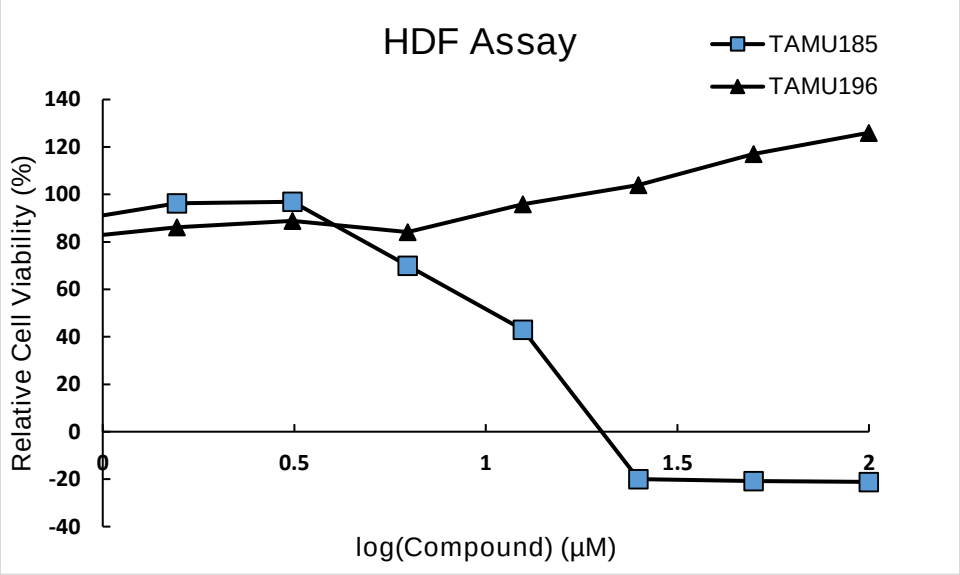


Figure 5. HDF assay results. TAMU196 shows no cytotoxic activity against human cells at MIC_{50} concentrations <100 µM. TAMU185 shows high cytotoxic activity.

TAMU196 showed low toxic effects against human cells as more than 100 µM concentration is needed to kill 50% of the cells. TAMU185, however, showed high cytotoxicity with an IC_{50} of 12.5 µM, making TAMU196 a safer compound for *in vivo* testing and selection as a lead molecule for drug discovery.

Active Pks13 TE Inhibitors from a Compound Library

High Throughput Screening

High Throughput Screening is used to test many molecules in a plate or a set of plates denominated a compound library, against the organism of interest, i.e. protein or cell, to find active inhibitors [9]. This experiment was done previously with a random compound population to determine the molecules within this population with the ability of inhibiting Mtb growth. The compounds with activity against Mtb were selected and saved for further characterization. In this experiment, a secondary assay was done using the selected population to determine which, if any, could inhibit the Pks13 TE domain specifically. Because the tested population was a biased one taken from the actual random population, percent inhibition, and not normal population statistics, were used as a method of identification.

The assay consisted in having the same Pks13 TE domain and MUH concentration as in the IC_{50} assay, but without a range of compound concentration, for all the wells in the 384-well compound library plates, except for the ones left for the controls, contained a different compound each. Therefore, the only compound concentration tested against the Pks13 TE domain was at 10 μM as a singlet. In contrast with the IC_{50} experiment, there was no dose-response of agonist vs antagonist as only one concentration was tested. Therefore, for data analysis, the controls were used with the sample data to produce 1-percent activity, i.e., percent inhibition. The percent activity was calculated using the relative fluorescence unit (RFU) values of the Pks13 TE-compound complex divided by the RFU values of the Pks13 TE activity. Only 8 out of 924 compounds, showed a fluorescence signal below the threshold of the sample mean [9], deviating a $n\sigma_x$ from the sample $\bar{x}$ (See Figure 6). This compounds also gave a 100% inhibition when

calculated, and thus with their location with respect to the sample mean and controls, and %Inhibition results, these were considered possible active compounds, or hits.

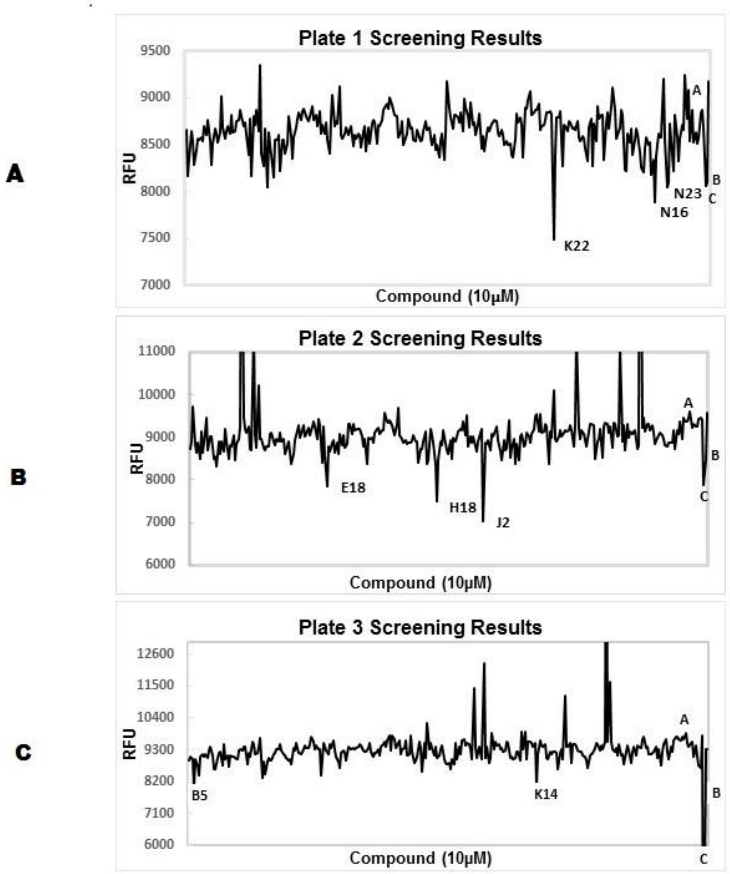


Figure 6. Screening Results. Hits at 10 μM after 2 hrs of incubation with 100% inhibition. A-C are controls: A is the Pks13 TE domain with MUH positive control, B is the Pks13 TE domain with a potent IMTB-28 compound, and C is the MUH only negative control(A) Portrays the results in plate 1 where three possible active inhibitors (K22, N16, N23), or hits, were identified as they are below the sample mean along with the antagonist MUH-only. (B) shows E18, H18, and J2 and (C) shows only B5 and K14. Even though Normal populations statistics are used here to portray the character of these molecules, % inhibition was used for assessment instead of Z-factor as this population is not a random population, but a biased population taken from the random population.

The structures of these compounds cannot be disclosed, but these are molecules with low MW, most have an amine as the central core with aromatics as R groups, with halogens such as Cl and S connected to these aromatics, with a structure similar to frontline TB drug Isoniazid, and are commercially available. However, even though these hits appear to be active against the Pks13 TE domain, an overall screening data contains plenty of variability [9] as these compounds were tested only as singlet at 10 μM. The screening results graphs, however, allowed

the visualization of compounds deviating from the sample mean and allowed pinpointing them for further dose-response IC_{50} analysis. Therefore, the assay was successful in identifying compounds appearing to be active against the Pks13 TE domain.

CHAPTER IV

CONCLUSION

From the IMTB-28 series, two compounds, TAMU185 and TAMU196, were found to be potent Pks13 TE inhibitors with IC_{50} values less than 1.5 μM. They were also found to be potent Mtb killing compounds with MIC_{50} in single and multiple carbon source , with values less than 2.3 μM, although target specificity that allows for their killing activity within the Mtb cell needs to be further determined as they were also active against IMTB-28 resistant Mtb $Mc^{2}7000$ cells. TAMU196 proved to be a safer potent inhibitor for antitubercular drug discovery as it is not cytotoxic against human cells in concentrations lower than 100 μM. Multiple commercially available hits were also found to be active against the Pks13 TE domain. Overall, this study successfully identified Pks13 TE domain inhibitors with 100% inhibition, and potent Pks13 TE domain inhibitors within the IMTB-28 analog series, which can be used as scaffolds for structural modification in the path to becoming a novel antitubercular drug.

REFERENCES

[1] *Center for Disease Control and Prevention*. http://www.cdc.gov/tb/topic/drtb/ (Accessed December 17, 2015).

[2] *World Health Organization*. http://www.who.int/tb/areas-ofwork/drug-resistant-tb/en/ (Accessed March 24, 2016).

[3] Daffé, M., Crick, D. C. & Jackson, M. Genetics of Capsular Polysaccharides and Cell Envelope (Glyco)lipids. *Microbiology Spectrum* **2**, (2014).

[4] Marrakchi, H., Lanéelle, M.-A. & Daffé, M. Mycolic Acids: Structures, Biosynthesis, and Beyond. *Chemistry & Biology* **21**, 67–85 (2014).

[5] Ioerger, T. R. *et al.* Identification of New Drug Targets and Resistance Mechanisms in Mycobacterium tuberculosis. *PLoS ONE* **8**, (2013).

[6] Lazareno, S. & Birdsall, N. Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoíf equations. *British Journal of Pharmacology* **109**, 1110–1119 (1993).

[7] Schmidt, L. The MIC50/MIC90: assessments of in vitro activities of antimicrobial agents that facilitate comparative agent-agent and agent-species susceptibility comparisons. *Antimicrobic Newsletter* **4**, 1–8 (1987).

[8] Takii, T. *et al.* Simple Fibroblast-Based Assay for Screening of New Antimicrobial Drugs against Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy* **46**, 2533–2539 (2002).

[9] Zhang, J.-H. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of Biomolecular Screening* **4**, 67–73 (1999).