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Testing of Indazole Inhibitors of KasA, a Vital Enzyme of M. tuberculosis

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Testing of Indazole Inhibitors of KasA,

a Vital Enzyme of *M. tuberculosis*

By:

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Submitted in partial fulfillment of the requirements for Honors in Biology at the University of Mary Washington.

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This Thesis by Karissa Highlander is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

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Biography

Karissa Nicole Highlander was born November 4th, 2000, in Bozeman, Montana. She attended the University of Mary Washington (UMW) in Fredericksburg, Virginia from the fall 2019 until the spring 2023. While at UMW, she obtained a bachelor's degree of science majoring in biomedical sciences and minoring in chemistry. Throughout her undergraduate years, she earned placement on the Dean's List by achieving a grade point average of 3.5 or higher. She was a member of the university's Honors Program and a part of the second cohort of Jepson Scholars. Along with participation in those programs, she was also involved in Chi Beta Phi, a scientific honorary for undergraduates, and Chi Alpha Sigma, an athletic honorary recognizing her accomplishments as a student-athlete on the women's basketball team.

Acknowledgements

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I would also like to acknowledge the research efforts of Lindsey Jones and Alexander Priest, whose previous projects provided inspiration and guidance throughout the course of this research project.

Through the academic support of my research advisors, financial support of the NSF and the UMW Undergraduate Research Grant (UGR), and emotional support of my family, I have been able to accomplish two summers and two semesters worth of research during my academic career at the University of Mary Washington. Without their continuous support, this presentation and my presentation at an American Society of Microbiology (ASM) Virginia branch meeting would not have been possible.

Abstract

Tuberculosis is a disease that affects the lungs caused by *Mycobacterium tuberculosis (M. tuberculosis)*. Although drug treatment options exist, increased rates of antibiotic resistant strains have become more prevalent in recent years, driving a need for new treatment approaches. KasA, a β-ketoacyl synthase, has been found to synthesize parts of the cell wall and been identified as an attractive drug target. Previous medicinal chemistry research has been completed to synthesize six effective competitive inhibitors of KasA that would potentially block the enzyme from binding the substrate, preventing elongation of the backbone and creation of the mycolic fatty acids that form the mycobacterial cell wall, ultimately killing the bacterium. With the sulfonamide and amine derivatives fully synthesized, theses were tested by means of the microdilution broth panel method using 96-well and 24-well titration plates, as well as through SPOTi assays to determine their effectiveness as potential drug candidates. Due to *M. tuberculosis* being highly contagious and infectious upon contact, the surrogate model *Mycobacterium aurum (M. aurum)* was used since it has the same target enzyme as in *M. tuberculosis*. Based on the high strength of the ligand-receptor binding energy values obtained from AutoDock Tools, a molecular binding simulation software, it was concluded that the six derivatives were suitable candidates for growth inhibition. Despite complications with the microdilution broth panel with the 96-well and 24-well titration plates, there is partial evidence through the SPOTi assay to support that the N-1-methyl-6-indazolyl benzene sulfonamide derivative potentially reduces mycobacterial growth on the plates containing *M. aurum.*

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Statement of Problem

A medicinal chemistry experiment was performed looking more into the structure of KasA. KasA is an enzyme that has been identified as an attractive drug target in *Mycobacterium tuberculosis (M. tuberculosis).* From previous research, DG167 has been found to be an inhibitor of KasA and has been used as a scaffold model for the disease (Kumar 2018). Utilizing a molecular binding simulation software, AutoDock Vina, it was determined that DG167 could block normal binding in KasA.

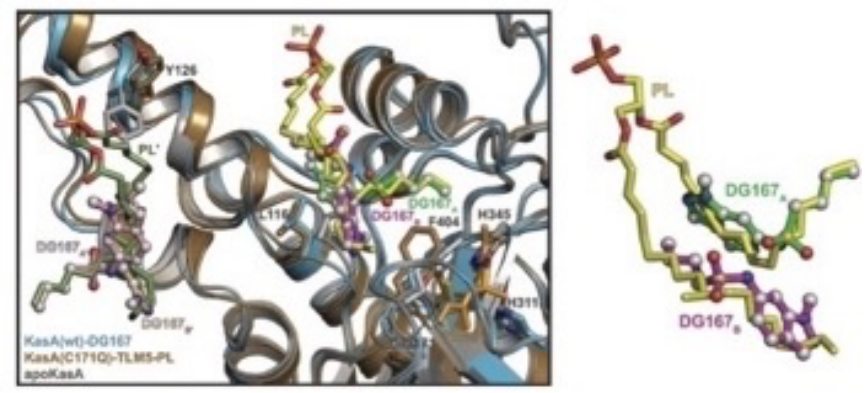


Figure 1. The image on the left shows how DG167 blocks binding in KasA. The image on the right shows the structure of DG167 (shown in green and purple) superimposed on a lipid shown in yellow. Obtained from Kumar 2018.

The competitive inhibition of KasA, in which a drug binds to the enzyme preventing its normal function, is known as substrate gating. The substrate gating for this enzyme is suspected to inhibit the normal function of the KasA enzyme by preventing the development of the fatty acid chains that play a role in forming the cell wall, leading to the inhibition of tuberculosis growth.

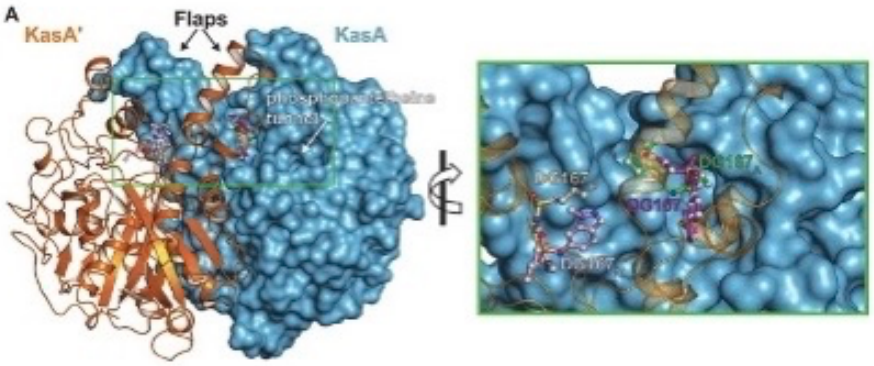


Figure 2. Shows the docking interaction between the inhibitor, DG167, and the binding site for the substrates in KasA. Obtained from Kumar 2018.

Applying this information, experimental research was conducted in which various drug derivatives were synthesized in efforts to find an alternative drug treatment option that would act similarly to DG167 by blocking binding in KasA.

Using the findings from the previous experiment, the purpose of this research was to investigate the effects of mycobacterial growth in the presence of the various drug derivatives in hopes of discovering novel and effective treatment options for the antibiotic resistant strains of the disease that are becoming increasingly prevalent. *M. aurum* on plates treated with the newly synthesized drug derivatives is expected to have reduced mycobacterial growth rates similar to what has previously been observed on plates treated with the current antibiotic treatment options, isoniazid or rifampin. Previous studies have been conducted using drug derivatives with different substituent groups and alkyl chains than will be used in this research. The goal of this research is to reduce mycobacterial growth in the presence of newly synthesized drug derivatives that act similarly to DG167 and other derivatives used in previous studies, but that could potentially be more effective in the antibiotic resistant strains of TB.

Introduction

Tuberculosis (TB) is a disease affecting the lungs, caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), and is the second leading infectious killer falling right after COVID-19 (World Health Organization 2021). Although drug treatment options exist, there have been increased rates of antibiotic resistant strains due to the bacteria becoming more immune to the current TB treatment options driving a need for newer treatment approaches (Namouchi et al. 2017). KasA, a β-ketoacyl synthase, has been found to synthesize parts of the bacterial cell wall and has been identified as an attractive drug target (Bhatt 2005). An effective competitive inhibitor of KasA would block the enzyme from binding the substrate, preventing elongation of the backbone and creation of the mycolic fatty acids that form the mycobacterial cell wall, ultimately killing the bacterium (Bhatt 2005). In this investigation, various indazole inhibitors of KasA that I synthesized will be tested as a way to assess mycobacterial growth in order to provide evidence of potential novel treatment options for antibiotic resistant strains of TB.

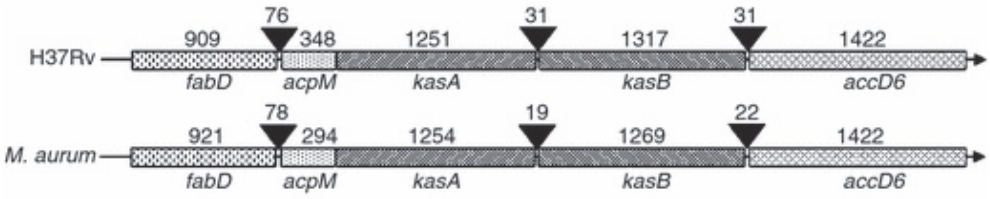
M. tuberculosis requires containment in a fully equipped biosafety level 3 (BSL-3) laboratory due to being highly contagious and infectious upon contact; however, these laboratories are scarce due to the financial responsibilities that arise with the necessary maintenance for protection of employees (van Soolingen et al. 2014). For this reason, a surrogate model with the same target enzyme in *M. tuberculosis* is needed in order to perform the experimental conditions in a safe manner. *Mycobacterium aurum* (*M. aurum*) can be handled in an undergraduate biosafety level 2 (BSL-2) laboratory and has shown a comparable resistance profile to that of *M.*

tuberculosis for several anti-TB drugs, revealing it to be a feasible surrogate (Namouchi et al. 2017).

M. aurum is an environmental, non-pathogenic strain of the mycobacterial family that has been discovered to grow in the gut of humans as part of their microbiota (Namouchi et al. 2017). In the study conducted by Namouchi and colleagues, the first complete genome sequence of *M. aurum* was reported, revealing a lot of information regarding its phenotypic and genomic comparisons to *M. tuberculosis*. It was determined that the minimum inhibitory concentration (MIC), which is the lowest concentration of a chemical that can prevent visible growth of a bacterium, was very similar between *M. aurum* and *M. tuberculosis* for half of the antibiotics tested (Namouchi et al. 2017). In addition to having similar MIC profiles, it was determined that both bacteria have similarities in over 80% of their genomes sharing around 2300 orthologous genes and around 50 identical genes (Namouchi et al. 2017). Not only do they share genes, but the complete genome alignment of *M. aurum,* reported by Namouchi and colleagues, also specifically reports having the enzyme KasA. Having this enzyme is a critical piece for this investigation because KasA is essential for mycobacterial growth and as a result has been identified as an attractive drug target (Bhatt 2005). All of this information provides substantial evidence for *M. aurum* being a reliable surrogate, since the organism's basic genetic information is relatively similar, as well as contains the target enzyme.

KasA is known to impact the fatty acid synthase type II pathway (FAS-II) because it assists in the elongation of the fatty acids which contributes to mycobacterial growth (Gupta 2008). However, inhibitors of KasA prevent elongation of the mycolic fatty acids that form the

mycobacterial cell wall (Bhatt 2005). For this investigation, the attractive drug target will be KasA. Inhibiting KasA will prevent the bacterium from initiating its elongation pathways in an effort to stop its overall growth, ultimately killing the bacterium.



	fabD		IGR	*acpM*		IGR	*kasA*		IGR	*kasB*		IGR	*accD6*	
	ntds	aa	ntds	ntds	aa	ntds	ntds	aa	ntds	ntds	aa	ntds	ntds	aa
H37Rv	909	302	76	348	115	overlap	1251	416	31	1317	438	31	1422	473
M. aurum	921	306	78	294	97	overlap	1254	417	19	1269	422	22	1422	473
% similarity	78	82		93	95		79	86		77	85		83	91

Figure 3. The organization of the Kas operon gene in *M. tuberculosis* (H37Rv) and *M. aurum*. Here it can be observed that the KasA gene is located within a five-gene operon in both bacteria at approximately the same spot. The comparative profile between the two reveals comparable organizational patterns that can be observed by the similar nucleotide number above each gene. Obtained from Gupta 2008.

As seen in figure 3, the Kas operon gene in *M. tuberculosis* and *M. aurum* are organized similarly where the promoter upregulates the reporter gene expression only once acted upon by treatment of a drug that inhibits the FAS-II pathway (Gupta 2008). Focusing on drug derivatives that will decrease activity of the FAS-II pathway seems to be the most effective method of testing since they will yield the lowest growth concentrations.

In previous research, isoniazid (INH), rifampin (RIF), and other structural derivatives of antitubercular drugs have been tested to observe whole cell growth patterns of *M. aurum* (Ambrożkiewicz et al. 2020). Isoniazid and rifampin are believed to be antibiotics that target KasA and are used to prevent the elongation of the mycolic fatty acids (Ambrożkiewicz et al. 2020). The study performed by Ambrożkiewicz and colleagues (2020) revealed that isoniazid

and rifampin are in fact successful antibiotic drugs in *M. aurum*, providing evidence that having a successful inhibitor of KasA can reduce mycobacterial growth and act as a treatment option. Using this information, combinations of these known antibiotic drugs with various indazole derivatives synthesized in a previous medicinal chemistry experiment will be used to observe growth patterns and to potentially find new treatment approaches.

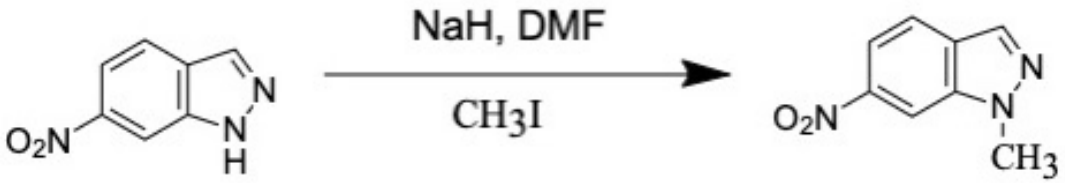
As tuberculosis continues to impact lives around the world, the bacteria are becoming more biologically immune to the treatments currently available to combat the disease (Namouchi et al. 2017). Because of this, the existing treatment options are no longer as effective as they once were. As a result of their decreased efficiency, there has been an increased need for further research into novel treatment options. The scientific community has been working together to discover different possibilities of antibiotics that will be as successful as the current drugs, be cost efficient, and produce limited side effects in humans. All of this work has been implemented in order to find a successful avenue to cure this disease before it turns into an even greater concern. Overall, the need for this research stems from the desire to save as many lives as possible of people who are affected by tuberculosis worldwide.

Experimental Design

Derivative Synthesis

During the summer of 2021, a chemical experiment, under the mentorship of Dr. Davis Oldham, was performed in which I converted the compound 6-nitroindazole into three sulfonamide and three amine derivatives. The procedure for this was adapted from a section, entitled "3.3 General method for alkylation of 7-nitroindazole derivatives," of a report published in 2005 by Bouissane

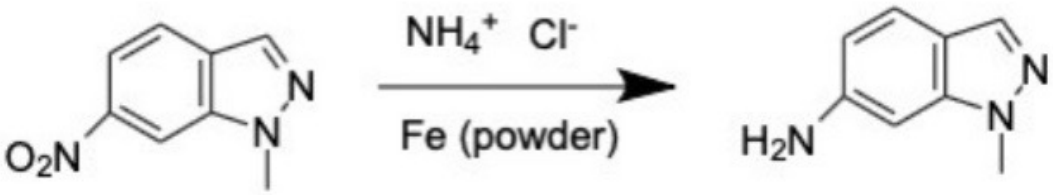
and colleagues (Bouissane 2005). To begin, 6-nitroindazole underwent an alkylation reaction, which methylated a nitrogen forming the compound 1-methyl-6-nitroindazole. In order to carry out this reaction, 2.2 g of sodium hydride (NaH) combined with 50 mL of dimethylformamide (DMF) was first added to a round bottom flask at 0° C. From there, 3.00 g of 6-nitroindazole was slowly added in the DMF solution and stirred at 0° C for two hours until being completely dissolved. Next, 3.44 mL of iodomethane (CH_3I) was added dropwise to the solution. After being stirred at room temperature for 18 hours, the solution was diluted with water and extracted with ethyl acetate (EtOAc). Then, the organic layer was washed with 25 mL of brine and dried over Celite. The ethyl acetate was removed by rotary evaporation. Finally, the solution was purified by means of column chromatography producing the 1-methyl-6-nitroindazole compound to be used in the next reaction.



Scheme 1. Alkylation reaction converting 6-nitroindazole into 1-methyl-6-nitroindazole by methylating one of the nitrogen atoms.

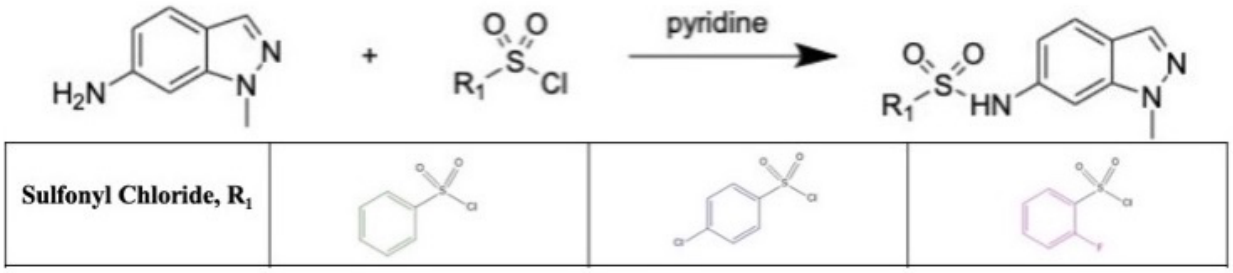
Once the 1-methyl-6-nitroindazole was purified, it was reduced using iron powder converting the original nitro group into an amino group forming the compound 1-methyl-6-aminoindazole. The procedure for this was adapted from a section on page 2348, entitled "1-(2,2-Dimethoxy-ethyl)-1H-indazol-5-yl amine," of a report published by Kym and colleagues (Kym 2006). In order to carry out this reaction, 1.15 g of 1-methyl-6-nitroindazole from the previous reaction was combined with 0.174 g of ammonium chloride (Na_4Cl) and 57.4 mL of 80% ethanol (EtOH) into a round bottom flask. Then, 3.575 g of iron (Fe) powder was added to the solution and slowly heated to 85-90° C to reflux. The reaction was stirred for an hour and then cooled to room

temperature. Next, the solvents in the solution were removed by rotary evaporation. Finally, the residue was taken up in a 10:1 ethyl acetate (EtOAc):triethylamine (Et_3N) mixture and filtered through column chromatography producing the 1-methyl-6-aminoindazole compound to be used in the next reactions.



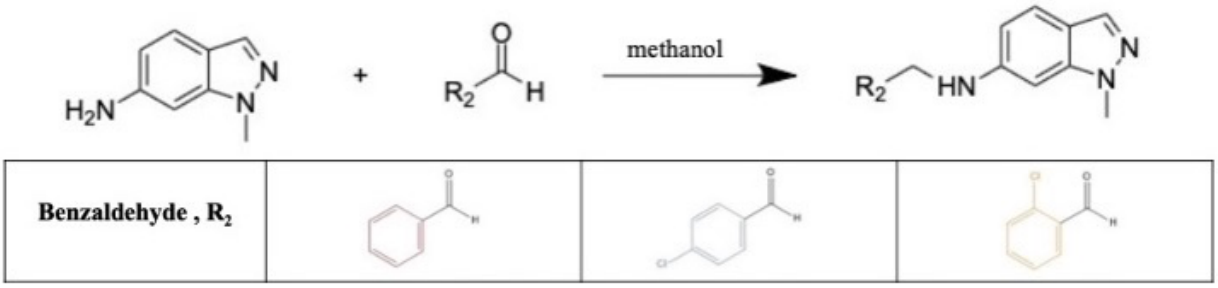
Scheme 2. Reduction reaction converting 1-methyl-6-nitroindazole into 1-methyl-6-aminoindazole by using an iron powder to change the original nitro group into an amino group.

After the 1-methyl-6-aminoindazole was formed, two additional reactions took place to synthesize the various derivatives. One reaction was the synthesis of sulfonamides where the 1-methyl-6-aminoindazole was reacted with various sulfonyl chlorides to produce the sulfonamide derivatives. The procedure for this was adapted from a section, entitled "3.4 Synthesis of compounds 2(a-d) and 3(a-d) *General method*" of a report published in 2005 by Bouissane and colleagues (Bouissane 2005). This reaction was carried out by following the same procedure using one of three various sulfonyl chlorides including benzene sulfonyl chloride, 4-chlorobenzene sulfonyl chloride, and 2-fluorobenzene sulfonyl chloride. To begin, 1.10 mmol of one of the sulfonyl chlorides was added to a solution of 0.147 g of the 1-methyl-6-aminoindazole from the previous reaction and 6.35 mL of pyridine to a round bottom flask. After the solution was stirred at room temperature for 16 hours, it was diluted with EtOAc then the organic layer was washed and extracted with sodium bicarbonate ($NaHCO_3$) followed by water and brine. The organic layer was dried with magnesium sulfate ($MgSO_4$), filtered, and then evaporated via rotary evaporator. Finally, the product was purified by means of column chromatography to produce the three sulfonamide derivatives.



Scheme 3. The synthesis of the three sulfonamide derivatives. The top section shows the reaction in which the 1-methyl-6-aminoindazole was reacted with the sulfonyl chlorides to produce the derivatives. Underneath the reaction is a table that reveals the specific sulfonyl chlorides that were used with the various R_1 functional groups attached.

The other reaction was the reductive animation of amines where the 1-methyl-6-aminoindazole was reacted with various benzaldehydes to produce the amine derivatives. The procedure for this was adapted from a section, entitled "Synthesis of N-Substituted Anilines," of a report published in 2008 by Chakrabarty and colleagues (Chakrabarty 2008). This reaction was carried out by following the same procedure using one of three various benzaldehydes including benzaldehyde, 4-chlorobenzaldehyde, and 2-chlorobenzaldehyde. To begin, 0.147 g of 1-methyl-6-aminoindazole was added to a solution of 1.05 mmol of one of the benzaldehydes and 1 mL of methanol (MeOH) to a round bottom flask. Three drops of acetic acid were then added to the solution at room temperature and stirred for three hours. After at least a 95% conversion to imine, 0.0378 g of sodium borohydride ($NaBH_4$) was added in small portions to the solution and left to sit for an hour to allow for gas evolution to cease. Then, the solvents in the solution were evaporated via rotary evaporator. The solution was then washed with a 25:10 mL of EtOAc:deionized water (ddH_2O) mixture and the aqueous layer was extracted with 25 mL of EtOAc. The organic layer was washed with 25 mL of brine, dried with $MgSO_4$, and then evaporated via rotary evaporator. Finally, the product was purified by means of column chromatography to produce the three amine derivatives.



Scheme 4. The reductive animation of the three amine derivatives. The top section shows the reaction in which the 1-methyl-6-aminoindazole was reacted with the benzaldehydes to produce the derivatives. Underneath the reaction is a table that reveals the specific benzaldehydes that were used with the various R_2 functional groups.

AutoDock Tools, an automated docking computer program, was then used to test the interactions between the inhibitor and each derivative in order to predict suitability of the drug candidate. With the six derivatives fully synthesized, they will eventually be tested to determine their effectiveness as potential drug candidates.

Microdilution Broth Panel with 96-Well Titration Plates

During the fall 2022 and spring 2023 semesters, a biological experiment, under the mentorship of Dr. Lynn Lewis, was conducted in which I utilized the previously synthesized derivatives to test for mycobacteria growth inhibition. All of the solutions, including the previous antibiotics and the various drug derivatives, utilized in this section of the procedure were prepared in advance and used throughout the experiment. To begin, an antibiotic stock solution was prepared for each of the known antibiotics and various drug derivatives. This solution was made by dissolving 300 µL of the antibiotic or derivative into 2 mL of dimethyl sulfoxide (DMSO). A serial dilution was performed utilizing the original stock solution from which 10 µL of the stock was added into a microcentrifuge tube with 990 µL of DMSO. From the previous solution, 10 µL was taken and added to a different microcentrifuge tube with 990 µL of phosphate buffered saline (PBS)

producing the working solution. When all antibiotic and derivative solutions were prepared, 50 µL of their working solutions were transferred into the corresponding wells on the titration plate.

In order to determine the amount of mycobacterial growth in the presence of each derivative, the microdilution broth panel method using 96-well titration plates was applied. The procedure for this was adapted from a section, entitled "3.4.1. Antimycobacterial In Vitro Activity Screening Against *Mycobacterium tuberculosis H37Ra*, *Mycobacterium smegmatis*, and *Mycobacterium aurum*," of a report published by Ambrożkiewicz and colleagues (Ambrożkiewicz 2020). The culturing medium was Middlebrook 7H9 (M7H9) broth. For growth of the bacterium, a culture of *M. aurum* (ATCC 23366) in the M7H9 broth was incubated at 37° C for a week prior to setup. Into the titration plates, 200 µL of deionized water was added to the perimeter wells to minimize the evaporation of the M7H9 during incubation. In the 2nd through 11th columns of rows B through G, 50 µL of M7H9 broth was added. The 10th column of row B through G was utilized as the negative control, containing no *M. aurum* and no drugs. The 11th column of row B through G was utilized as the positive control, containing *M. aurum* but no drugs. In the 2nd column of rows B and C, 50 µL of the isoniazid and rifampin was added, respectively. In the 2nd column of rows D through G, 50 µL of one of the sulfonamide or amine drug derivatives was added. To perform the dilution, 50 µL from column 2 was transferred to column 3 from rows B though G creating a 1:2 serial dilution. The transferring of 50 µL from well to well was continued until reaching the 9th column. At that point, 50 µL was discarded from the 9th column of rows B through G in order to maintain consistent volumes in each well. From there, 50 µL of *M. aurum* was added to the 2nd through 9th and the 11th columns of rows B through G.

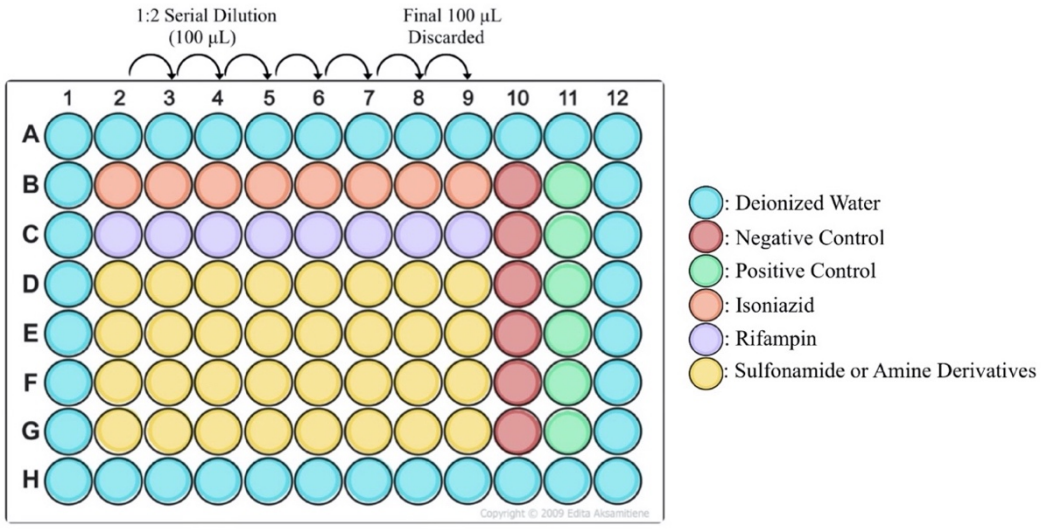


Figure 4. 96-well titration plate template for experimental design. Perimeter wells contain 200 µL of deionized water. Columns 2 though 9 of rows B through G contain 50 µL of M7H9 and 50 µL of *M. aurum.* In addition, columns 2 through 9 of rows B and C contain 50 µL previous antibiotics and rows D through G contain 50 µL of the various drug derivatives. Negative control wells contain 100 µL of M7H9 only. Positive control wells contain 50 µL of M7H9 and 50 µL of *M. aurum*.

After the set-up of the microtitration plates, they were incubated at 37° C for five to seven days. The plates were then placed into a plate reader to determine the MIC and to answer the question of whether or not the drug derivatives resulted in reduced mycobacterial growth.

Microdilution Broth Panel with 24-Well Titration Plates

In attempts to reduce evaporation within the wells during the period of incubation, larger volumes of the culturing medium, *M. aurum,* previous antibiotics, and various drug derivatives were used in a 24-well microtitration plate. Like with the 96-well microtitration plate setup, there was a perimeter of 400 µL of deionized water surrounding the wells containing the liquids to minimize evaporation during the period of incubation. The culturing medium will remain as M7H9 broth. The 4th column in row B was utilized as the negative control well, which contained 400 µL of M7H9 only. The 5th column of row B was utilized as the positive control well, which contained 200 µL of M7H9 and 200 µL of *M. aurum.* The 2nd column of row B contained 200 µL

of isoniazid, 200 µL of M7H9, and 100 µL of *M. aurum*. The 3rd column of row B of contained 200 µL rifampin, 200 µL of M7H9, and 100 µL of *M. aurum.* In row C, 200 µL of one of the various drug derivatives, 200 µL of M7H9, and 200 µL of *M. aurum* were added. Unlike with the 96-well titration plate setup, there is no 1:2 serial dilution because the wells in row C each contained a different drug derivative.

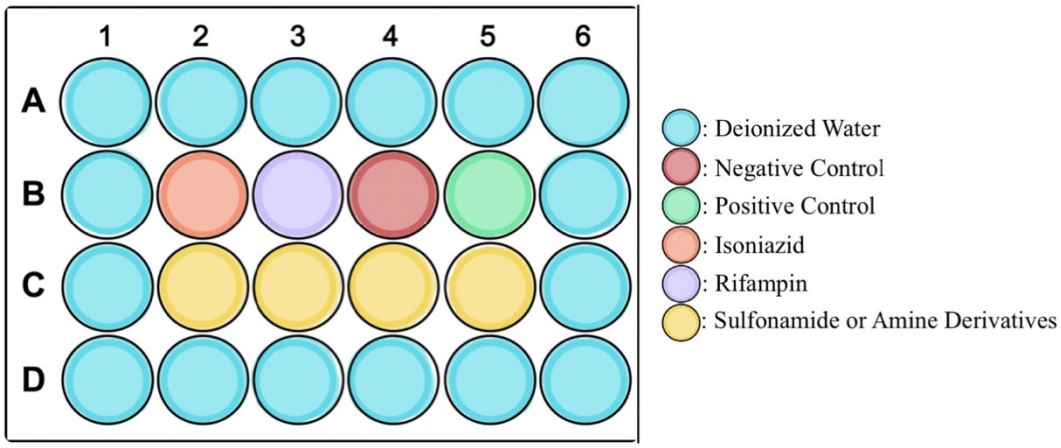


Figure 5. 24-well titration plate template for experimental design. Perimeter wells contain 400 µL of deionized water. Columns 2 and 3 of row B contain 200 µL of the previous antibiotics (isoniazid and rifampin, respectfully), 200 µL of M7H9, and 200 µL of *M. aurum.* Columns 2 through 5 of row C contain 200 µL of one of the various drug derivatives, 200 µL of M7H9, and 200 µL of *M. aurum.* Negative control wells contain 400 µL of M7H9 only. Positive control wells contain 200 µL of M7H9 and 200 µL of *M. aurum.*

After the setup of the microtitration plates, they were incubated at 37° C for five to seven days. The plates were then placed into a plate reader to determine the MIC and to answer the question of whether or not the drug derivatives resulted in reduced mycobacterial growth.

SPOTi Assay

As a method to visualize the effects of the various drug derivatives and determine the MIC, a spot culture growth inhibition assay (SPOTi assay) was conducted. The procedure for this was adapted from a report published in 2010 by Evangelopoulos and Bhakta (citation) . To begin, AD supplement was freshly made for this method utilizing 2.0 g of dextrose, 0.85 g of sodium

chloride (NaCl), 5.0 g of albumin, and 95.0 mL of ddH_2O. The albumin, NaCl, ddH_2O were added to a beaker then stirred until completely dissolved. Then, the dextrose was added and stirred until completely dissolved. Once all components were added, the solution was filter sterilized and then stored at 4° C until ready for use in the M7H9 agar.

Next, the M7H9 agar was made utilizing 1.9 g of M7H9 agar, 1.25 mL of glycerol, 88.75 mL of ddH_2O, 10 mL of the AD supplement, 0.1 mL of previously prepared CB stock, and 0.1 mL of previously prepared CHX stock. The M7H9 agar was first mixed with the ddH_2O. Then, the glycerol was added, mixed thoroughly, and the solution was heated for 1 minute while stirring continued. After, the solution was autoclaved at 121° C for 15 minutes then removed and cooled to 55° C in a water bath. Once at the appropriate temperature, the AD supplement was added followed by the CB and CHX stocks then mixed thoroughly. The final step included pouring the agar into the 6-well titration plates and stored at 4° C. (Note, this procedure made 0.1 L of M7H9 agar and prepared six 6-well titration plates.)

After agar has set, the SPOTi assay was conducted. The 1^{st} and 4^{th} wells contained 100 µL of *M. aurum* only. The 2^{nd} and 3^{rd} wells contained 100 µL of *M. aurum* and 100 µL of isoniazid and rifampin, respectfully. Finally, the 5^{th} and 6^{th} wells contained 100 µL of *M. aurum* and 100 µL of one of the various drug derivatives.

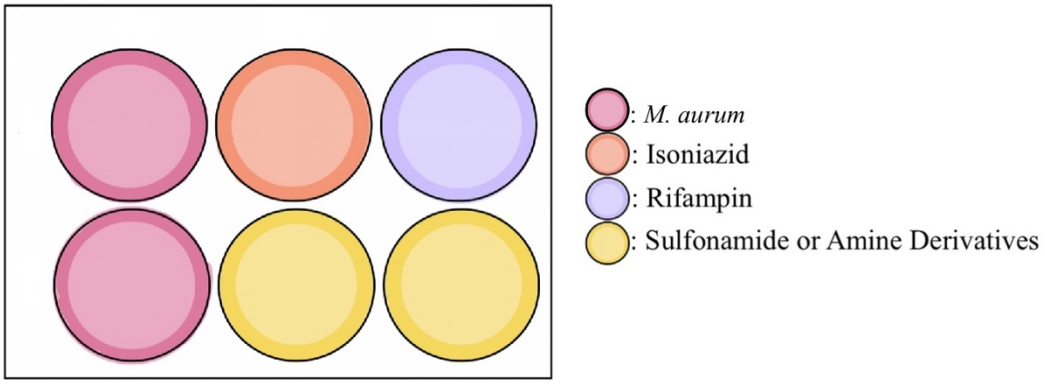


Figure 6. SPOTi assay template for experimental design. In wells 1 and 4, 100 µL of *M. aurum* only was added. In wells 2 and 3, 100 µL of *M. aurum* and 100 µL of isonazid and rifampin, respectfully, were added. Finally, in wells 5 and 6, 100 µL of *M. aurum* and 100 µL of one of the various drug derivatives were added.

The plate was then incubated at 37° C for five to seven days. Once the incubation period was compete, the plates were observed for mycobacterial growth to determine the MIC. The number of colonies in the wells with the *M. aurum* only will be compared to the number of colonies in the wells with the previous antibiotics and various drug derivatives in order to determine whether or not the drug derivatives resulted in reduced mycobacterial growth.

Results and Analysis

Derivative Synthesis

From the alkylation reaction that converted 6-nitroindazole to 1-methyl-6-indazole through the methylation of a nitrogen atom, a mixture of products resulted from which the major product was produced in 49% yield. After the purification of 1-methyl-6-nitroindazole, the compound was reduced using iron powder converting the original nitro group into an amino group forming the compound 1-methyl-6-nitroindazolamine in 85% yield. Utilizing the reduced compound, two reactions were performed to synthesize the final drug derivatives. The first reaction was the synthesis of sulfonamides, and the second reaction was the reductive animation of amines. From

the two reactions, six products were produced with range between 36% and 84% yield amongst all derivates.

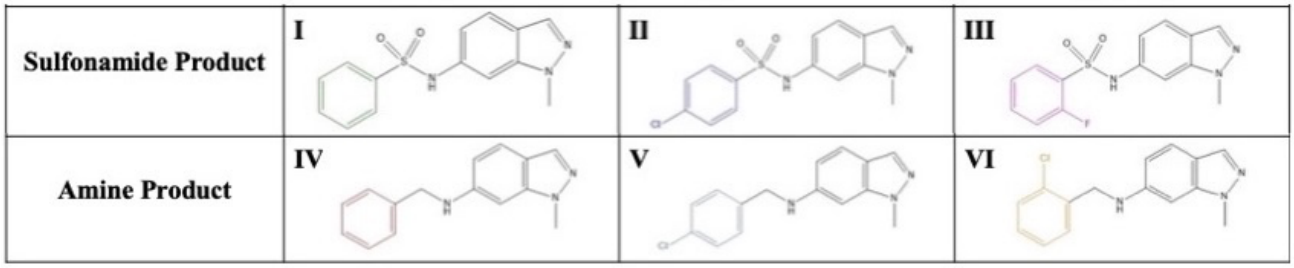


Figure 7. The six potential drugs that resulted from the derivative synthesis reactions. The top row reveals the sulfonamide products that resulted from the synthesis reaction with the various sulfonyl chlorides. I is N-1-methyl-6-indazolyl benzene sulfonamide. II is N-1-methyl-6-indazolyl 4-chlorobenzene sulfonamide. III is N-1-methyl-6-indazolyl 2-fluorobenzene sulfonamide. The bottom row reveals the amine products that resulted from the reductive amination with the various benzaldehydes. IV is N-benzyl-1-methyl-6-indazolamine. V is N-4-chlorobenzyl-1-methyl-6-indazolamine. VI is N-2-chlorobenzyl-1-methyl-6-indazolamine.

Each of the derivatives was run on AutoDock Tools to determine their potential effectiveness as a drug candidate. The results from AutoDock Tools revealed capability of strong binding affinity between the ligand and receptor for each of the derivatives. Despite all six derivatives having strong binding affinities, only four out of the six derivatives were used in further experimentation. The four that were used are labeled I, II, III, and IV (figure 7). The reason for this occurring is because these four derivatives generated a large enough percent yield to be able to produce potential results in the subsequent steps. The other two derivatives, V and VI (figure 7), did not yield enough mass to be thought to warrant any viable results in the subsequent steps.

Microdilution Broth Panel with 96-Well Titration Plates

Despite multiple efforts with varying techniques, there are no results to report from the microdilution broth panel with the 96-well titration plates. The wells in a 96-well titration plate are small holding roughly around 300 µL of liquid. Because most of the wells required the

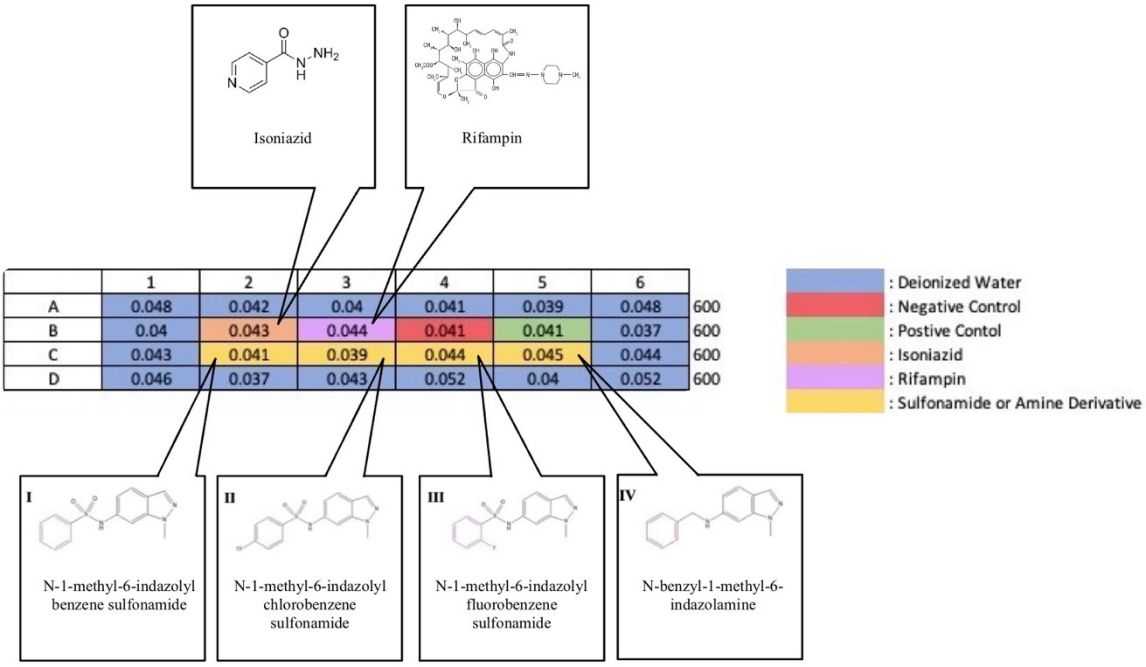
addition of three components – the M7H9 broth, *M. aurum*, and previous antibiotic or drug derivative – there was a limited amount of each that was able to be effectively added into the wells without overflowing. As mentioned previously, the bacterium requires incubation for five to seven days making it a fairly slow process. Such little volume inside of each well in correspondence with an extended amount of time being incubated to allow time for the *M. aurum* to grow led to nearly complete evaporation of all liquids during the incubation time.

In order to try to eliminate the amount of evaporation that occurred during the incubation several different techniques were employed. One technique was to add slightly more ddH_2O in the perimeter wells. The purpose of having water around the perimeter of the plate was to minimize evaporation, so there was hope that adding more than the original 200 μL would help to resolve the evaporation issue. Another technique was to add a tray of water to the bottom of the incubator and to cover the titration plate with another tray. The goal of this method was to generate a humid atmosphere that would prevent the sample from drying out. This would allow the *M. aurum* the sufficient amount of time to grow while not causing all the liquid in the wells to evaporate during the process. However, neither of these techniques proved to be beneficial because other trials with the 96-well titration plates had the same results as the first. A different method was needed that would utilize larger volumes of all of the liquids to minimize evaporation, so the procedure was adapted to fit into a 24-well titration plate.

Microdilution Broth Panel with 24-Well Titration Plates

The 24-well titration plates hold approximately four times as much liquid as the 96-well titration plates can. A similar procedure was followed for this method with two major differences: there

was four times as much liquid in the wells and there were fewer wells. The advantage of having more liquid in the wells was that there would not be complete evaporation after the duration of incubation. This means that at the end of the incubation period there would still be enough liquid in the wells that could be run through a plate reader to determine the concentration inside each of the wells. After the plate was read, the question of whether or not the drug derivatives reduced mycobacterial growth could be answered by observing lower concentration in the wells containing the derivatives compared to the wells containing the *M. aurum*. The disadvantage of having fewer wells to work with is not many concentrations of the derivatives and known antibiotics could be tested. No serial dilution was performed, so it is not possible to find the minimum inhibitory concentration solely based off of these plates. The goal of this method was to determine which, if any, of the four derivatives reduced mycobacterial growth.



	1	2	3	4	5	6	
A	0.048	0.042	0.04	0.041	0.039	0.048	600
B	0.04	0.043	0.044	0.041	0.041	0.037	600
C	0.043	0.041	0.039	0.044	0.045	0.044	600
D	0.046	0.037	0.043	0.052	0.04	0.052	600

Figure 8. Results of the 24-well titration plate run on the plate reader at a wavelength of 600 nm. Similar absorbance readings are present in every well indicating there to be no evidence of mycobacterial growth. The perimeter wells of the plate all contain only ddH_2O. The well B2 contained the known antibiotic, isoniazid. The well B3 contained the known antibiotic, rifampin. The well B4 was the negative control which contained only M7H9. The well B5 was the positive control which contained only M7H9 and *M. aurum*. The wells in row C contain one of the four

derivatives. The well C2 contained N-1-methyl-6-indazolyl benzene sulfonamide. The well C3 contained N-1-methyl-6-indazolyl 4-chlorobenzene sulfonamide. The well C4 contained N-1-methyl-6-indazolyl 2-fluorobenzene sulfonamide. The well C5 contained N-benzyl-1-methyl-6-indazolamine.

As figure 8 shows, there are no significant results to report from the microdilution broth panel with 24-well titration plates. All wells appear to have similar absorbance readings indicating there to be no evidence of any mycobacterial growth. Because wells containing the known antibiotics did not show reduced mycobacterial growth as they should have and the wells containing the bacterium were similar in concentration as the wells containing just ddH_2O, it is suspected that this method was not reliable at this moment in time. It is unclear whether the results of this method were flawed due to inefficient binding to the target enzyme making the derivatives used unsuitable as potential drug candidates or if caused by other errors during the procedure. Due to the uncertainties regarding the reasoning behind these observations, a new method was proposed to optimize time and resources.

SPOTi Assay

In order to obtain a more direct visualization of the effects the drug derivatives have on the *M. aurum*, a SPOTi assay was conducted. Through this method the overall goal is that colonies of the bacterium will not grow on the M7H9 agar when exposed to any of the sulfonamide or amine derivative during the incubation period, however they will grow in the absence of them. Due to the limited number of wells, only one of the four derivatives was tested on a singular titration plate rather than testing multiple as the previous methods did.

The N-1-methyl-6-indazolyl benzene sulfonamide was the first derivative to be selected for testing. There were two main reasons behind this selection, the first being its structure and the

second being its abundance. As can be observed in figure 7, the N-1-methyl-6-indazolyl benzene sulfonamide has a fairly simple structure in comparison to some of the other derivative options. Unlike the other sulfonamide derivatives that have larger substituents with the chlorine and fluorine elements, the N-1-methyl-6-indazolyl benzene sulfonamide has a basic phenol ring with no additional elements attached as part of its substituent group. This structural difference may allow for the derivative to bind to the target enzyme more efficiently, resulting in a higher likelihood for reduced mycobacterial growth, which is partially the reason it was chosen to be the first derivative tested. The second reason was that during the derivative synthesis part of the experiment, the N-1-methyl-6-indazolyl benzene sulfonamide produced one of the higher percent yields. Having a larger amount of the derivative to work with lowered concerns for running out of or wasting any of the product during experimentation.

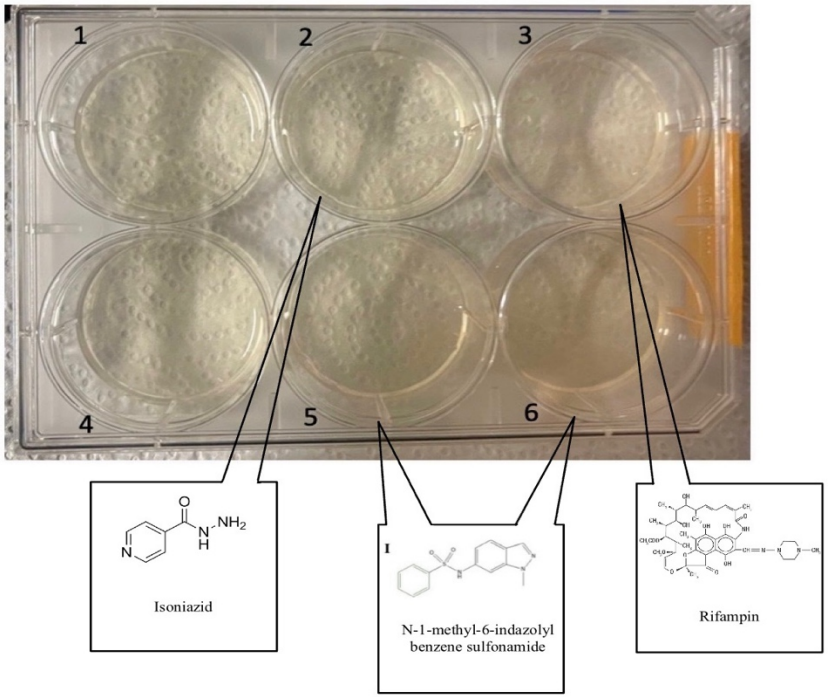


Figure 9. Results from the first SPOTi assay attempt. No colony growth can be observed on the plate after seven days of incubation at 37° C. Wells number 1 and 4 contain only 100 µL of *M. aurum*. Well number 2 contains 100 µL of *M. aurum* and 100 µL of isoniazid. Well number 3 contains 100 µL of *M. aurum* and 100 µL of rifampin. Wells number 5 and 6 contain100 µL of *M. aurum* and 100 µL of the N-1-methyl-6-indazolyl benzene sulfonamide derivative.

As seen in figure 9, no colony growth can be observed on the plate after seven days of incubation at 37° C for the first SPOTi assay attempt. The culture of *M. aurum* utilized on this plate was the same culture used during the previous method of the microdilution broth panel with 24-well titration plates. A combination of having no significant results after being read on the plate reader and having no growth in the wells that excluded any antibiotic or derivative raise concerns of an ineffective culture of the bacterium. For this reason, a new culture of *M. aurum* was cultivated to better the chances of getting bacterial growth for the next trial.

There were complications with growing the *M. aurum,* so the process was extended and took around two weeks instead of the anticipated one week. Once finally able to cultivate a workable culture, the same process as before was followed with N-1-methyl-6-indazolyl benzene sulfonamide being used as the derivative.

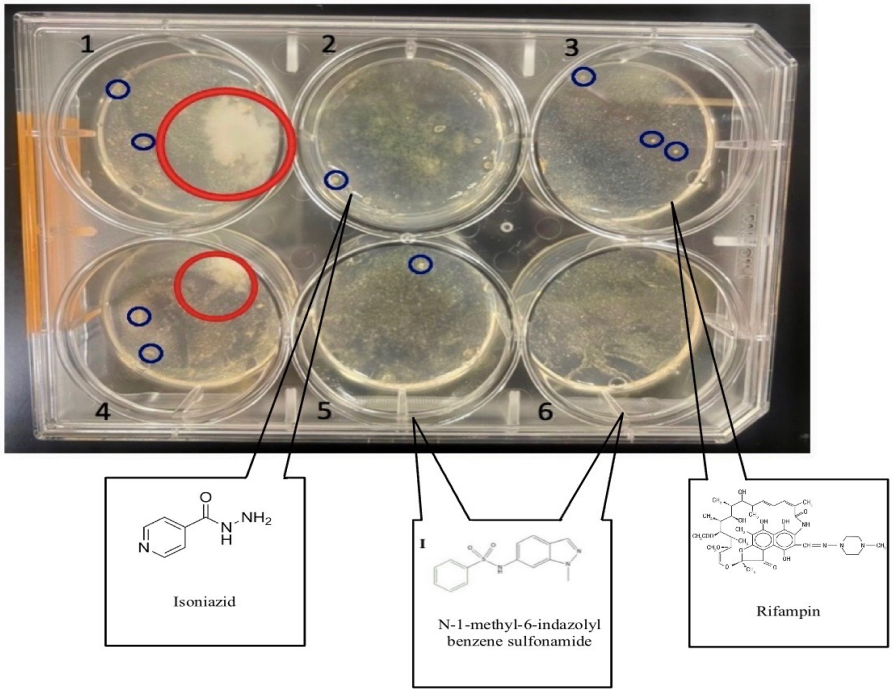


Figure 10. Results from the second SPOTi assay attempt. Colony growth can be observed on the plate after seven days of incubation at 37° C indicated by the blue circles, however there is

apparent contamination of the plate which can be observed within the red circles. Wells number 1 and 4 contain only 100 µL of *M. aurum*. Well number 2 contains 100 µL of *M. aurum* and 100 µL of isoniazid. Well number 3 contains 100 µL of *M. aurum* and 100 µL of rifampin. Wells number 5 and 6 contain 100 µL of *M. aurum* and 100 µL of the N-1-methyl-6-indazolyl benzene sulfonamide derivative.

Unlike the first attempt, the second SPOTi assay attempt produced results. Colony growth was observed on the plate after seven days of incubation at 37° C, which is indicated by the blue circles on figure 10. The colonies are very small in size, orange in color, and limited in number with less than five colonies in any of the wells. However, there is apparent contamination in wells 1 and 4 indicated by the red circles on figure 10. These two wells contain only the culture of *M. aurum* and no antibiotic or drug derivative. It is unclear why this contamination occurred, however, there is possibility that the complications during the original growth period may have caused it. Despite having colony growth inside the wells, the results are invalidated due to the contamination driving a need for a new culture of the bacterium to be cultivated and the process to be repeated.

For a second time during this part of the experiment, a new culture of *M. aurum* was required to continue on with the research process. Due to the possible contamination on the culture utilized in the second attempt, a new culture was cultivated in hopes of resolving the issue and preventing any further contamination during the next attempt. The process took the expected one week to grow in the incubation, so there was reason to believe the culture would produce better results than the second attempt. With the new culture of *M. aurum*, the same process as before was followed with N-1-methyl-6-indazolyl benzene sulfonamide being used as the derivative.

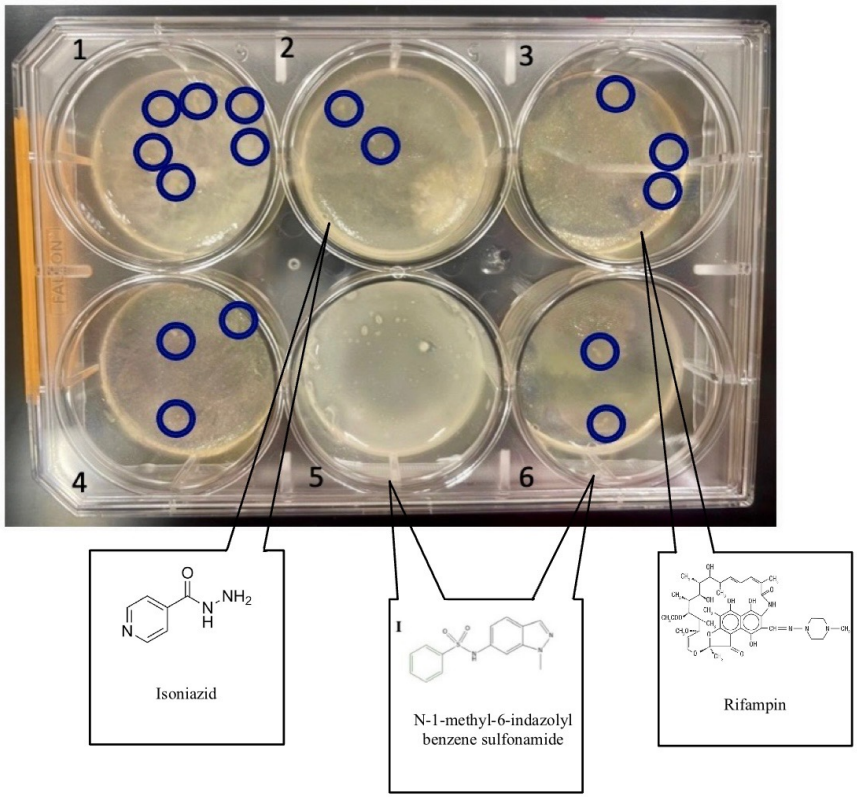


Figure 11. Results from the third SPOTi assay attempt. Due to the contamination on the second attempt, a new culture of the *M. aurum* was cultivated in hopes of resolving the issue. Although not clearly shown in the picture, colony growth can be observed on the plate after seven days of incubation at 37° C. Rather than circling every colony due to their size being very small, clusters of them are indicated by the blue circles. Wells number 1 and 4 contain only 100 µL of *M. aurum*. Well number 2 contains 100 µL of *M. aurum* and 100 µL of isoniazid. Well number 3 contains 100 µL of *M. aurum* and 100 µL of rifampin. Wells number 5 and 6 contain 100 µL of *M. aurum* and 100 µL of the N-1-methyl-6-indazolyl benzene sulfonamide derivative.

As seen in figure 11, there are significantly more results from the third SPOTi assay attempt. Clusters of colony growth can be observed on the plate after seven days of incubation at 37° C indicated by the blue circles. The colonies are very small in size, most around a millimeter in diameter, and very light orange in color. On the titration plate it appears that in the first well there are approximately 42 colonies, in the second well there are approximately 6 colonies, in the third well there are approximately 11 colonies, in the fourth well there are approximately 15 colonies, in the fifth well there are no visible colonies, and in the sixth well there are approximately 7 colonies of *M. aurum* that grew. The first well, containing only the bacterium,

had nearly four times the numbers of colonies in comparison to the other wells. Based on this data, it can be concluded that there is partial evidence for reduced mycobacterial growth in the presence of the known antibiotics and newly synthesized derivatives.

Although both wells containing the *M. aurum* showed signs of colony growth, there is clear indication that growth was significantly more obvious in the first well compared to the fourth well. This observation provides evidence that there are still some issues in the growing process because expectations would be that both wells would more closely resemble each other. Further experimentation would need to be conducted to determine if colony growth in the wells actually decreased in number as a result of the derivative binding to the target enzyme and inhibiting the elongation of the backbone and creation of the mycolic fatty acids or as a result of ineffective bacterial growth during the initial step. As for now, there is reason to believe that the N-1-methyl-6-indazolyl benzene sulfonamide drug derivative does show signs of potential reduced mycobacterial growth and should be experimented with further to determine for certainty and to establish its MIC.

Future Directions

For future work, there are three main objectives that would need to be accomplished. For starters, more research regarding *M. aurum* would need to be conducted in order to discover a more effective and efficient method of growing the bacterium. During the entirety of this experiment the *M. aurum* was grown in an incubator at 37° C for a week. However, it was proven through the results that there was not much successful growth under these conditions in the laboratory space utilized. It would be beneficial for the project as a whole to find an alternative method for

cultivating a culture of *M. aurum*. The main way an answer to the question of whether or not the drug derivatives can reduce mycobacterial growth is to have a viable culture of *M. aurum* that will allow for the drug derivatives to bind to the target enzyme and inhibit the elongation of the backbone and creation of the mycolic fatty acids that form the mycobacterial cell wall, ultimately killing the bacterium.

Once having a working culture of *M. aurum*, the second objective would be to continue further experimentation. Experimentation should begin with the N-1-methyl-6-indazolyl benzene sulfonamide derivative due to the results from this project but should also be extended to include the other sulfonamide and amine derivatives, as well. Based on the fact that the plates used in the SPOTi assay appeared to have a few colonies of *M. aurum* growing, I believe this method has potential to successfully reduce mycobacterial growth. By following the experimental procedures of the SPOTi assay with multiple replicates and more time, I would be able to determine which derivatives, if any, can be used as a potential treatment option by observing if there are a reduced number of colonies in the wells containing the derivatives compared to the wells containing only the *M. aurum*. From there, the next step would be to perform either the microdilution broth panel with the 96-well or 24-well titration plates. This would allow for the MIC to be determined because of the varying concentrations that will be added into each well after the serial dilution takes place.

Finally, the last objective would be to either synthesize more of the derivatives that have already been determined to show capabilities of strong binding affinities or to revisit the chemical aspect of the research and synthesize new derivatives that would potentially work better. This step

would depend upon the results from the further experimentation. If the current derivatives show signs of reducing mycobacterial growth, then the first option would occur and more of these derivatives would be synthesized in order to be able to continue researching this topic. If the current derivatives continue to show no significant signs of reducing mycobacterial growth, then the second option would occur and different derivative options would need to be looked into before proceeding.

Time was a major deterrent in this project, so having more time would allow for the more in-depth experimentation and provide more results that would be able to determine the effectiveness of the drug derivative as potential treatment options. With all this being said, there are a number of additional steps that would need to be carried out in order to come to a definite answer to the proposed question.

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Appendix I

Materials

- 6-nitroindazole
- Sodium hydride (NaH)
- Dimethylformamide (DMF)
- Iodomethane (CH_3I)
- Ethyl acetate (EtOAc)
- Brine
- Celite
- Ammonium chloride (Na_4Cl)
- 80% ethanol (EtOH)
- Iron (Fe) powder
- Triethylamine (Et_3N)
- Benzene sulfonyl chloride
- 4-chlorobenzene sulfonyl chloride
- 2-fluorobenzene sulfonyl chloride
- Pyridine
- Sodium bicarbonate ($NaHCO_3$)
- Magnesium sulfate ($MgSO_4$)
- Benzaldehyde
- 4-chlorobenzaldehyde
- 2-chlorobenzaldehyde
- Methanol (MeOH)
- Acetic acid
- Sodium borohydride ($NaBH_4$)
- Deionized water (ddH_2O)
- Dimethyl sulfoxide (DMSO)
- Phosphate buffer saline (PBS)
- Middlebrook 7H9 (M7H9) broth
- *M. aurum*
- Isoniazid
- Rifampin
- AD supplement (dextrose, sodium chloride (NaCl), and albumin)
- M7H9 agar
- Glycerol
- CB stock
- CHX stock