5-16-2016

Inhibitory Effects of Unique Sulfonamides on Leishmania tarentolae and Potential Pathway of Inhibition

Jade M. Katinas
Illinois State University, katinajm@gmail.com

INHIBITORY EFFECTS OF UNIQUE SULFONAMIDES ON LEISHMANIA TARENTOLAE AND POTENTIAL PATHWAY OF INHIBITION

Jade M. Katinas

80 Pages

Leishmaniasis is an endemic disease caused by the protozoan parasite *Leishmania*. Current treatments for the parasite are limited by cost, availability, and drug resistance as the worldwide occurrence of leishmaniasis continues to be more prevalent. Sulfonamides are a class of compounds with medicinal properties that have been used to treat bacterial and parasitic diseases via various pathways. In this study, newly synthesized, unique structural analogs of sulfonamide compounds were assessed for their impact on *Leishmania* cell viability and potential pathways for inhibition were evaluated. *Leishmania tarentolae* (ATCC Strain 30143) axenic promastigote cells were grown in BHI medium and treated with varying concentrations of the unique sulfonamide compounds. Light microscopy and viability tests were used to assess the cells with and without treatment. Recombinant *Leishmania major* dihydrofolate reductase-thymidylate synthase (DHFR-TS) bifunctional enzyme was expressed in *E. coli*, extracted, and purified. DNA sequencing at University of Illinois, Urbana-Champaign (UIUC) confirmed the gene was expressed correctly, but the recombinant protein did not exhibit catalytic activity. Future work should include evaluation of the inhibitory effects of the sulfonamides on the recombinant DHFR-TS.

The conclusions of this work will help determine potential applications for tested sulfonamides improving future medicinal therapies for this disease.

KEYWORDS: Leishmania, Leishmaniasis, Sulfonamide, Potential Treatment, Promastigote

INHIBITORY EFFECTS OF UNIQUE SULFONAMIDES ON LEISHMANIA TARENTOLAE AND POTENTIAL PATHWAY OF INHIBITION

JADE M. KATINAS

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Chemistry

ILLINOIS STATE UNIVERSITY

2017

INHIBITORY EFFECTS OF UNIQUE SULFONAMIDES ON LEISHMANIA TARENTOLAE AND POTENTIAL PATHWAY OF INHIBITION

JADE M. KATINAS

COMMITTEE MEMBERS:

Marjorie A. Jones, Chair

Jon Friesen

Christopher Hamaker

ACKNOWLEDGMENTS

I would first like to thank my research advisor and mentor, Dr. Jones, for providing her leadership and guidance through my career at Illinois State University. Dr. Jones' contributions to my education and encouragement to pursue my dreams will always be remembered and appreciated.

I would also like to thank Dr. Friesen for guiding me through the gene and protein expression methodology, and providing direction and knowledge into data analysis and interpretation. Dr. Friesen also kindly reviewed this thesis and provided valuable input.

Dr. Hamaker and his lab provided the sulfonamide compounds tested in this study, making this thesis possible. He also welcomed me into his lab to perform my own synthesis and gain experience in a different area of chemistry. His input and guidance have been greatly appreciated.

I would like to acknowledge the faculty, staff, my fellow students, and lab peers at Illinois State University for providing a supportive, accepting, and challenging environment in which to learn and grow, as well as the lab space, equipment, instrumentation, and materials for this thesis. I would specifically like to thank Dr. Weitzel for kindly delivering samples to UIUC, Jacob Walker for sharing his protein assay data and lab support, and my friends for providing empathy and encouragement along the way.

In addition, this work could not have been completed without the support of the Kurz Fellowship and the Illinois State Chemistry Club, which provided summer funding to support work toward completing this thesis.

Finally, I want to express gratitude to my family and husband for providing me with unfailing love and support as I took on this adventure. This accomplishment would not have been possible without them.

J. M. K.

CONTENTS

TABLES

FIGURES

CHAPTER I

INTRODUCTION

LEISHMANIASIS AND *LEISHMANIA*

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. Leishmaniasis is transmitted by sandflies and is often found in areas of Africa, Central and South America, the Mediterranean regions, Central and East Asia, and Southeastern Europe The disease is endemic in over eighty countries worldwide and is considered a dangerous public health concern with an incidence of 1.3 - 2 million affected each year with approximately 310 million at risk of infection.[1] The disease can present in three main ways: cutaneous, mucocutaneous, or visceral leishmaniasis.[2]

Cutaneous leishmaniasis, the most common form, causes painful sores that may start as papules or nodules and end as ulcers that may develop within a few weeks or months after parasite transmission to the host. Species of *Leishmania* known to cause cutaneous leishmaniasis include *Leishmania tropica*, *L. major*, *L. aethiopica*, *L. infantum*, and *L. donovani* in the Old World (Eastern Hemisphere) and include the *L. mexicana* species and the subgenus *Viannia* species in the New World (Americas).[3]

Mucocutaneous, or mucosal, leishmaniasis is a consequence of the *Viannia* subgenus of *Leishmania* infecting the mucous membranes of the nose, mouth, or throat, typically after years of cutaneous infection. This type of leishmaniasis can cause severe physical damage to the infected areas.[3]

Visceral leishmaniasis is the most life threatening form of the disease, as it affects internal organs such as the spleen, liver, and bone marrow. Hosts typically develop the disease months to years after the initial infection, which can cause fever,

swelling of the spleen and liver, and reduced red blood cell, white blood cell, and platelet counts.[4] Visceral leishmaniasis is usually caused by the *L. donovani, L. infantum* or *L. chagasi* species.[3]

Studies have shown that *Leishmania tarentolae*, a species isolated from the gecko *Tarentolae annularis*, is a suitable model for *in vitro* screening of anti-leishmanial compounds. Because this form of *Leishmania* is not infectious to humans, the axenic form of the parasite is considered suitable for preliminary potential anti-leishmanial compound studies.[5]

Leishmania are digenic and can exist as two cell stages: amastigotes and promastigotes. As can be seen in Figure 1,[3] the promastigote stage of *Leishmania* is transferred to an alternative host upon feeding by an infected sandfly.[6] A macrophage or other phagocytic cells in the immune system phagocytize the cells. Once in the phagocytic cell, the *Leishmania* transform to the amastigote stage, which is characterized by a more spherical shape and loss of flagellum. Amastigotes replicate in the phagocytic cell until its rupture and the subsequent release of amastigotes thus can lead to infection of other phagocytic cells. The amastigote stage of the *Leishmania* has several characteristics different from the promastigote stage, including changes in gene expression leading to enzymatic changes that allow the cells to survive in the acidic environment of a phagocytic cell.[7]

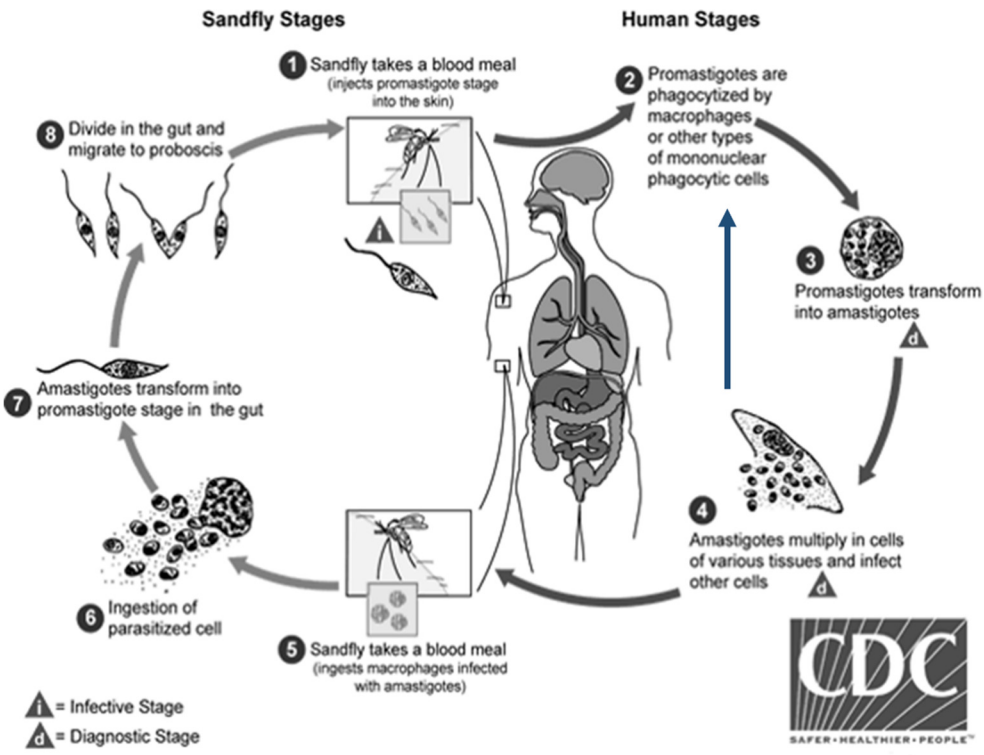


Figure 1. Life cycle of *Leishmania*. *Leishmania* present as different cell stages within their lifecycle, with different cell morphology and genes expressed (modified from Centers for Disease Control and Prevention[3]).

The diversity in presentation of leishmaniasis owing to the many different species of the parasite make it a difficult treatment target. In addition, the life cycle of the parasite leads to changes in gene and enzyme expression in the different hosts, presenting additional complexity in identifying and designing small molecules for specific drug targets. With the disease affecting nearly 2 million people annually, identifying new therapeutics has become more pertinent in preventing spread and improving quality of life for those affected.

CURRENT TREATMENTS FOR LEISHMANIASIS

Treatments exist today for the three types of leishmaniasis;[2] however, due to the infections primarily occurring in developing countries with weak infrastructure and impoverished economies, access to these treatments is costly and complicated. Advancement of known and new treatments is largely ignored and the World Health Organization (WHO) has considered leishmaniasis a "neglected" disease.[1] Common treatments for leishmaniasis generate increasing concerns over variable responses in different species of the infective parasite, as well as failure in treatment due to resistance from overuse.

Pentavalent antimonial (Sb^V) drugs are typically the first line of defense for all types of leishmaniasis infections, though studies have shown Sb^V drugs to have three- to fivefold greater sensitivity in *L. donovani* and *L. brasilensis* species compared to *L. major*, *L. tropica*, and *L. mexicana* species.[8] However, many scientists believe that because of widespread overuse, the acquired resistance to the treatment has increased and some species of the parasite have become refractory to the treatment.[9]

Amphotericin B, a polyene antibiotic, has been used in areas with Sb^V drug resistance with some success. However, side effects are reported to be a concern. [10] Clinical resistance to amphotericin B is currently rare, but with the increasing use of the treatment in place of antimonial drugs, it is expected that resistance could become an issue.[9] Resistant clones of *L. donovani* promastigotes have been identified in cell cultures and have been seen to express mutations with changes in the sterol profile of their plasma membrane.[11]

The first oral agent approved for treatment of leishmaniasis is miltefosine, with cure rates of 94% for visceral leishmaniasis[12]; however, the drug maintains subtherapeutic levels due to its slow clearance in the host that may lead to greater opportunity for drug resistance.[13] Studies have shown variation in sensitivity of amastigote and promastigote forms of different species of *Leishmania* to miltefosine, with *L. donovani* being the most sensitive and *L. major* being the least.[14]

Other therapies are also available, but the opportunity for resistance in different species of the parasite as a function of use and application is high.[9] In 2010, the World Health Organization identified high cost of medicines, treatment availability, lack of updated protocols for application, and non-continuous supply of the most effective treatments to be the largest hurdles for treatment of the disease in the most highly impacted regions of the world.[15] Availability of treatments with greater ease of use, lower treatment rates, and less sensitivity to resistance would be of great benefit globally.

SULFONAMIDES AND THEIR INHIBITORY EFFECTS

One possible treatment option for leishmanisis is the development of unique sulfonamides that target parasite cell function and inhibit cell viability and proliferation while causing minimal negative side effects for the host. Sulfonamide drugs are characterized by the presence of a $-S(=O)_2-NH_2$ functional group in the compound. Sulfonamides became popular antibacterial agents in the 1940s, though the first sulfonamide drug, Prontosil (Figure 2) was first patented in 1932.[16] The drug was

synthesized by Josef Klarer and Fritz Mietzsch and found effective against bacterial infections in mice by Gerhard Domagk in 1932, and in 1939 Domagk was awarded the Nobel Prize in Medicine for his work with the first medicine that could treat a range of bacterial infections in humans.[17] Sulfonamides, or sulfa drugs, are commonly used as chemotherapeutic and preventative medicines for various diseases, with over 30 such drugs being in clinical use as of 2014.[18] Sulfa drugs have been used as antihypertensive,[19] antibacteria,l[20] antiprotozoal,[21] antifungal,[22] and anti-inflammatory agents.[18] Sulfonamide derivatives have also been used to treat urinary, intestinal, and ophthalmic infections, burns, ulcerative colitis,[23] rheumatoid arthritis,[24] and more recently, cancer,[25] Alzheimer's disease,[26] and HIV.[27]

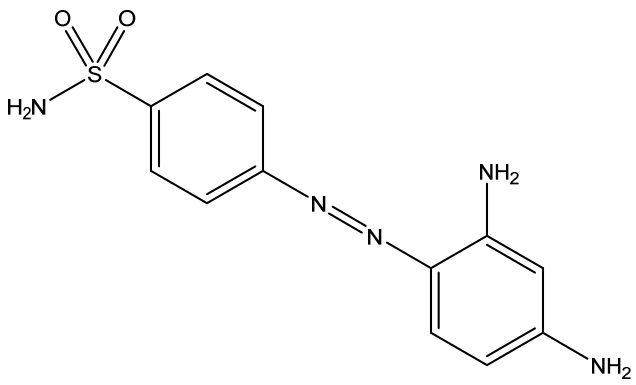


Figure 2. Chemical structure of Prontosil.[16]

Potential side effects of sulfa drugs include skin rash, itching, headache, dizziness, diarrhea, tiredness, nausea or vomiting, pale skin, joint pain, and sensitivity to light, and allergies to sulfonamides and other sulfa drugs are common.[28] The side effects of anti-leishmanial Sb^V drugs often include nausea and vomiting, joint pain, hepatitis, pancreatitis and cardiac dysrhythmias and require frequent monitoring for

toxicity.[29] Clinical studies with amphotericin B have shown infusion reactions and renal impairment while miltefosine, in the active oral form, has some reproductive toxicity as well as gastrointestinal side effects, which can cause nausea and vomiting.[29] Severity of side effects are dependent on treatment concentrations and accumulation in patients. Though sulfonamides have been seen to form renal crystals, this can be managed by monitoring treatment concentrations and could potentially be reduced by improving water solubility.[30]

Studies have identified the primary pathway of sulfonamide inhibition as competitive inhibitors of the enzymes dihydropteroate synthase (EC 2.5.1.15) and dihydrofolate reductase (EC 1.5.1.3; DHFR), enzymes involved in folate metabolism.[31] Folate synthesis and the products of the folate pathway are important in the synthesis of purines and DNA. This pathway of inhibition is also a primary method of treatment of cancers using sulfonamide drugs,[32] as it prevents the replication of DNA and cell proliferation. Other modes of inhibition induce disruption of microtubule activity, thereby decreasing cell division.[33] Studies have also shown that the inhibition of DHFR can allow for an accumulation of NADPH, which would have an inhibitory effect on glucose-6-phosphate dehydrogenase in the oxidative phase of the pentose phosphate pathway.[34] The pentose phosphate pathway has two primary functions: regeneration of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) for use in multiple pathways and generation of pentoses, which are necessary for nucleic acid synthesis.[35]

Sulfonamides are typically prepared by reacting a sulfonyl chloride compound containing the desired side chain with ammonia or an amine containing the second

desired side chain, making the process fairly simple and low cost.[18] Due to the stability of these compounds, if stored properly, they can have a shelf life of approximately two years.[36] Sulfa drugs can be applied orally, topically, or intravenously,[37] allowing them to be good treatments for all three types of leishmaniasis.

FOLIC ACID METABOLIC PATHWAYS

As shown in Figure 3, the various intermediary metabolites of folate have a variety of roles, as cofactors, in a variety of pathways, especially in one-carbon transfers.[38] Thus, amino acid metabolism, choline derivatives (such as phosphatidylcholine formation from phosphatidylethanolamine), as well as nucleic acid metabolism can be affected as folic acid metabolism is affected.[39]

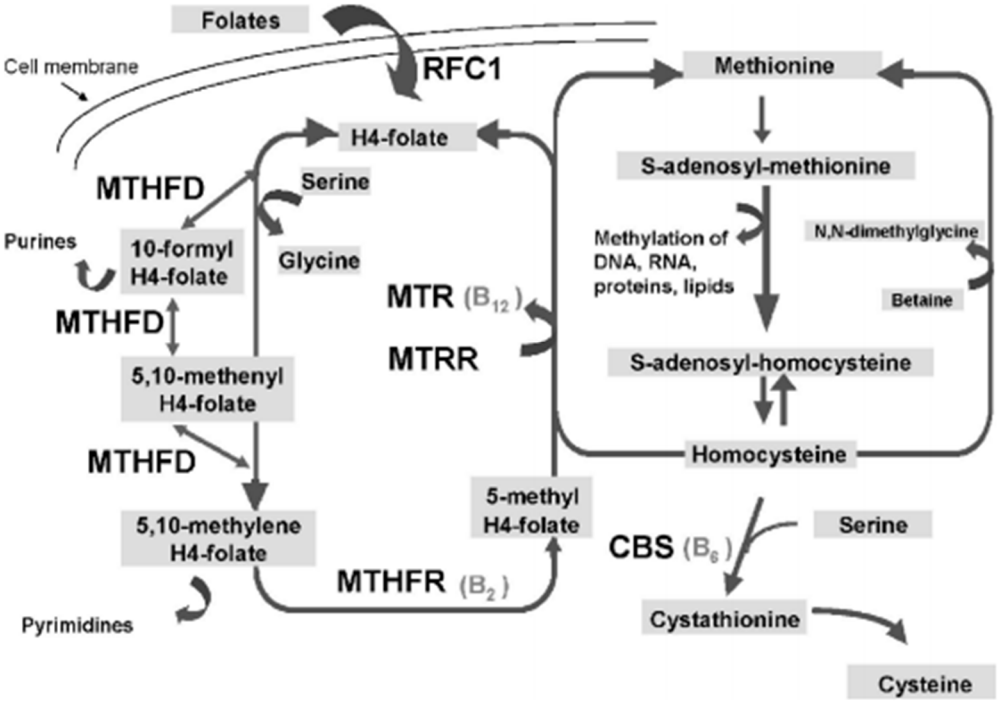


Figure 3. The folate metabolic pathway.[39]

Sulfonamides have been used to treat bacteria and *Leishmania* species perhaps by mainly inhibiting the enzymes dihydropteroate synthetase (DPS) and dihydrofolate reductase (DHFR).[7] These enzymes are essential in the production and metabolism of folate, affecting the synthesis of DNA. DHFR, specifically, allows for the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) while oxidizing the NADPH to $NADP^+$ (Figure 4).[40]

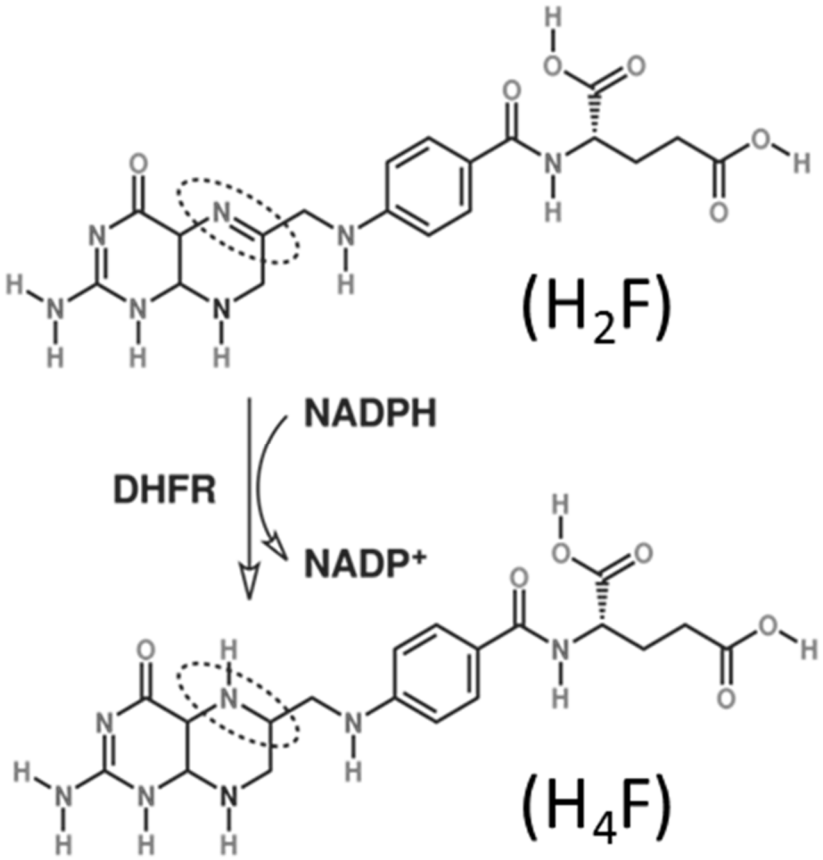


Figure 4. Reaction catalyzed by DHFR. Dihydrofolic acid (H_2F) is reduced to tetrahydrofolic acid (H_4F) with the oxidation of NADPH to $NADP^+$.[40]

The proposed mechanism for the DHFR enzyme, from *Mycobacterium tuberculosis*, is shown in Figure 5.[41] The primary coordinating amino acid of the dihydrofolate binding pocket is an aspartate or glutamate, dependent on species, as the carboxyl group of the amino acid is important in hydrogen bonding to the pterin ring of the dihydrofolate. Water is also necessary to ensure a proton transfer occurs along with the hydride transfer from NADPH, allowing for the final product of tetrahydrofolate (H_4F).

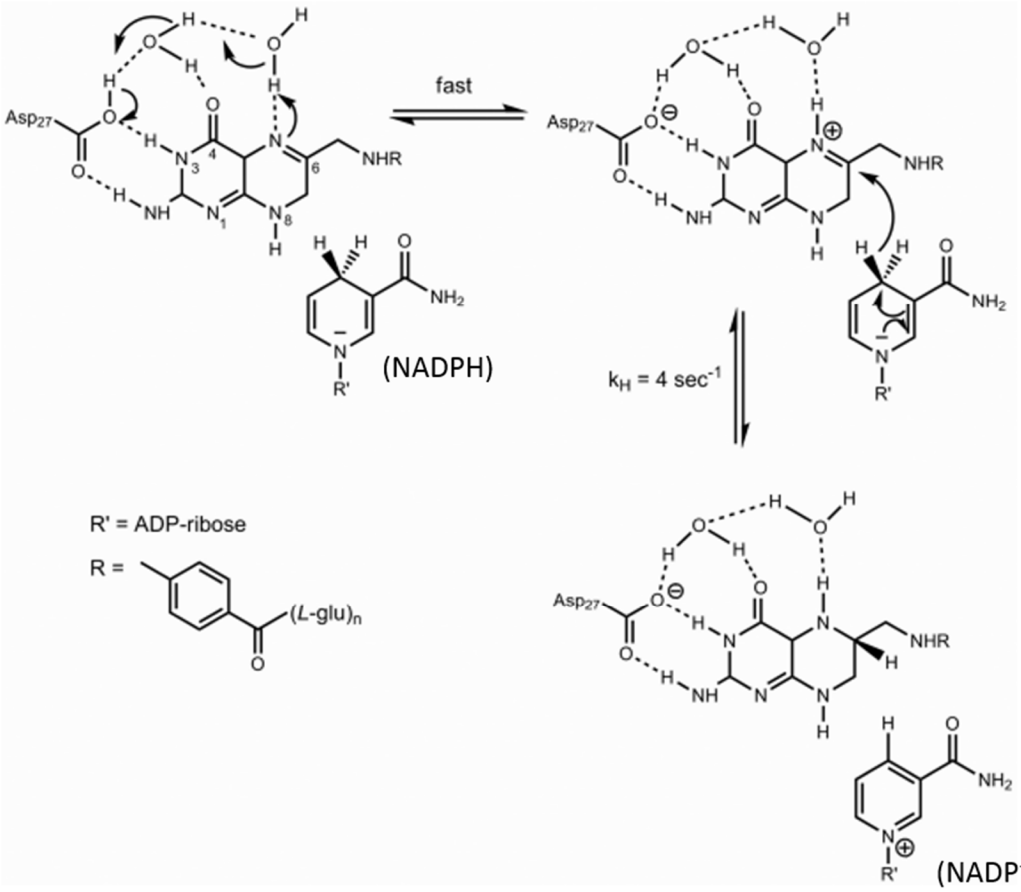


Figure 5. Proposed catalytic mechanism by DHFR from *Mycobacterium tuberculosis.*[41]

DHFR activity is the major endogenous source for H_4F, and is important in maintaining the cellular pools of H_4F and its derivatives for the synthesis of purines and thymidylate among other highly important products. Without DHFR, cells are unable to grow and proliferate without addition of exogenous H_4F.[40] Because of this, DHFR is a primary target for treatment against rapidly growing cells, such as bacteria, parasites, and even cancers.

It is important to note, however, that *Leishmania* have a more complex pathway of folate metabolism than bacteria. *Leishmania* contain a bifunctional polypeptide form

of DHFR that also encodes thymidylate synthase (EC 2.1.1.45) (TS), meaning the enzyme, DHFR-TS, is reversible in the presence of N^5, N^{10} – methylene tetrahydrofolate (CH_2=H_4F) and deoxyuridine monophosphate (dUMP). Although the TS enzyme is present ubiquitously among cells, it is typically only found in the bifunctional form in protozoa and plants.[42] In addition, a second enzyme, pteridine reductase 1 (EC 1.5.1.33) (PTR1), has been identified to reduce folates and unconjugated pteridines in the folate pathway, as seen in Figure 6.[43] PTR1 can act as a metabolic bypass of DHFR inhibition drugs solely targeting tetrahydrofolate production by the bifunctional DHFR-TS enzyme and halting single carbon metabolic pathways.[44]

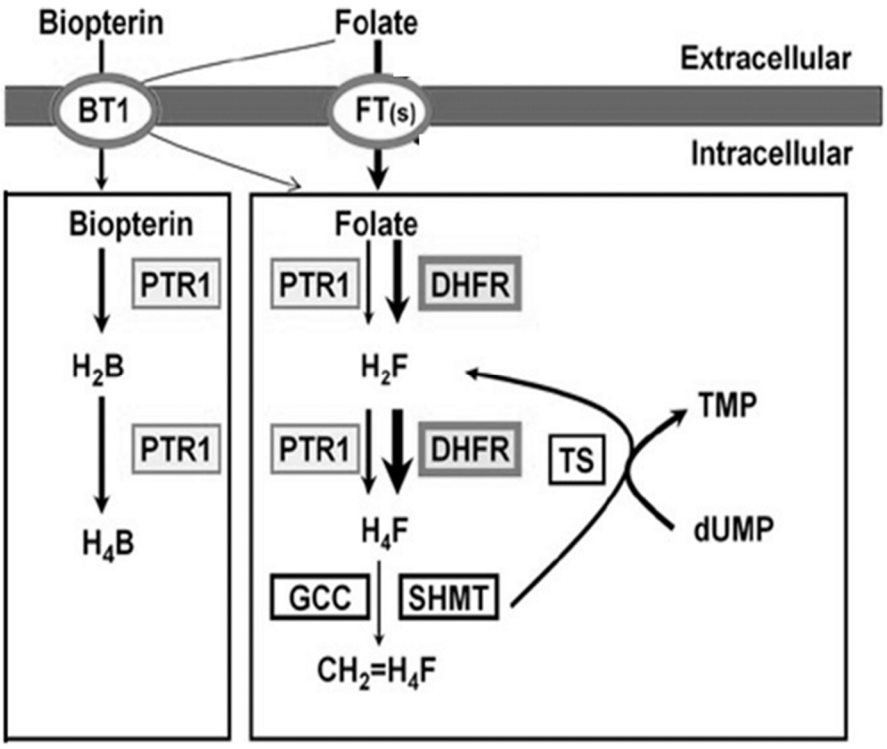


Figure 6. The folic acid metabolic pathway for *Leishmania*. The proposed metabolic pathway for folate and biopterin in *Leishmania,* showing PTR1 can catalyze both the reduction of folate and dihydrofolate (H_2F) to tetrahydrofolate (H_4F), which are essential steps in the synthesis of DNA.[43]

PTR1 plays an important role in the salvage and oxidation of pterins.[44] Studies have shown that DHFR-TS has no role in the reduction of pterines, but PTR1 can have significant activity with folates, increasing the opportunity for DHFR drug target resistance in *Leishmania.*[44] Though the role of biopterin in trypanosomatids is not well established, PTR1 and quinoid dihydropteridine reductase (qDPR) have been identified as important for recycling quinonoid dihydrobiopterin (Figure 7).[44] This recycling may also have an important role in the biosynthesis of tyrosine for these parasites (Figure 7). Studies have also shown that mutants lacking PTR1 are unable to survive, even in the

presence of folate or dihydrofolate[45] and, therefore, play an important role in *Leishmania* growth and viability. Because of this additional enzyme complexity, determining the pathways of inhibition of specific treatments is important in determining the efficacy of a drug and likelihood for species resistance.

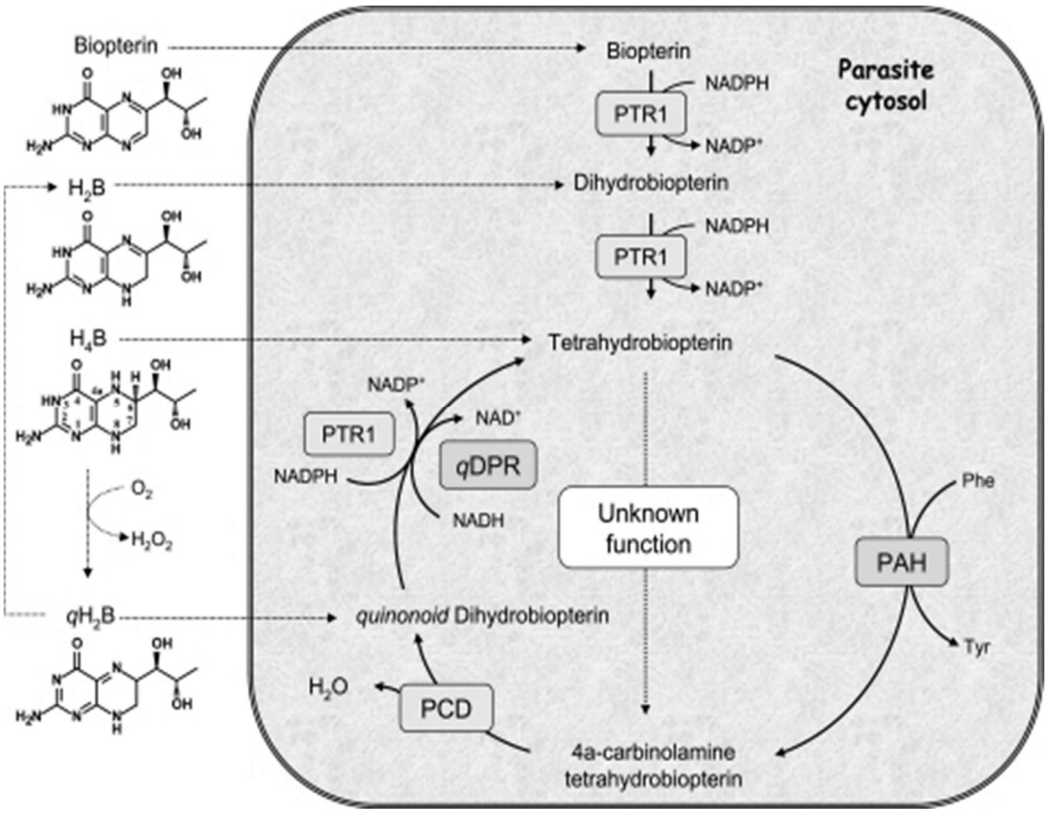


Figure 7. Metabolic pathway of biopterin in *L. major.*[46]

In this thesis, newly synthesized, unique sulfonamide compounds were assessed for their impact on *Leishmania tarentolae* cell viability and potential pathways of inhibition were investigated. Due to the known inhibitory effects of sulfonamides on DHFR, the folate pathway was a focus of interest. Axenic promastigotes (ATCC Strain

30143) were grown in brain heart infusion (BHI) medium and treated with varying concentrations of the synthesized sulfonamides. Light microscopy and viability tests were used to assess the cells with and without treatment. The folic acid pathway was probed by adding folic acid to the cells and evaluating the effects with and without treatment. Recombinant DHFR-TS from *L. major* was expressed in *E. coli*, extracted, and purified. Enzyme activity was evaluated by measuring the oxidation of NADPH to $NADP^+$ using change in absorbance at 340 nm.[47] However, catalytic activity was not exhibited by the enzyme and additional work is needed to evaluate the effects of the unique inhibitory sulfonamides on DHFR-TS from *Leishmania*. Understanding the form and inhibitory function of these sulfonamide compounds would be helpful in developing treatments for *Leishmania* that may overcome the many complexities of their infective capability.

CHAPTER II

MATERIALS AND METHODOLOGY

PROMASTIGOTES

Leishmania tarentolae promastigotes (ATCC Strain 30143, American Type Culture Collection) were grown in sterile conditions in brain heart infusion (BHI) medium supplemented with penicillin and streptomycin (100 units and 0.1 mg per mL respectively) and hemin (10 µM) in flasks (Falcon, polystyrene) following the method of Morgenthaler et al.[48] Promastigote forms of the *Leishmania* cells were transferred during the log phase of the growth curve to maintain cell cultures. Work with promastigotes was performed in a sterile hood to prevent contamination.

MTT ASSAY

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate promastigote viability at different points in the growth curve and after treatments. The MTT assay is a colorimetric assay which assesses cell viability based on the activity of oxidoreductase enzymes.[49] These enzymes reduce the yellow tetrazolium reagent to an insoluble, purple formazan. One hundred microliters of cells were transferred to wells of a 96 well plate (Fisher, polypropylene), 4 replicates to allow for calculation of mean and standard deviation, then 10 µL of MTT reagent (Sigma, 5 mg per 1 mL water) was added to each well with gentle mixing. Cells and reagent were allowed to incubate for 1 hour before the addition of the stopping agent which consists of 10% Triton X-100 (v/v), 225 mL isopropanol, and 2 mL 0.14 M hydrochloric acid. A

96 well plate reader spectrophotometer (BIO RAD, iMark Microplate Reader) was then used to obtain absorbance data at 595 nm. BHI medium, without cells, treated with dimethyl sulfoxide (DMSO) or the same concentration of sulfonamide in DMSO being tested were used as blanks as appropriate. Corrected absorbance at 595 nm is then assumed to correlate with the amount of viable cells present.

MICROSCOPY

Light microscopy (JENCO, Model CP-2A1) was used to qualitatively assess cells for shape, clumping, and cell motility. Cells were evaluated in the flasks, without fixation, since this is an inverted microscope. Pictures and videos were obtained with a Kodak EasyShare C743 camera.

SULFONAMIDE SYNTHESIS AND CHARACTERIZATION

Starting materials were obtained from commercial sources and used as received. Dr. Hamaker synthesized *N*-(2'-methylthiophenyl)-1-napthalene sulfonamide (Compound A), *N*-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), and *N*-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C) by reaction of 1-naphthalene sulfonyl chloride with the appropriate aniline in pyridine. A 500 MHz NMR was used to acquire ^{1}H spectra for samples in DMSO-*d*6. The generic reaction is shown in Figure 8.[50]

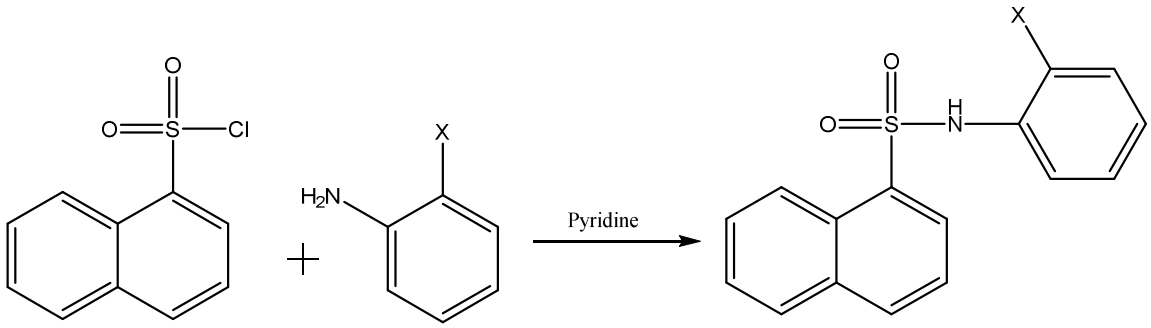


Figure 8. Generic reaction to synthesize sulfonamides. Sulfonamides A, B, and C were synthesized when 1-napthalene sulfonyl chloride was mixed with the appropriate aniline in pyridine.

To synthesize *N*-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D), 30 mL of aqueous 1 M sodium carbonate containing 2.200 g (9.710 mmol) of 1-napthalenesulfonyl chloride and 1.528 g (10.11 mmol) 3-methyl-4-aminobenzoic acid was prepared in a 250 mL Erlenmeyer flask. The solution was allowed to stir for 24 hours and then acidified with 6 *M* hydrochloric acid. The precipitate was filtered and washed with water. The precipitate that formed was collected by vacuum filtration, washed with water, and air dried overnight to yield 1.765 g (47.33%) of Compound D as a light pink solid. ^{1}H NMR (500 MHz, $(CD_3)_2SO$): 12.73 (br s, 1H, COO*H*); 10.16 (s, 1H, N*H*); 8.73 (d, 1H, aromatic); 8.23 (d, 1H, aromatic); 8.09 (m, 2H, aromatic); 7.69 (m, 2H, aromatic); 7.59 (m, 3H, aromatic); 7.20 (d, 1H, aromatic); 1.95 (s, 3H, -C*H*$_3$) (Appendix A).[50]

To synthesize *N*-(4-carboxyl)-1-naphthalene sulfonamide (Compound E), 25 mL of aqueous 1 *M* sodium carbonate in a 125 mL Erlenmeyer flask was charged with 1.371g (10.00 mmol) of anthranilic acid and 2.269 g (10.01 mmol) 1-naphthalenesulfonyl chloride. The solution was stirred for 20 hours and acidified with

6 M hydrochloric acid. The precipitate that formed was collected by vacuum filtration, washed with water, and air dried overnight to yield 2.588 g (74.01%) of Compound E as a white solid. ^{1}H NMR (500 MHz, $(CD_3)_2SO$): 13.90 (br s, 1H, COO*H*); 8.62 (d, 1H, aromatic); 8.31 (d, 1H, aromatic); 8.19 (d, 1H, aromatic); 8.04 (d, 1H, aromatic); 7.79 (d, 1H, aromatic); 7.69 (t, 1H, aromatic); 7.63 (m, 2H, aromatic); 7.34 (m, 2H, aromatic); 6.88 (t, 1H, aromatic) (Appendix B).[50]

EVALUATION OF SULFONAMIDE TREATMENT OF PROMASTIGOTES

The sulfonamides were dissolved in DMSO and added to promastigotes grown in BHI medium. A large volume (60 mL) of cells were grown in a single flask (Falcon, 75 cm^2 polystyrene) and transferred to smaller flasks, 5 mL (Falcon, 12.5 cm^2 polystyrene) or 10 mL (Falcon, 25 cm^2 polystyrene) for treatment. This was done in an effort to reduce variation in number and age of cells in each test flask. Promastigotes were treated with varying concentrations of the sulfonamides (between 10 µM and 100 µM) dissolved in DMSO, ensuring cells were exposed to no more than 1% (v/v) DMSO, at different points in the growth curve for the cells (Figure 9). Untreated cells and cells treated only with the same final volume DMSO were used as controls for these studies. Blank samples were used to calculate the corrected average absorbance of the cellular samples by subtracting the absorbance values of the blanks from the cellular values. MTT assays were used to quantitatively evaluate cell viability, while light microscopy was used to qualitatively assess cells for shape, clumping and cell motility. Each experiment was performed in four wells in at least three experiments to confirm

repeatability and a statistical Students t-test was used to determine statistical significance for the results ($p < 0.05$ was considered significantly different). Cultures determined to be severely inhibited were transferred to fresh medium to determine if they were in early or late senescence phase and could recover. Those without recovery (measurable MTT response) over 3 days were considered fully inhibited (dead).

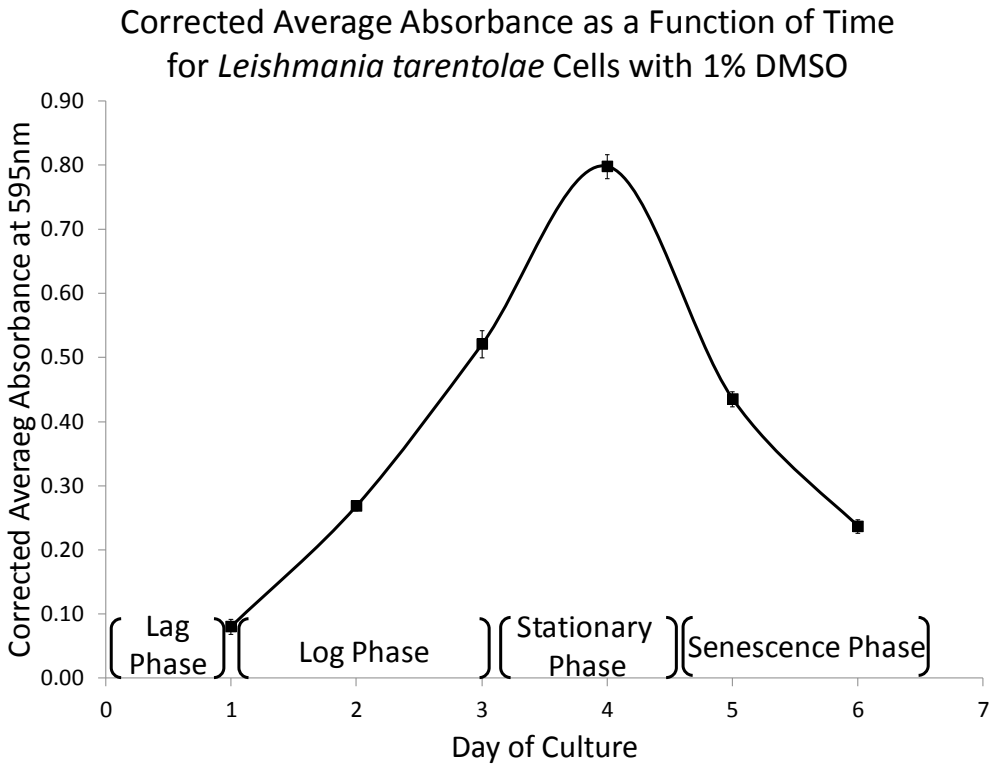


Figure 9. Typical growth curve for *Leishmania tarentolae* promastigotes. The graph shows the typical growth curve and phases for *Leishmania tarentolae* promastigotes, where absorbance at 595 nm is representative of cell viability as a function of day in culture. Each point is a mean ± standard deviation of n = 4.

Cells shape, motility, and clumping were assessed with inverted light microscopy daily. The results were documented subjectively and photography or video were used as necessary for further documentation.

POTENTIAL PATHWAY OF INHIBITION INVESTIGATION

Potential pathway of inhibition in the presence of those sulfonamides with effects on cell viability was evaluated by adding folate to the medium in concert with the sulfonamide addition. If the pathway of inhibition is related to the folic acid metabolic pathway, the cells should have improved viability when folate is added in the presence of the sulfonamide. If folate does not protect the cells there is likely a different pathway(s) of inhibition.

Cells were grown in the same manner as described above. Promastigotes were treated with the most effective concentration of the sulfonamides shown to have a negative effect on cell viability (100 µM) and the same concentration of folate. Both were dissolved in the same vial of DMSO, and cells were treated with the combination, ensuring cells were exposed to no more than 1% (v/v) DMSO, in the log phase of cell growth. Untreated cells, cells treated only with 1% volume DMSO, cells treated with 100 µM folate, and cells treated with 100 µM sulfonamide were used as variables for these studies. MTT assays were used to quantitatively evaluate cell viability, while light microscopy (JENCO, Model CP-2A1) was used to qualitatively assess cells for shape, clumping and cell motility. Each experiment was performed at least three times to

confirm repeatability and a statistical Students t-test was used to determine statistical significance for the results ($p < 0.05$ was considered significantly different). Cultures determined to be severely inhibited were transferred to fresh medium to determine if they were in early or late senescence phase and could recover. Those without recovery (measurable MTT response) over 3 days were considered fully inhibited (dead).

DNA AMPLIFICATION BY PCR

To evaluate the enzyme DHFR-TS from *L. major,* the gene encoding DHFR-TS was cloned using polymerase chain reaction (PCR) DNA amplification and the encoded protein was expressed in *E. coli* cells. *Leishmania major* was chosen for this work, as it is one of the human infective forms of the parasite, so studies related to therapeutic inhibition of DHFR-TS would be applicable to a medically relevant form of the parasite. Also, the genome for this species has been sequenced (Genbank ID: M12734).[51] Standard methods used were adapted from Sambrook and Russell, Molecular Cloning – A Laboratory Manual.[52]

Single-stranded DNA primers (Sigma) complementary to the 5'- and 3'- ends of the gene were designed to amplify the gene encoding *L. major* DHFR-TS (Genbank ID: M12734). When the primers are present in solution with genomic DNA from *L. major*, the primers hydrogen bond with the complementary DNA of the gene of interest. PCR (MJ Research, PTC-200 Peltier Thermal Cycler) was used to amplify the gene of interest between the primers. After incubation at 94°C for 5 minutes, the PCR

amplification, using the PCR Master Mix (Promega), involved the following program: 94℃ for 15 seconds, 50℃ for 30 seconds, and 72℃ for 1 minute; 30 complete cycles were performed. At 94℃ the double stranded DNA template, from *L. major*, denatures to form single strands of DNA to which the primers anneal at 50℃. This is then heated to 72℃, where the OneTaq DNA polymerase (New England BioLabs, 2x concentration) elongates the primers from the 5'-3' direction. The volumes of materials used in the PCR amplification are shown in Table 1. Deoxy ribonucleotide (present in the PCR MasterMix, Promega, 2x concentration) addition occurs on both strands, and after 30 cycles 2^{30} DNA strands, in theory, were synthesized.

Nuclease Free (Autoclaved) Water	PCR MasterMix (Promega, 2x)	5' Oligonucleotide (Sigma, 100 µM)	3' Oligonucleotide (Sigma, 100 µM)	Template *L. major* Genomic DNA	Magnesium Sulfate (New England BioLabs, 50 mM)
22 µL	25 µL	1 µL	1 µL	1 µL	0
20 µL	25 µL	1 µL	1 µL	1 µL	2 µL

Table 1. Materials and volumes used for PCR amplification of the DHFR-TS gene.

The primers were designed to include start and stop codons for the gene, the appropriate nucleotide sequence to code for restriction enzyme target regions for BamHI and HindIII, and 17 nucleotides of the 5'- sequence of the DHFR-TS gene, and 22 nucleotides of the 3'- sequence of the DHFR-TS gene, as seen in Figure 10. The primers were ordered from Sigma-Aldrich.

Figure 10. Primer sequences designed for PCR amplification of the *L. major* DHFR-TS gene. The BamHI restriction enzyme sequence (GGATCC) is underlined in the 5'-strand of the primer, and the HindIII restriction enzyme sequence (AAGCTT) is underlined in the 3'- strand. The start codon, ATG, and stop codon, CTA, are shown in bold, and the nucleotides of the gene are in the boxed region.

Agarose gel (1.4% (w/v)) electrophoresis was used to analyze the amplified DNA. The gel was prepared by adding 1.4 g agarose (ISC BioExpress, GenePure LE Quick Dissolve Agarose) to 100 mL 0.5x TBE Buffer (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA, purchased from Fisher) and microwaving until dissolved. After cooling to approximately 50℃, 3 µL SYBR safe (Invitrogen) was added as an intercalating fluorescent dye for visualization of DNA under UV light. The mixture was poured into an agarose gel mold.

The PCR material was purified using a PCR purification kit (PureLink™ Quick PCR Purification Kit, Invitrogen™). The PCR samples and pET-45b plasmid were digested with BamHI (New England BioLabs, 20,000 U/mL) and HindIII (New England BioLabs, 20,000 U/mL) enzymes for 4 hours using volumes shown in Table 2. The pET-45b plasmid encodes a six histidine tag (His-tag) within the reading frame for the inserted gene; therefore, the expressed protein should contain a His-tag that can be used for protein purification on a cobalt or nickel resin column. The plasmid also encodes a gene for ampicillin resistance.

	PCR Material	pET-45b Plasmid	Nuclease Free Water	SmartCut Buffer (New England BioLabs, 10x concentration)	BamHI Restriction Enzyme	HindIII Restriction Enzyme
PCR sample	16 µL	0 µL	0 µL	2 µL	1 µL	1 µL
pET-45b	0 µL	5 µL	11 µL	2 µL	1 µL	1 µL

Table 2. Respective volumes for DNA digestion of PCR products and plasmid.

To 15 µL of sample from the restriction enzyme digestion, 5 µL Blue/Orange Loading Dye (Promega, 6x) was added. A standard 1 kilo-base pair (Kb) ladder (Promega) was used as a molecular weight standard for comparison (Figure 11). Five µL of the standard and 15 µL of sample were loaded onto separate lanes of the gel. Then 150 voltage was applied for approximately 45 minutes. Results were visualized using a fluorescent gel imaging system (Kodak, Gel Logic 200 Imaging System) and imaging software (Kodak ID 3.6).

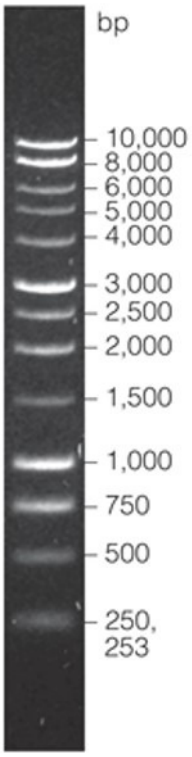


Figure 11. Promega 1 Kb Ladder standard in 0.7% agarose gel.[53]

RECOMBINANT GENE LIGATION

To improve the likelihood of a successful ligation, DNA with the expected number of base pairs (approximately 1500 base pairs) for the DHFR-TS gene and pET-45b plasmid were cut directly from the gel and purified using a DNA purification kit (DNA Purification System, Promega). Ligation of the amplified DNA and the pET-45b plasmid was performed with different volumes of plasmid and PCR material, as shown in Table 3. The materials were incubated at 15°C for approximately 24 hours.

	pET-45b Plasmid	PCR material	T4 DNA Ligase Buffer (New England BioLabs, 10x concentration)	T4 DNA Ligase (New England BioLabs, 400,000 U/mL)	Nuclease Free Water
DHFR-TS 1	1 µL	8 µL	1 µL	0.5 µL	0 µL
DHFR-TS 2	4 µL	4 µL	1 µl	0.5 µL	0 µL
Control - No DHFR-TS	1 µL	0 µL	1 µL	0.5 µL	8 µL

Table 3. Ligation mixture volumes for DHFR-TS gene with pET-45b plasmid. The ligation was performed with two different volumes of pET-45b plasmid and PCR material as the optimal amount of each needed for ligation was unknown. The sample labeled DHFR-TS 1 contained 8 µL PCR material, while the sample labeled DHFR-TS 2 contained 4 µL PCR material.

DNA TRANSFORMATION IN *E. COLI*

Once the ligation was complete, the DHFR-TS gene should have been inserted into the plasmid (referred to as ligation mixture). To transform *E. coli* to express the gene to be used for protein expression, 50 µL of methyltransferase deficient, chemically competent *E. coli* cells suitable for replication of a plasmid free of Dam and Dcm methylation (dam^-, dcm^- *E. coli* cells; New England BioLabs) were added to a 14 mL polystyrene test tube with a lid (Fisher Brand, Fisher). Five µL of the ligation mixture (DHFR-TS 1, DHFR-TS 2, or Control – No DHFR-TS from Table 3) was added and incubated on ice for 30 min, placed in a 42℃ water bath for 30 sec, and placed back on ice for 2 min. One mL of Lysogeny Broth (LB) medium (GeneMate, LB Miller Broth Powder) was added and the mixture was incubated with agitation (225 rpm) at 37℃ for 30 min. The cells were centrifuged (Force Micro, Force1618) for 1 min, 10,000 rpm (8,944x g) in a microcentrifuge (Fisher Brand, 1.5 mL polypropylene) tube to pellet the cells, which were re-suspended in a minimal volume (50-100 µL) of LB medium. The

suspension was dispersed onto an LB agar plate containing 100 µg/mL ampicillin and the cells were allowed to grow overnight at 37°C while shaking at 225 rpm. Colonies that grew were assumed to contain the ampicillin resistance provided by retaining the pET-45b plasmid containing the DHFR-TS gene.

To grow large amounts of the cells that should contain the plasmid, a single colony of cells was removed and added to 2 mL of LB medium in a polystyrene test tube (Fisher Brand, Fisher) with lid containing 100 µg/mL ampicillin per 1 mL of LB medium. These were allowed to grow overnight at 37°C with 225 rpm agitation. The DNA from these cells was purified using the Wizard Plus SV Miniprep DNA Preparation System kit (Promega).

A digestion, as described above (Table 2) using BamHI and HindIII restriction enzymes, was performed on the purified DNA and agarose gel electrophoresis was used to confirm the plasmid contained a DHFR-TS gene. The DHFR-TS gene consists of approximately 1500 base pairs.

PROTEIN EXPRESSION IN *E. COLI*

For protein expression, 1 µL of purified plasmid DNA containing the DHFR-TS gene was added to 50 µL of rare codon isoleucine, proline, leucine (RIPL) *E. coli* cells (Agilent Technologies), and the DNA transformation procedure, as previously discussed, was used to transform the plasmid into the RIPL *E. coli*. RIPL *E. coli* cells are often necessary for expression of genes that contain rare codons for isoleucine,

proline, and leucine that are more common in non-*E. coli* species. The cells were allowed to grow for 1 hour at 37°C with agitation (225 rpm) and were dispersed onto an agarose plate containing 100 µg/mL ampicillin (AMP) and 34 µg/mL chloramphenicol (CM). The agarose plate was incubated at 37°C overnight. Any colonies that grew were assumed to contain the plasmid with the DHFR-TS gene insertion.

A single colony was removed from the plate and placed in 5 mL of LB media containing 100 µg/mL AMP and 34 µg/mL CM. After 24 hours of growth at 37°C and 225 rpm agitation, these cells were transferred to 50 mL LB containing 100 µg/mL AMP and 34 µg/mL CM and allowed to grow for 3 hours at 37°C, 225 rpm. Finally, the 50 mL cell growth was transferred to 1200 mL LB media containing 100 µg/mL AMP and 34 µg/mL CM and grown at 37°C, 225 rpm for 24 hours. A 1 mL sample was removed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. To the rest of the cells, 0.0500 g of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for a final concentration of 0.175 mM. After 3 hours of growth, the cells were pelleted in 250 mL aliquots by centrifugation (5000x g for 5 min, Beckman, Avanti J-25 I). Cells were re-suspended in 50 mM Tris, 100 mM NaCl pH 7.5 buffer as 20 mL aliquots and stored/frozen in 50 mL conical tubes (Corning, polypropylene centrifuge tubes).

PROTEIN EXTRACTION AND PURIFICATION

To harvest the recombinant protein, the frozen cell pellet was placed on ice to thaw and 500 µL protease inhibitor cocktail (Sigma) was added. The protease inhibitor cocktail consists of 2 mM 4-(2-Aminoethyl)benzensulfonyl fluoride hydrochloride

(AEBSF), 0.3 µM Aprotinin, 116 µM Bestatin, 14 µM E-64, 1 µM Leupeptin, and 1 mM EDTA[54], all of which inhibit different classes of proteases. A French Pressure Cell Press (Spectronic Instruments, SLM-AMINCO) was used to lyse the cells at 10,000 psi to release soluble proteins. The lysed material (crude extract) was collected and centrifuged at 31,000x g for 20 min at 4°C. A sample of the pre-centrifuged material and a sample of the supernatant were saved for SDS-PAGE analysis. The supernatant was collected for protein purification and the pellet was saved for protein analysis.

An affinity column was prepared using TALON® metal affinity resin, a cobalt containing resin. Approximately 3 mL of resin was added to a column and equilibrated with approximately 45 mL of Tris buffer (50 mM Trizma, 150 mM NaCl, pH 7.5). The supernatant was applied to the column and material that passed through the column was collected (column flow-through). Because of the His-tag present on the amino terminal of the recombinant protein, it was expected that any expressed DHFR-TS protein would bind to the cobalt column due to Lewis acid-Lewis base interactions. The column was washed with approximately 45 mL Tris buffer, to remove non-adhered protein in the column, and this was collected. Approximately 20 mL of Tris buffer containing 10 mM imidazole was added to the column to remove (wash) any loosely bound protein by competitive binding of imidazole to the resin; the wash was collected. Finally, 10 mL of Tris buffer containing150 mM imidazole was added, and 1 mL fractions were collected in microcentrifuge tubes. The 150 mM imidazole buffer was expected to remove any DHFR-TS tightly bound to the resin by the His-tag.

BIO-RAD ASSAY FOR PROTEIN CONCENTRATION DETERMINATION

The Bio-Rad reagent (Bio-Rad) was used for protein concentration determination in each fraction collected. The Bio-Rad Protein Assay causes a shift in absorbance from 465 nm to 595 nm when the reagent-dye is bound to protein.[55] A standard protein curve was developed using known concentrations of bovine serum albumin (BSA) and absorbance was measured at 595 nm. The following table (Table 4) was used to develop the standard protein curve (provided by J. Walker, Jan., 2015), while Figure 12 shows the standard curve and linear equation.

Tube	Volume of 0.1 mg/mL BSA	H_2O	Bio-Rad Reagent (Bio-Rad, Bio-Rad Protein Assay Dye Reagent Concentrate)	Protein Amount (µg)	Absorbance at 595 nm
1	0 µL	800 µL	200 µL	0	0.002
2	20 µL	780 µL	200 µL	2	0.133
3	40 µL	760 µL	200 µL	4	0.134
4	80 µL	720 µL	200 µL	8	0.344
5	120 µL	680 µL	200 µL	12	0.540
6	160 µL	640 µL	200 µL	16	0.634
7	200 µL	600 µL	200 µL	20	0.776

Table 4. Data for standard BSA protein curve provided by J. Walker.

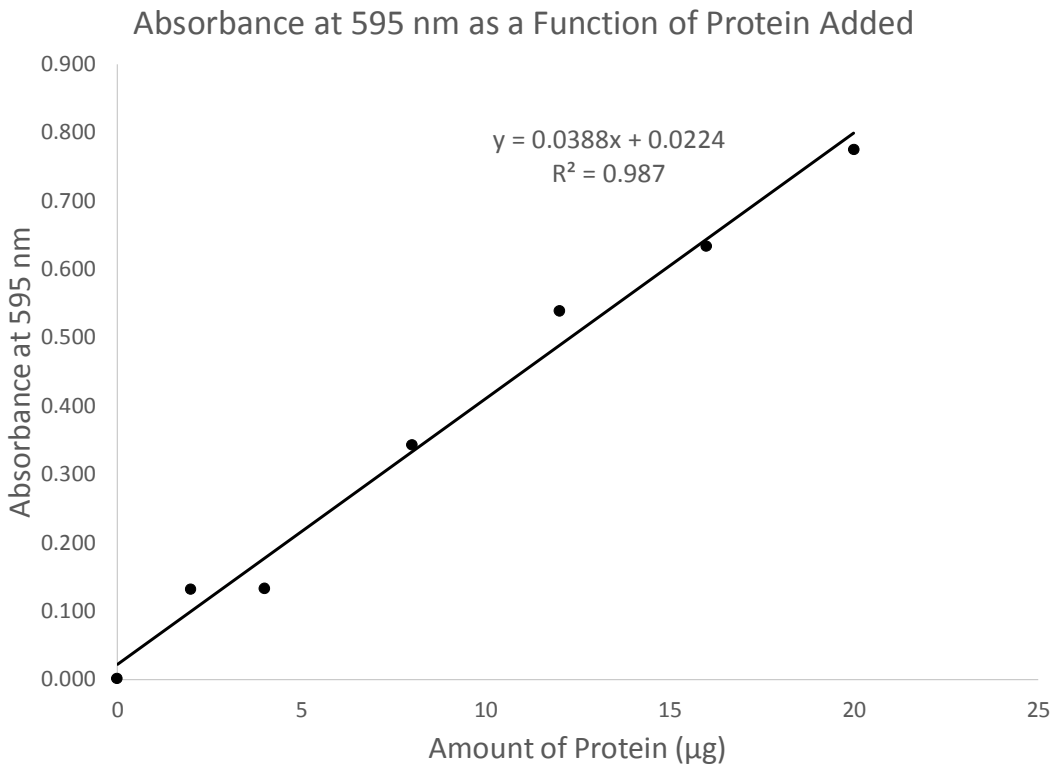


Figure 12. Standard protein curve for protein concentration determination.

Table 5 shows the volumes from each fraction used for protein determination. The absorbance was then measured at 595 nm using visible spectroscopy (Hewlett Packard, 8453 UV-Vis Spectrometer). Protein concentration was determined based on a standard absorbance as a function of concentration of bovine serum albumin (BSA) curve with the line equation y = 0.0388x + 0.0224, where "y" is absorbance at 595 nm and "x" is the apparent protein mass (µg). Protein concentration was calculated by dividing the amount of protein by the volume used in the assay.

Sample:	BioRad Reagent (Bio-Rad, Bio-Rad Protein Assay Dye Reagent Concentrate)	H_2O	Protein Sample
Blank	200 µL	800 µL	0 µL
Crude Extract	200 µL	798 µL	2 µL
Supernatant	200 µL	798 µL	2 µL
Column Flow-Through	200 µL	790 µL	10 µL
Buffer Wash	200 µL	700 µL	100 µL
10mM Imidazole	200 µL	700 µL	100 µL
150 mM Imidazole Fraction	200 µL	750 µL	50 µL

Table 5. Volumes of reagents used for Bio-Rad protein assay. Per the product information from Sigma-Aldrich, solutions containing less than 200 mM imidazole and 2.0 M Tris do not interfere with absorbance at 595 nm.[56]

SDS-PAGE PROTEIN ANALYSIS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate samples for protein expression using the general method of Laemmli.[57] DHFR-TS was expected to have a molecular weight of approximately 60 kilo-Daltons (kDa). Using gel electrophoresis, proteins in a sample can be separated based on apparent molecular weight.

SDS running gels were prepared by mixing 2.15 mL deionized (DI) water, 1.5 mL 40% acrylamide (Sigma), 1.75 mL 1.5 M Tris pH 8.8 buffer, 0.05 mL 10% (w/v) SDS, 10% (w/v) freshly made ammonium persulfate (APS), and 2 µL tetramethylethylenediamine (TEMED) (Sigma). This mix was quickly pipetted between the glass plates of an SDS-PAGE mold apparatus (Bio-Rad, Mini PROTEAN® System Glass Plates) as the protein resolving gel. Isopropanol was added to create a level

interface for the stacking gel portion. Once the protein resolving gel polymerized, the isopropanol was removed and the stacking gel, consisting of 1.5 mL DI water, 0.25 mL 40% acrylamide, 0.25 mL 1 M Tris pH 6.8 buffer, 0.02 mL 10% SDS, 20 µL APS, and 2 µL TEMED, was added and the well comb was inserted. SDS gels were typically made in batches of 2-4 and were stored in 1x SDS-buffer solution at 5℃ for later use.

SDS-PAGE protein samples were prepared with no more than 15 µg of protein for non-purified samples and no more than 2.5 µg of protein for purified samples to be loaded on the gel. The appropriate volume of protein solution and 4 µL 4x SDS dye were combined, with Nanopure water being added to a final volume of 20 µL. Pellet samples were resuspended in 20 µL 1x SDS dye. The samples were boiled for 5 min to denature the proteins before loading. Precision Kaleidoscope Standard (Bio-Rad, Precision Plus Protein™ Kaleidsocope™ Prestained Protein Standards; 5 µL) was used to evaluate protein molecular weights (Figure 13). The 1x SDS-PAGE Running Buffer (25 mM Trizma, 250 mM glycine, 0.1% (w/v) SDS, pH 8.3) was added to the SDS-PAGE chamber. Then, 150 V was applied and the current was allowed to pass through the gel until the blue dye front ran off the gel. Coomassie blue stain was used to visualize the protein bands, and de-stain, composed of 45% methanol, 10% acetic acid, and 45% water (v/v/v), was applied to remove stain from regions of the gel devoid of proteins. Gels were preserved by lamination as necessary.

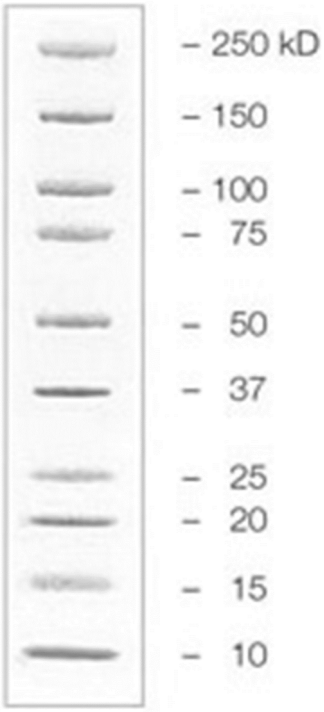


Figure 13. Precision Plus Protein™ Kaleidsocope™ Prestained Protein Standards.[58]

DHFR ENZYME ACTIVITY ASSAY

In a UV transparent cuvette, purified *L. major* DHFR-TS protein was incubated with 400 µM final concentration NADPH (Sigma) in pH 6.5 Tris, 150 mM imidazole buffer at room temperature, after blanking with pH 6.5 Tris, 150 mM imidazole buffer. After approximately 5 min of incubation, 50 µM final concentration dihydrofolic acid was added. Absorbance was measured at 340 nm utilizing the kinetic mode (Hewlett Packard 8453 UV-Vis spectrophotometer, HP 8453A software). Because NADPH absorbs at 340 nm, if the DHFR-TS enzyme is active, a reduction in absorbance at that

wavelength would be expected with the oxidation of NADPH to $NADP^+$ in a stoichiometric reduction of dihydrofolate to tetrahydrofolate.[59]

CHAPTER III

RESULTS AND DISCUSSION

LEISHMANIA GROWTH CURVES WITH AND WITHOUT SULFONAMIDES

The promastigote form of axenic *Leishmania tarentolae* was grown to the log phase of the growth curve (day 2) then treated with a 100 µM concentration of three sulfonamides (Figure 14): *N*-(2'-methylthiophenyl)-1-napthalne sulfonamide (Compound A), *N*-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), and *N*-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C) (provided by Dr. Hamaker).

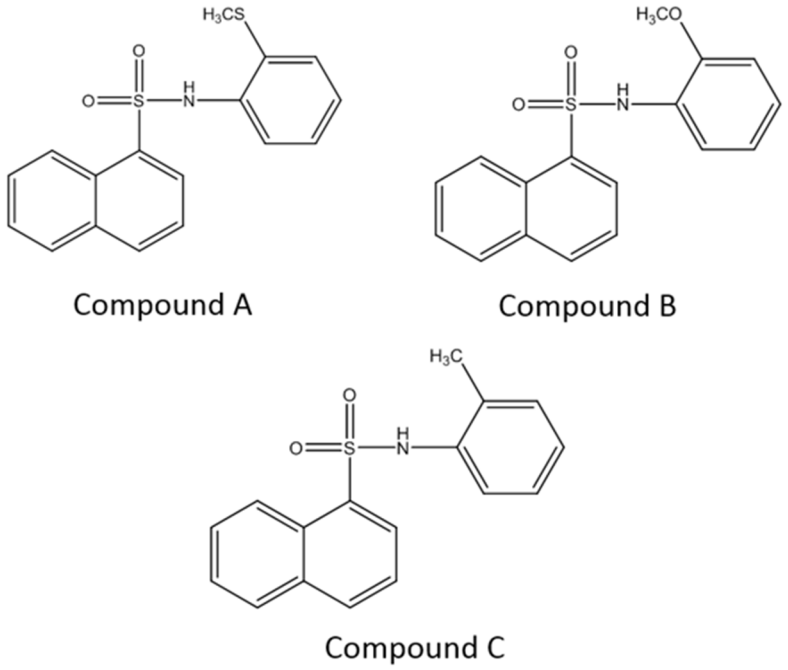


Figure 14. Chemical structures of *N*-(2'-methylthiophenyl)-1-napthalene sulfonamide (Compound A), *N*-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), and *N*-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C).

Upon addition to cells grown in BHI, water solubility issues were seen at this concentration for the three sulfonamides with crystals forming within 24 hours after addition. Significant inhibitory effects on cell viability were seen with a 100 µM addition of Compound C, but were not seen with the other two sulfonamides (Figure 15). Within 24-48 hours after addition of Compound C, an 85-95% reduction in cell viability was observed. Cells transferred to fresh medium were unable to recover over three days, which was an indication of cell death.

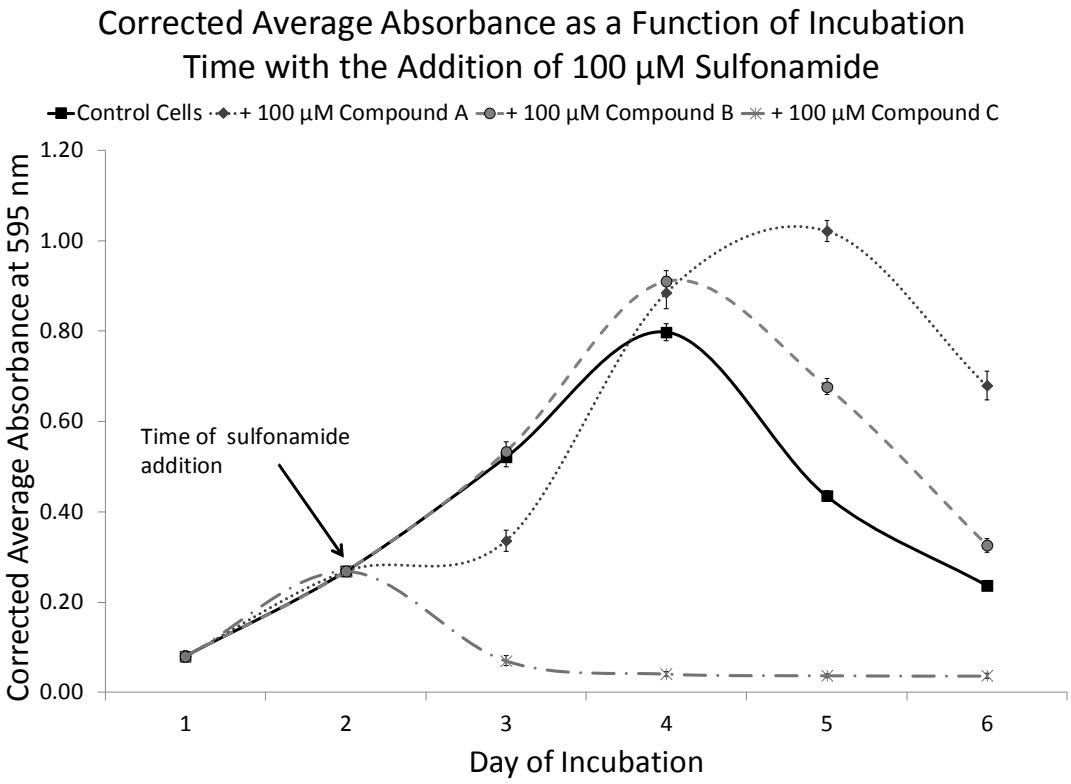


Figure 15. MTT cell viability results after addition of 100 µM sulfonamides for representative experiment. One percent DMSO was added to control cells. Values are the mean ± standard deviation for n = 4 replicates.

Microscopy was also used to evaluate the cells in the presence of the sulfonamides. Addition of 100 µM Compound C caused cells to maintain typical shape, but, per the MTT cell viability assay, lose viability. This is different than typical senescence phase cell death, which is visually characterized by cells becoming spherical in shape. This effect on cell shape was noted for each 100 µM Compound C experiment. Compounds A and B did not negatively affect viability or motility, whereas cells lost motility in the presence of Compound C. It appeared that Compounds A and B had a positive effect on the *Leishmania*, increasing the viability and extending the culture lifetime relative to control cells.

Because of the water solubility issues with Compound C, it was not possible to know the effective concentration (EC_{50}) of Compound C that caused negative cell responses. To determine if lower concentrations could provide similar results 10 µM or 50 µM concentrations of Compound C were added to cell culture and these cells were evaluated for viability. As seen in Figure 16, lower concentrations of Compound C did not have the same inhibitory effect without recovery over time. The 10 µM concentration appeared to have an activating effect on cells, more like that seen for Compounds A and B. The 50 µM concentration appeared to have some negative effect on cell viability, but crystal formation was still seen within 24 hours of addition, meaning the cells were likely not exposed to the full 50 µM concentration. The water solubility issues also prevented multi-day compound additions.

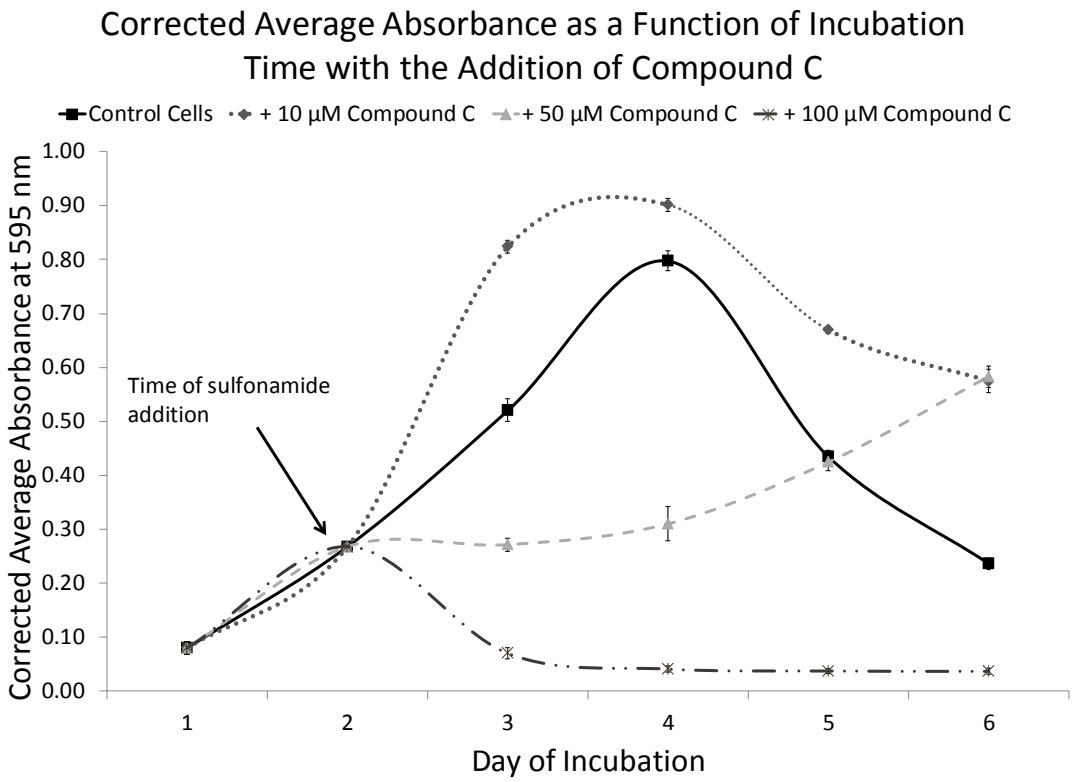


Figure 16. MTT cell viability of cells with 10 µM, 50 µM, or 100 µM additions of Compound C. One percent DMSO was added to control cells. Values are the mean ± standard deviation for n = 4 replicates.

SYNTHESIS OF WATER SOLUBLE STRUCTURAL ANALOGS AND INHIBITION TESTING

Because insolubility of compounds in water can affect how cells access and utilize compounds, water solubility can be an important characteristic for therapeutics. When compounds re-crystalize in the growth medium, the optimal treatment concentration cannot be easily evaluated and multiple doses cannot be utilized. To address the water solubility concerns seen with the most inhibitory compound, two water-soluble analogs of Compound C were synthesized (Figure 17). Because the group ortho to the amide in Compounds A, B, and C differentiated between cellular

inhibition, a structural analog of Compound C with a carboxyl group para to the amide was synthesized (Compound D) and a second compound without the functional group at the 2'-phenyl position but with a carboxyl para to the amine (Compound E) was also synthesized and characterized.

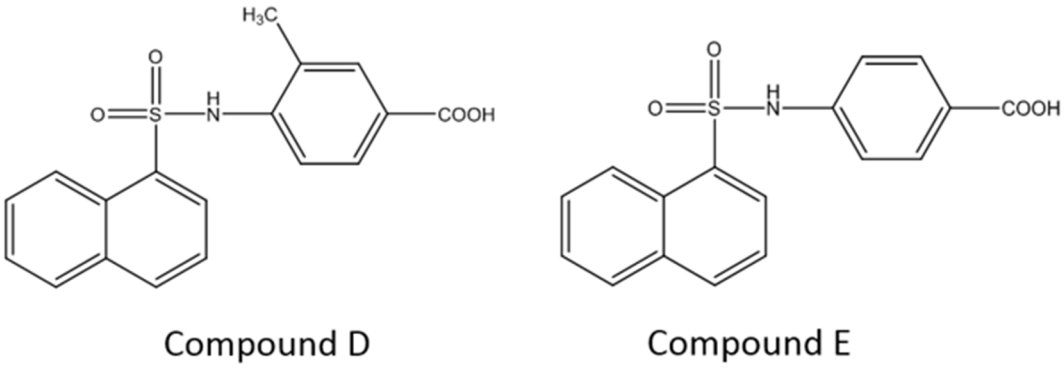


Figure 17. Chemical structures of *N*-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D) and *N*-(4-carboxyl)-1-napthalene sulfonamide (Compound E).

Compounds D or E were added to cells at 100 µM concentrations and cell viability was evaluated with an MTT assay. The results in Figure 18 were plotted as a percentage of control cells (1% DMSO added), and show Compound E had only modest inhibition of cell viability and was only tested for 2 days after addition, while Compound D had some inhibitory effect on cell viability at days 1-3 after addition (approximately 70% inhibition) with viability recovery four days after addition. Where results were compared to Compound C, less inhibition and more apparent cell recovery was

observed over the 4 days, unlike results with Compound C (approximately 90-96% inhibition without recovery). However, because Compound D is water soluble, multiple additions of the compound can be applied to the cells.

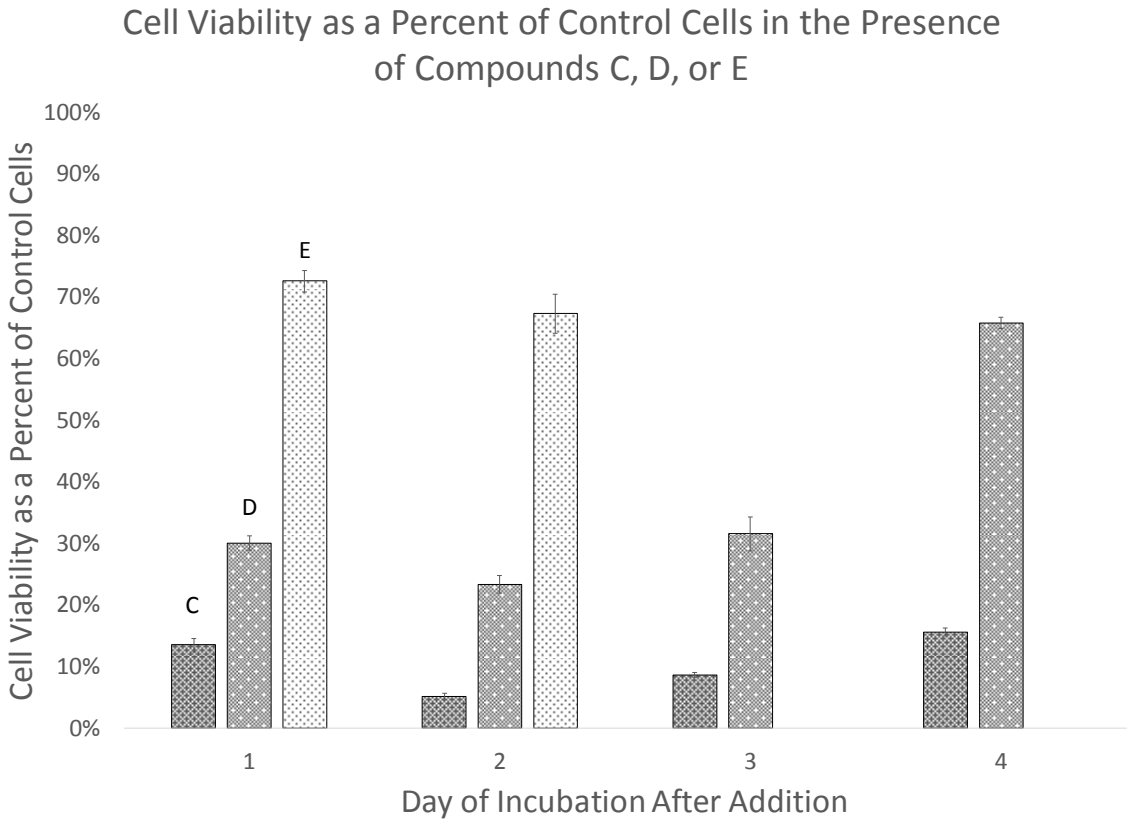


Figure 18. Cell viability as a percent of control cells in the presence of 100 µM Compound C, D, or E. Values are the mean ± standard deviation for n = 4 replicates.

To determine if inhibitory effects could be accumulative for Compound D, two sequential doses of 50 µM Compound D were added to cells and evaluated for effect on

cell viability. As seen in Figure 19, two sequential doses of 50 µM Compound D were equivalent on day 5 to a single 100 µM addition of Compound D.

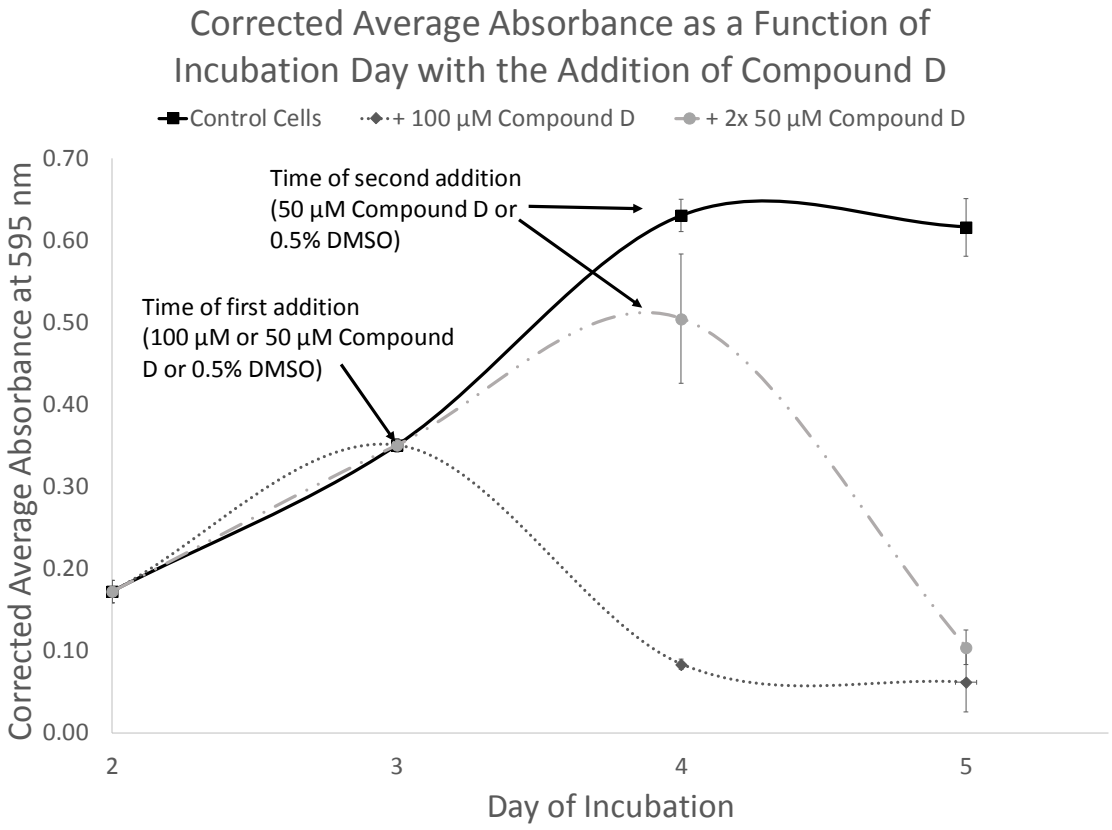


Figure 19. Representative growth curve showing the impact of two doses of 50 µM Compound D added on day 3 and day 4 on cell viability compared to single dose of 100 µM Compound D added on day 3. One percent DMSO was added to control cells. Values are the mean ± standard deviation for n = 4 replicates.

When evaluated by microscopy, the cells treated with Compound D had a difference appearance than those treated with Compound C. Cells treated with

Compound C appeared to maintain a typical cell shape but with greatly reduced motility, though MTT cell viability results indicated cell inhibition. Cells treated with Compound D presented a cell shape more similar to typical senescence. The visual difference in cells after treatment implies different pathways of inhibition for these two sulfonamides.

Half maximal effective concentration (EC_{50}) is the concentration of a treatment that induces a response that is 50% of the maximum response after a specific exposure time. EC_{50} was calculated for Compound D by plotting the percent cell viability (percent of control cells) after 24 hours of exposure as a function of concentration added. As can be seen in Figure 20, the EC_{50} was determined to be approximately 74 µM for this study.

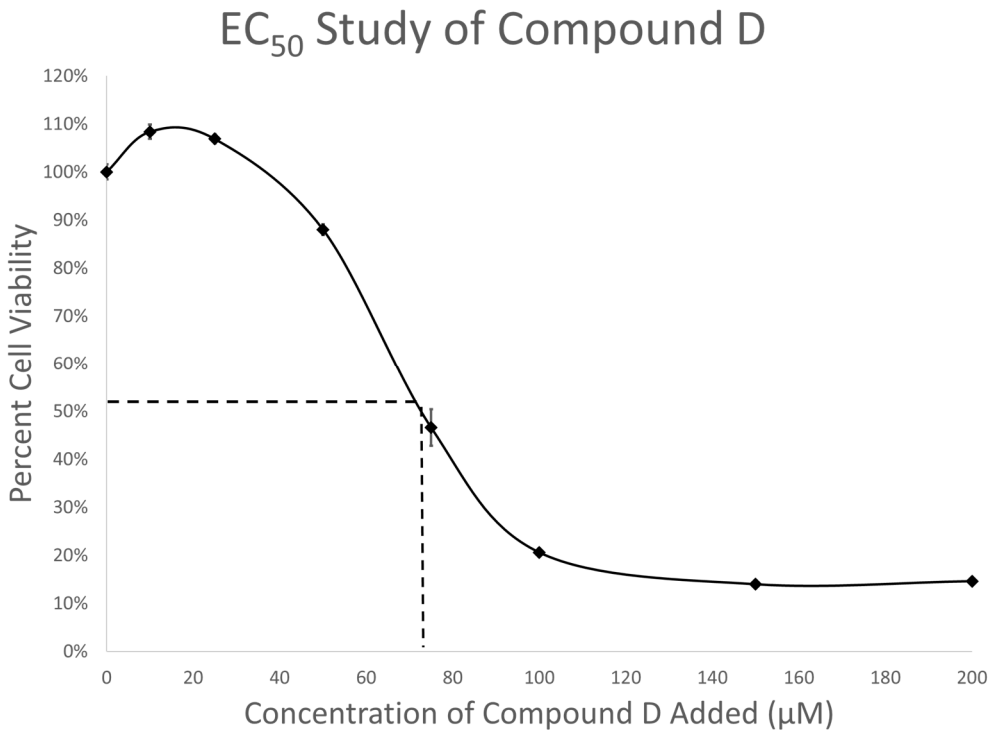


Figure 20. Determination of approximate EC_{50} for Compound D. EC_{50} was calculated to be approximately 74 µM.

EFFECTS OF FOLATE ON CELL VIABILITY WITH AND WITHOUT SULFONAMIDES

Sulfonamides are known inhibitors of dihydrofolate reductase (DHFR), an enzyme in the folic acid cycle of bacteria, preventing folate from being reduced to dihydrofolate and dihydrofolate from being reduced to tetrahydrofolate.[7] To determine if Compounds C or D could be inhibitors of DHFR, 100 µM folic acid and 100 µM Compound C or D were added to cells in the log phase and evaluated for cell viability compared to control cells.

As can be seen in Figure 21, addition of an equivalent concentration of folic acid as Compound C provided approximately 50% more cell viability than cells treated with Compound C alone. However, cells treated with an equivalent amount of folic acid and Compound D did not exhibit protection. This also indicates the likelihood of different pathways of inhibition for these two sulfonamides. A Students two tail statistical t-test, where $p < 0.05$ is considered significant, confirmed that mean cell viability as a percent of the control cells indicated statistically different responses with the addition of Compound C only or Compound C with 100 µM folic acid. However, there was no statistical difference with the addition of Compound D only or Compound D with 100 µM folic acid.[50]

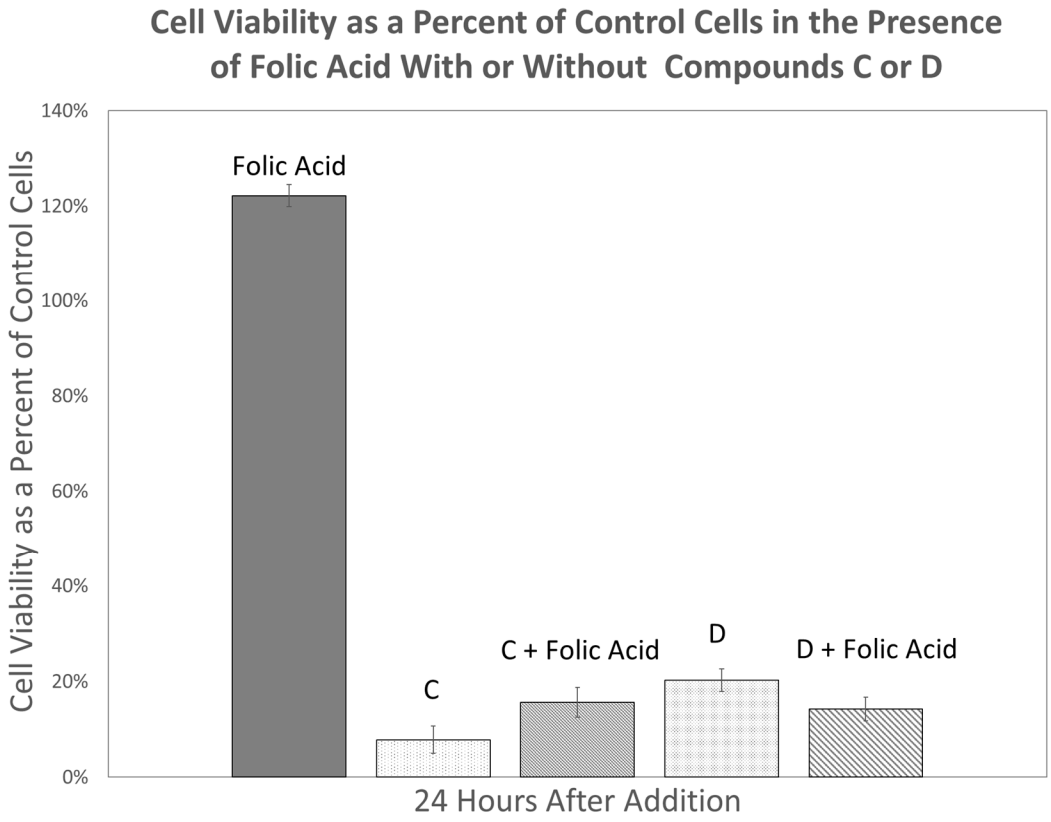


Figure 21. Effects of folic acid or folic acid with Compound C or D on cell viability as a percent of control cells. Values are the mean ± standard deviation for n = 4 replicates.

RECOMBINANT DHFR-TS EXPRESSION AND EVALUATION

To evaluate the effects of the sulfonamide inhibitors on DHFR function, recombinant DHFR-TS from *L. major* was expressed, extracted, purified, and activity evaluated by measuring the rate of NADPH oxidation to $NADP^+$. Using PCR, recombinant DNA for *L. major* DHFR-TS was synthesized. Agarose gel electrophoresis suggested the recombinant DNA contained the proper number of base pairs expected for the gene. The nucleotide sequence of the DHFR-TS gene, containing 1563 nucleotides, is shown in Figure 22.[51]

5′–
atgtccagggcagctgcgaggtttaagattccgatgccggagacgaaggcagactttgctttcc
cctccctgcgcgccttctccatcgtcgtggccctcgatatgcagcacggcatcggcgacggcga
gtcgatcccgtggcgggtgccggaggacatgacgttttcaagaaccagacgacgctgctgcgc
aacaagaagccgccgacggagaagaagcgcaacgccgtcgtgatgggccgcaagacttgggaga
gcgtcccggtaaagttccgaccactcaagggacggctgaacatcgtgttatcctcgaaggccac
cgtcgaggagcttctggcgccgctgccggagggacagcgcgcggcggcggcgcaggatgtggtg
gtggtgaacggcggtctggccgaggcgctccgcctcctcgcacgcccgctgtactgcagctcca
tcgagacagcgtattgcgtcggtggtgcgcaggtttacgcggacgccatgctgtcgccgtgcat
cgagaaactgcaggaagtgtacctgacccgcatctacgcgacggcgcctgcgtgtacgcgcttc
tttccgtttccgcccgagaacgcggccacggcgtgggacctggcgtcgtctcagggacgccgca
agagcgaggcggagggcctcgagttcgagatctgcaagtacgtgccgcgcaaccacgaggagcg
gcagtaccttgagctgattgaccgcatcatgaagacggggatcgtgaaggaggaccgcaccggc
gtgggcaccatcagcctcttcggcgcccagatgcgcttctccctacgcgacaaccgcctgccgc
tgctgacgacgaagcgtgtcttctggcgcggcgtgtgcgaggagctgctgtggttcctgcgcgg
ggagacgagtgcgcagctgctggcagacaaggacattcacatctgggacggcaacggttcgcgc
gagtttctcgacagccgcggcttgacagagaataaggagatggacctcggccctgtctacggct
tccagtggcgccacttcggggcagattacaaggggtttgaagcgaactacgacggcgaaggggt
ggaccagatcaagctcatcgtggagaccatcaagacgaacccgaacgaccgccgcctcctagtc
actgcctggaacccgtgcgcgctgcaaaagatggcgctgccgccgtgccacttgcttgctcagt
tctacgtgaacacagacacgagcgagctatcctgcatgttgtaccagcgctcgtgtgacatggg
tcttggcgtccccttcaacattgcctcctacgcgctgctcaccatcctcattgccaaggcgacg
ggtctgcggcctggtgagcttgtgcacaccctcggcgacgcccacgtctaccgcaaccacgttg
atgccctcaaggcgcagctcgagcgagtcccgcacgcgttcccgaccctcatcttcaaggagga
gcggcagtacctcgaggactacgagttgacggacatggaggtgatcgactacgttccacacccg
gcgatcaagatggagatggccgtatag – 3′

Figure 22. Nucleotide sequence for the coding single strand of the *L. major* DHFR-TS gene.[51]

The agarose gel electrophoresis for the digested pET-45b plasmid and the recombinant DHFR-TS gene are shown in Figure 23. The DNA bands (circled in lanes 2 and 3) were cut from this gel for the pET-45b plasmid (lane 2) and DHFR-TS gene (lane 3) for ligation.

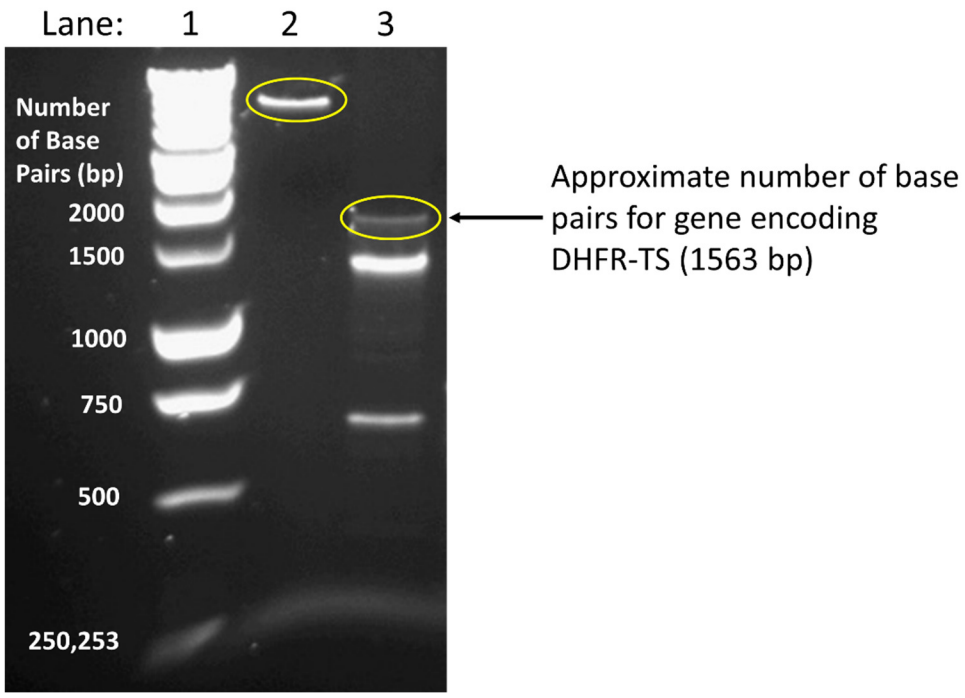


Figure 23. Agarose gel electrophoresis of digested pET-45b and recombinant DHFR-TS gene used for ligation. Lane 1 is the standard 1 kb Ladder, lane 2 is the digested pET-45b plasmid, and lane 3 is the digested recombinant DHFR-TS gene.

After ligation, to ensure the DHFR-TS gene was inserted into the plasmid and expressed in the dam$^-$ dcm$^-$ *E. coli* cells, a BamHI and HindIII digestion was performed on the purified DNA and an agarose gel was used to evaluate the digested components. The agarose gel is shown in Figure 24. The gel provided evidence that the gene was inserted into the pET-45b plasmid and could be transformed into *E. coli* cells to express protein.

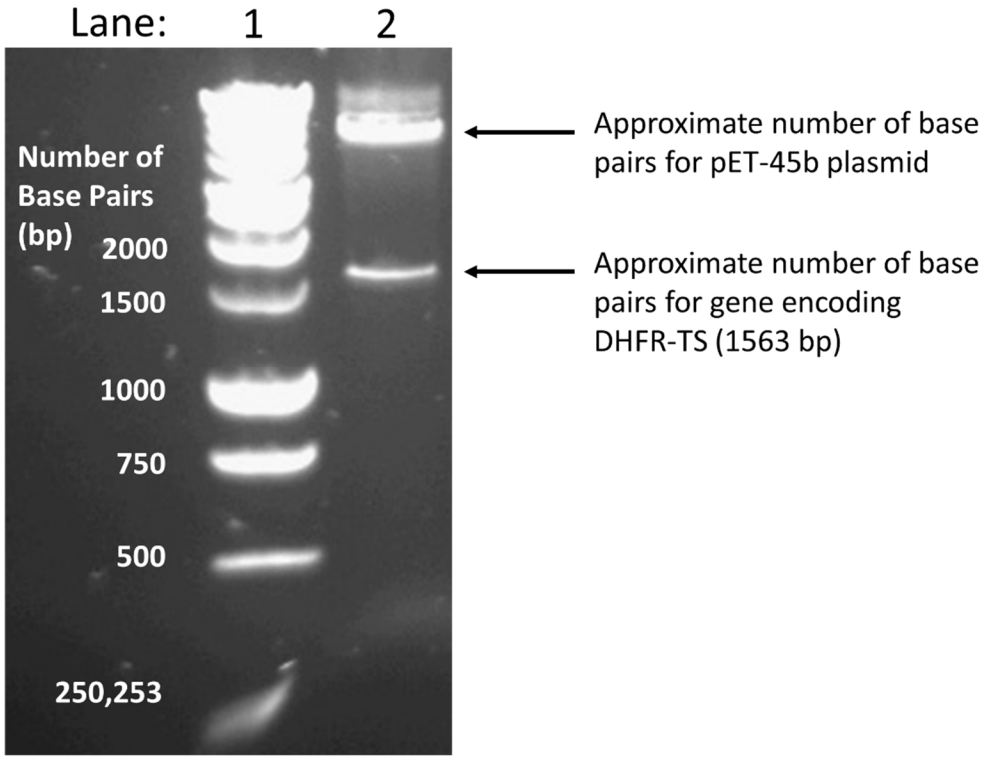


Figure 24. Agarose gel electrophoresis of digested plasmid DNA from dam$^-$ dcm$^-$ *E. coli*. Lane 1 is the standard 1 kb ladder and lane 2 is the digested pET-45b plasmid containing the DHFR-TS gene.

The concentration of the purified DNA, pET-45b with inserted recombinant DHFR-TS gene was determined using a NanoDrop 2000c Spectrophotometer (ThermoScientific) by measuring absorbance at 260 nm. The estimated concentration was found to be 25 ng/µL. Samples of the DNA were sent to the University of Illinois Urbana-Champaign (UIUC) Core Sequencing Facility in Urbana, IL for DNA sequencing from the promoter and terminal ends of the DNA for a more accurate output. The data from UIUC can be seen in Appendix C. Some nucleotides in both sequences could not be assigned automatically by the software, but manual review of the raw trace allowed

for some nucleotides to be replaced with the most likely option. An example trace is shown in Appendix D.

A Basic Local Alignment Search Tool (BLAST) search was performed to identify regions of similarity between the gene sequencing provided by UIUC and a vast database of nucleotide sequences from various species.[60] It is important to note that most sequencing methods can become less reliable around 700 nucleotides, so it is important to evaluate both the promotor and terminal directions of the DNA of interest. A 96% agreement was identified between nucleotides 190-816 of the sequenced promoter DHFR-TS gene and the sequence from which the primers were designed, Sequence ID M12734.1.[51] After the terminal sequence of the DHFR-TS gene was reversed and converted to its complimentary nucleotides, 97% agreement was found between nucleotides 399-1269 of the sequenced terminal DHFR-TS gene and the sequence from which the primers were designed.[51] However, 395 nucleotides of the recombinant DHFR-TS gene were inadequately sequenced. Two additional primers were designed to allow for more accurate sequencing of those nucleotides and data was, again, acquired from UIUC. The alignments are shown in Appendix E.

The sequenced data were combined, based on the alignment data, and the nucleotide sequences were translated to amino acids using the translation software from ExPasy.[61] Amino acid alignments were performed between the translated DNA sequences from the promoter and terminus gene sequences and DHFR-TS from *L. major* (M12734)[51] using Kalign Multiple Sequence Alignment [62] and BoxShade software.[63] The alignment is shown in Figure 25. There was 100% alignment between

the amino acid sequences of the *L. major* DHFR-TS from the Gene Bank and the recombinant *L. major* DHFR-TS.

```
DHFRTS          1 MSRAAARFKIPMPETKADFAFPSLRAFSIVVALDMQHGIGDGESIPWRVPEDMTFFKNQT
SeqRecGene      1 MSRAAARFKIPMPETKADFAFPSLRAFSIVVALDMQHGIGDGESIPWRVPEDMTFFKNQT

DHFRTS         61 TLLRNKKPPTEKKRNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGQR
SeqRecGene     61 TLLRNKKPPTEKKRNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGQR

DHFRTS        121 AAAAQDVVVVNGGLAEALRLLARPLYCSSIETAYCVGGAQVYADAMLSPCIEKLQEVYLT
SeqRecGene    121 AAAAQDVVVVNGGLAEALRLLARPLYCSSIETAYCVGGAQVYADAMLSPCIEKLQEVYLT

DHFRTS        181 RIYATAPACTRFFPFPPPENAATAWDLASSQGRRKSEAEGLEFEICKYVPRNHEERQYLEL
SeqRecGene    181 RIYATAPACTRFFPFPPPENAATAWDLASSQGRRKSEAEGLEFEICKYVPRNHEERQYLEL

DHFRTS        241 IDRIMKTGIVKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGVCEELLWFLRGE
SeqRecGene    241 IDRIMKTGIVKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGVCEELLWFLRGE

DHFRTS        301 TSAQLLADKDIHIWDGNGSREFLDSRGLTENKEMDLGPVYGFQWRHFGADYKGFEANYDG
SeqRecGene    301 TSAQLLADKDIHIWDGNGSREFLDSRGLTENKEMDLGPVYGFQWRHFGADYKGFEANYDG

DHFRTS        361 EGVDQIKLIVETIKTNPNDRRLLVTAWNPCALQKMALPPCHLLAQFYVNTDTSELSCMLY
SeqRecGene    361 EGVDQIKLIVETIKTNPNDRRLLVTAWNPCALQKMALPPCHLLAQFYVNTDTSELSCMLY

DHFRTS        421 QRSCDMGLGVPFNIASYALLTILIAKATGLRPGELVHTLGDAHVYRNHVDALKAQLERVP
SeqRecGene    421 QRSCDMGLGVPFNIASYALLTILIAKATGLRPGELVHTLGDAHVYRNHVDALKAQLERVP

DHFRTS        481 HAFPTLIFKEERQYLEDYELTDMEVIDYVPHPAIKMEMAV
SeqRecGene    481 HAFPTLIFKEERQYLEDYELTDMEVIDYVPHPAIKMEMAV
```

Figure 25. Amino acid alignment of recombinant *L. major* DHFR-TS gene to DHFR-TS gene from *L. major* (Genebank ID: M12734).[51] DHFRTS identifies the amino acid sequence of the *L. major* gene from the gene bank (M12734) and the SeqRecGene identifies the amino acid sequence of the combined, sequenced recombinant *L. major* amino acid sequence.

Because a crystal structure of the bifunctional enzyme, DHFR-TS, from *L. major* does not currently exist, a crystal structure from *Trypanosoma cruzi* (Protein Data Bank ID: 3HBB)[64] was used instead for identification of amino acids important to the active site and activity of the enzyme. Figure 26 shows the amino acid comparison of *L. major* and *T. cruzi*, where the amino acids believed to be associated with the active site are

highlighted in blue (dihydrofolic acid interaction) and red (NADPH interaction). The DHFR portion of the two enzymes does not align completely, but the active sites within the enzyme do share common amino acids, making the *T. cruzi* crystal structure beneficial in evaluating mutations that may be seen in the sequencing data.

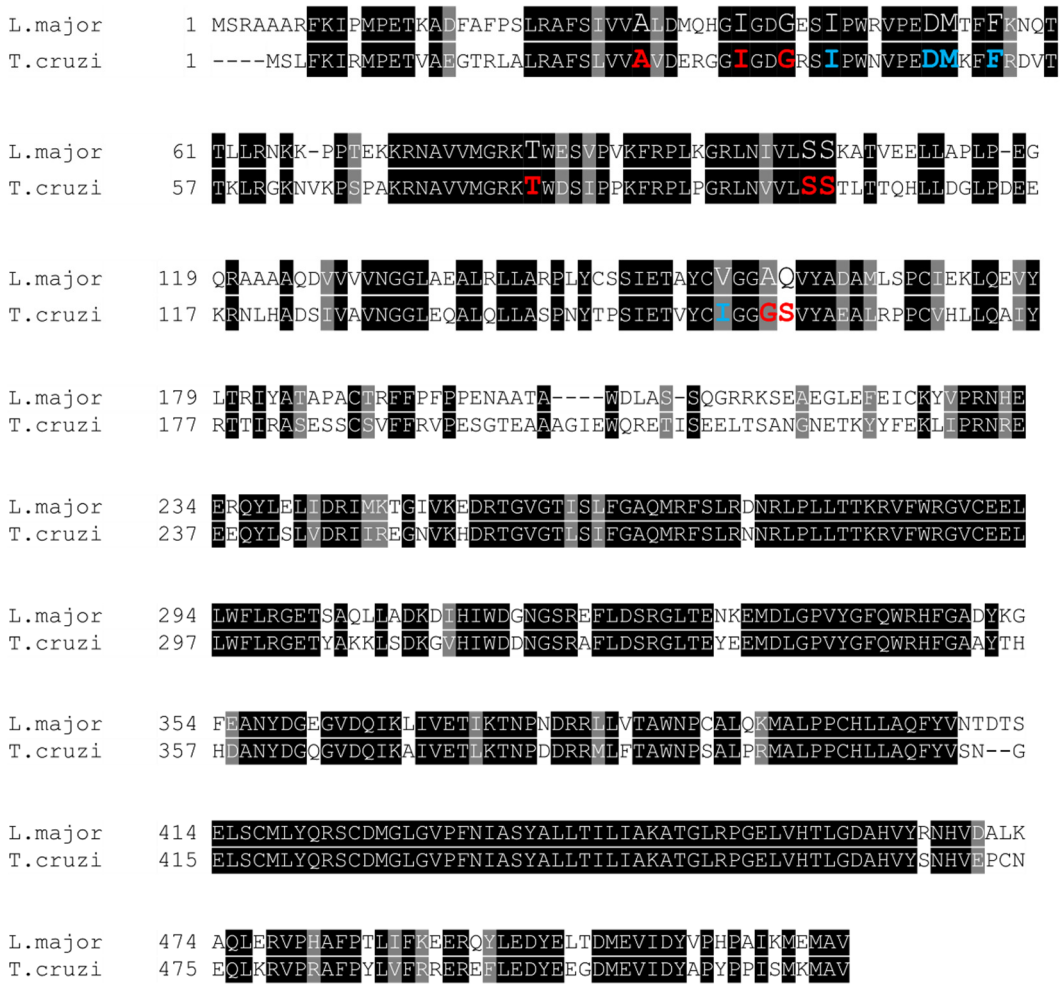


Figure 26. Amino acid alignment of *L. major* and *T. cruzi* DHFR-TS. Amino acids highlighted in blue were identified as binding to dihydrofolic acid and red were identified as binding to NADPH.

PROTEIN EXPRESSION, PURIFICATION, AND KINETICS ASSAYS

Once the DHFR-TS gene was inserted into the plasmid, the plasmid was transformed into competent RILP *E. coli* cells. The protein was extracted, purified, and protein concentration was calculated for each fraction using a Bio-Rad Assay. Calculated protein concentrations are shown in Table 6.

	Calculated Protein Concentration (µg/µL)
Crude	3.3-9.8
Supernatant	1.1-8.0
Column Flow-Through	0.5-2.2
Buffer Wash	0.12-0.21
150 mM Imidazole Fractions	0.04-0.2

Table 6. Calculated protein concentrations from protein purification.

SDS-PAGE was used to determine if the DHFR-TS protein was expressed in the *E. coli*. Aliquots from the lysate (crude extract), supernatant from the crude extract, column flow through, buffer wash, 10 mM imidazole wash, and 150 mM imidazole fractions from the cobalt affinity purification process were first evaluated for protein concentrations using the Bio-Rad assay for protein determination. Based on the results for this work, the appropriate concentration of each aliquot was prepared and SDS-PAGE was used to evaluate protein present, with specific interest in a band appearing at approximately 60 kD. The molecular weight of DHFR-TS is 58,689 g/mol without the

His-tag and 59,511 g/mol with the His-tag. A representative gel with expressed protein at the approximate molecular weight for DHFR-TS is shown in Figure 27.

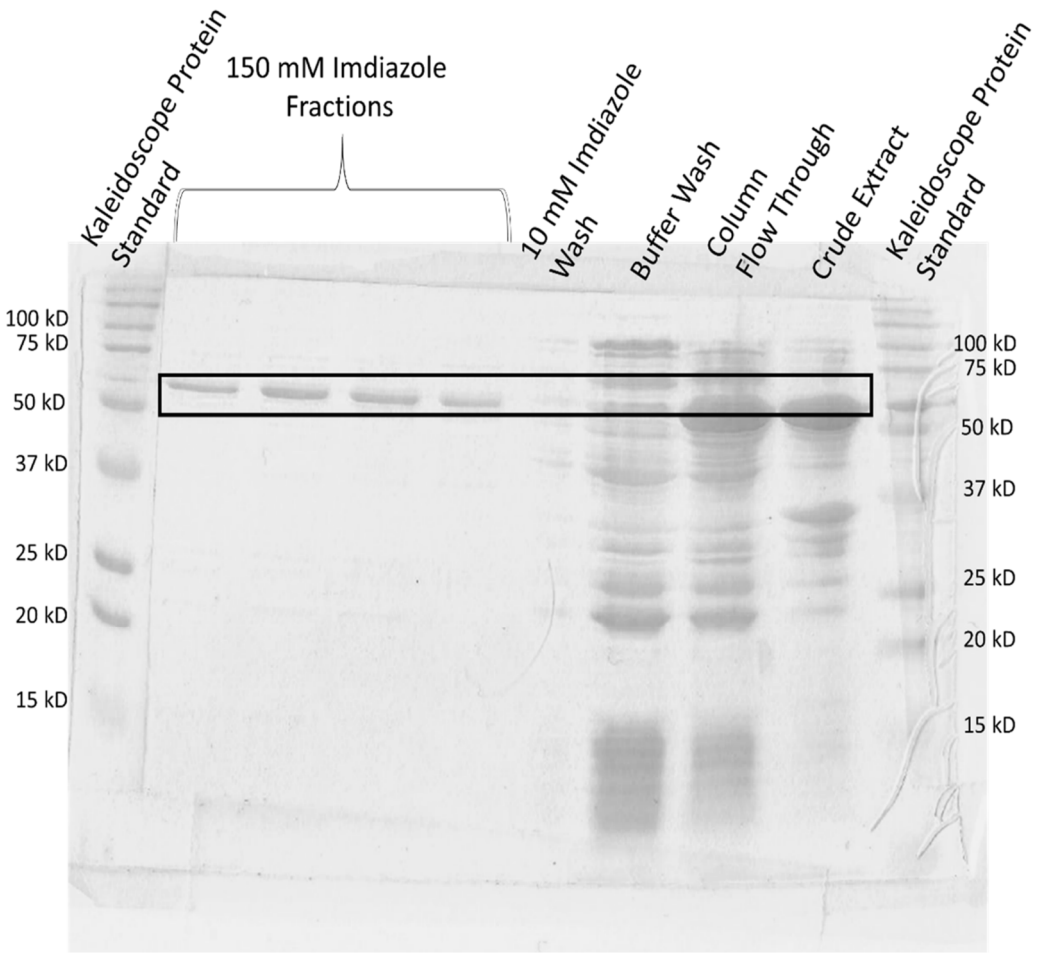


Figure 27. SDS-PAGE gel of crude lysed cells, column flow through, buffer wash, 10 mM imidazole wash, and 150 mM fractions containing protein. The DHFR-TS enzyme has a molecular weight of 59,511 g/mol (60 kD) with the His-tag. A band was identified at approximately 60 kD, indicating DHFR-TS was likely extracted and partially purified.

Because DHFR-TS is soluble and should contain a His-tag, the band shown in the fractions of 150 mM imidazole were expected to be the protein of interest. Fractions containing the protein of approximately the correct molecular weight of DHFR-TS were

evaluated for enzyme kinetic activity. The assay for DHFR activity involved measuring the absorbance reduction at 340 nm as NADPH was oxidized to $NADP^+$ in the presence of dihydrofolic acid. For all column fractions obtained in this experiment, enzyme assays were performed, but no catalytic activity was detected.

One possible reason for lack of enzyme activity could be instability of the DHFR-TS bifunctional enzyme. Studies to characterize the bifunctional DHFR-TS of *Trypanosoma brucei*, another protozoan parasite, have shown instability of the DHFR-TS complex typically caused by the more insoluble TS region of the protein.[65] To improve the stability of the bifunctional enzyme, the authors used an *E. coli* elongation factor, Ts, which was designed upstream of the His-tag. The addition of this elongation factor improved protein stability and allowed for activity of the DHFR and TS portions of the protein.[65] Elongation factors are proteins used in the synthesis of proteins and are present in large quantities in cells to help elongate peptide chains. Ts is a guanine-nucleotide exchange factor that catalyzes mediation of the displacement of a byproduct of elongation, guanosine diphosphate (GDP), by Tf (a highly abundant elongation factor). Then guanosine triphosphate (GTP), an energy source necessary for the peptide bond formation[66], is inserted allowing for potential improvement in protein solubility.

Lack of enzyme activity could also be due to protein misfolding. Chaperonins are proteins found in all cells that help fold proteins into their proper three-dimensional structure. GroEL and GroES are *E. coli* chaperonins that have been well characterized and studied, and have been found to help fold proteins which is accompanied by the

hydrolysis of adenosine triphosphate (ATP).[67] Chaperonins may work well with many proteins. However, when *E. coli* are forced to grow quickly and exhibit high protein expression, there is more opportunity for misfolding of heterologous proteins. Using a strain of *E. coli* that grows at low temperatures can improve the accuracy of folding activity in the cells. The *E. coli* strain, ArcticExpress competent cells, improves protein folding as the cells also express cold-temperature chaperonins Cpn10 and Cpn60.[68] Use of ArcticExpress cells can increase the amount of active, soluble protein present, improving the possibility of enzyme activity.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

SULFONAMIDE EFFECTS ON *LEISHMANIA* PROMASTIGOTE VIABILITY

Promastigote and amastigote forms of *Leishmania* differ by shape, motility, and gene expression. The amastigote form of the parasite is commonly found in a phagocytic cell, such as the macrophage, of the vertebrae host, while the promastigote form is considered the infective form of the parasite. Targeting the promastigote form of the life cycle could provide prevention of further infection of phagocytic cells and be an effective mechanism of treatment for the different forms of leishmaniasis.

In this study, newly synthesized sulfonamides and water-soluble analogs were evaluated for their inhibitory effects on the growth and viability of *Leishmania tarentolae* promastigotes. *N*-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C) was seen to inhibit cell viability 90-96% 24 hours after 100 μM addition, though crystallization was seen in the medium and effective concentration (EC_{50}) was not able to be determined. Other sulfonamides tested in this experiment, *N*-(2'-methylthiophenyl)-1-napthalene sulfonamide (Compound A) and *N*-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), with larger, more electronegative groups ortho to the amine group, exhibited no negative effect on cell viability or acted as a vitamin to the parasites, improving cell viability. These data provide insight into the interaction of the sulfonamide with the inhibitory target (enzyme, transport protein, or other), in that the small, non-polar functional group ortho to the amine must have some important interaction that affects function of the target.

The differences that structural changes have on inhibitory effects could also be seen with the addition of a carboxyl group para to the amine group. The water-soluble analog, *N*-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D), exhibited approximately 70% cell viability inhibition after 24 hours with 100 μM addition, while the water-soluble analog without a methyl group para to the amine group exhibited only approximately 30% inhibition with one addition of 100 μM. Furthermore, differences in cell behavior were also documented. Cells treated with 100 μM Compound C appeared to be paralyzed 24 hours after treatment, as microscopy indicated cells were without movement but maintained the promastigote cell shape. In contrast, cells evaluated 24 hours after treatment with 100 μM Compound D appeared to lose their flagella and were more circular in shape, a cellular change more commonly associated with cell death; however, both samples of cells were transferred to fresh medium and little to no cell recovery was noted. This observation leads to the assumption that there is a difference in inhibitory mechanism between the two compounds.

Because Compound C was insoluble in the cellular medium at concentrations from 10 μM to 100 μM, an EC_{50} value could not be determined. However, the EC_{50} value for Compound D obtained and compared to those previously reported[7] as seen in Table 7. The EC_{50} values show that a lower concentration of Compound D, with a shorter exposure time, is a more effective anti-leishmanial compound compared to other compounds reported by Peixoto and Beverley.[7]

Compound	EC_{50} after 48 hours
Sulfamoxole	150 µM
Sulfaquinoxaline	600 µM
Dapsone	600 µM
Sulfadimethoxine	>2 mM
Sulfanilamide	>2 mM
Sulfaguanidine	>2 mM
Sulfadiazine	>2 mM
Sulfabenzamide	>2 mM
Sulfamonomethoxine	>2 mM
Sulfamethoxazole	>2 mM
Sulfanitran	>2 mM
Sulfthiazole	>2 mM
Sulfamethizole	>2 mM
Sulfixsoxazole	>2 mM
N-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D)	74 µM*

Table 7. Comparison of EC_{50} values for previously published compounds compared to *N*-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D). All EC_{50} values were reported after 48 hours exposure[7], except for Compound D, which was calculated after 24 hours of exposure (* this thesis).

Peixoto and Beverley also reported that folate addition in the presence of the sulfonamides tested did not affect inhibition, which was different from the results of Compound C from this study, but not with Compound D. This, again, enforces the potential differences in mechanism of inhibition between the two tested compounds and possibly a different pathway of inhibition between Compound C and the sulfonamides tested by Peixoto and Beverley.[7]

Because Compound D is water-soluble, sequential treatments can be added to the cells with accumulative inhibition. Experimentation showed that the addition of two 50 μM concentrations of Compound D over 48 hours exhibited equivalent cell viability inhibition compared to one 100 μM addition 24 hours after exposure. Additive inhibitory effects could be beneficial as a treatment, allowing lower doses to be given to a patient to reduce potential side effects and/or manage toxicity to the patient.

Current treatments utilize varying treatment doses that have been shown to inhibit *Leishmania* promastigotes. Amphotericin B exhibited 100% inhibition of *L. donovani* promastigotes with addition of 0.3 μM[69], which was scaled-up to 0.75-1 mg/kg doses applied daily for 15-20 days for human trials.[70] However, at this treatment rate, side effects can be severe and close monitoring is necessary, leading to increased cost of therapy. Miltefosine required a 10 μM dose to inhibit 66% of *L. donovani* promastigotes after 48 hours.[71] In human studies, 50-100 mg doses were required over 28 days for a 94% cure rate, but, it is important to note, that the long half-life makes the compound vulnerable to drug resistance and the cost and need for monitoring makes the treatment expensive.[70] Antimonial drugs, like glucantime, have been shown to inhibit *L. braziliensis* and *L. guyanensis* in a dose-dependent manner over concentrations of 0.23-23 mM.[72] Human treatments require 20 mg/kg doses over 28-30 days[73]; however, again, side effects such as cardiotoxicity, renal failure, and pancreatitis, as well as reduced responsiveness to these treatment types have affected its use.[9] In comparison, *N*-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide could allow for a novel approach to treatment with potential for reduced parasitic resistance. As a water-soluble compound, it has the ability to be administered as a

topical agent, orally, or intravenously, once the pathway and mechanism of inhibition is more thoroughly evaluated.

DHFR-TS PATHWAY EVALUATION

Agarose gel electrophoresis confirmed that the approximate size of the *L. major* DHFR-TS gene was inserted into the pET-45b plasmid. The plasmid was transformed to competent RILP *E. coli* and, after protein purification, SDS-PAGE confirmed the approximate protein size was eluted from the affinity column. However, the DHFR portion of the enzyme has provided no measurable catalytic activity. The lack of activity could be due to enzyme instability caused by the more unstable TS portion of the bifunctional enzyme or by misfolding of the protein within the RILP *E. coli* used for protein expression.

Gene sequencing performed at UIUC confirmed the recombinant gene matches the *L. major* gene from which the primers were designed near the beginning and end of the gene sequence, but was not accurately sequenced near the middle of the gene. Primers were designed and submitted to UIUC for more accurate sequencing of the gene, as there are 395 nucleotides that were not sequenced. Because the recombinant gene aligns, it is likely a catalytically active form of the protein could be expressed.

If the gene has been properly expressed, future work should include the expression of recombinant DHFR-TS in ArcticExpress cells, as growth at lower temperatures with different chaperonin proteins could improve protein folding. If this is

not sufficient, it may also be beneficial to encode the Ts elongation factor into new primers, as this has been seen to improve the solubility of the TS portion of the enzyme. Expression of the DHFR portion of the bifunctional protein gene alone, without the TS portion of the enzyme, could also help express the catalytically competent DHFR enzyme for inhibition testing. The primers for the DHFR gene have been designed, but the recombinant gene was not successfully expressed without the TS gene. The method used to successfully ligate the recombinant DHFR-TS gene into pET-45b plasmid, in which the purified DNA was cut from the agarose gel after electrophoresis, purified, and used for ligation, should be used for future studies. Understanding the effect of the inhibitory sulfonamide compounds on DHFR kinetics would be helpful in identifying pathways of inhibition for the *Leishmania* promastigotes, as well as better understanding potential side effects in the host.

FUTURE WORK

Although a significant amount of work has been completed in the evaluation of sulfonamides synthesized at Illinois State University as inhibitors of *Leishmania*, there are still many areas of interest that should be investigated. *N*-(2'-chlorophenyl)-1-napthalene sulfonamide, previously tested by research students in the Jones lab (via personal communication), showed promising response at low concentrations (76% inhibition with 15 μM addition, after 24 hours). This compound was not available for these studies, but it would be very interesting to compare the inhibitory effects of this

compound with the inhibition demonstrated by Compounds C and D and what effects, if any, folate would have on the inhibition.

Also, because promastigotes and amastigotes are known to express different genes, testing the inhibitory effects of Compounds C and D on the amastigote forms of the *Leishmania* would help determine if the sulfonamides would inhibit both cellular forms, which could make for a potentially better treatment. Previous work has shown axenic amastigotes are acceptable screening methods for anti-leishmanial treatments.[5] This method should be used to evaluate Compounds C and D for inhibition of axenic amastigotes. Testing with *Leishmania* that infect humans is also essential in insuring inhibitory effects seen with *L. tarentolae* are applicable to the human pathogenic species. The sulfonamides of interest can be sent to collaborators in Colombia to perform this testing.

The folate pathway also involves other enzymes that could be evaluated in the presence of the inhibitory sulfonamides, including dihydropteroate synthase and pteridine reductase (PTR1). Dihydropteroate synthase is reported to be inhibited by some sulfonamides.[31] To test the effects of the sulfonamides on inhibition, the recombinant genes could be expressed using dam$^-$ dcm$^-$ competent *E. coli* and recombinant protein expressed in either RILP *E. coli* or ArcticExpress cells. The ability to inhibit PTR1 could be beneficial in inhibiting the amastigote form of the disease, as PTR1 has been reported to be more active in acidic environments such as that found in a phagocytic cell.[43] Also, because folate did not have an effect on the inhibitory performance of Compound D, evaluating the mechanism of inhibition for this compound

could be interesting. Other pathways of inhibition that may be useful to investigate include inhibition of carbonic anhydrase[74] and disruption of cell proliferation by disruption of microtubule activity. [33]

The promising effects of Compounds C and D for inhibition of *L. tarentolae* promastigotes at reasonable treatment concentrations within 24 hours indicates that these compounds may be good candidates for treatment of human infective species of *Leishmania*. Additional work is necessary to understand the true potential of the compounds of interest, including testing the compounds in the human infective forms of the parasite, evaluating the compounds' effects on the amastigote form of *Leishmania*, and identifying the pathway and mechanism of inhibition and developing a better understanding of potential effects in the host. With so many people affected globally, the need to develop better treatments for leishmaniasis is necessary, and the compounds in this study could become prospective treatments with additional investigation.

REFERENCES

1. World Health Organization. Leishmaniasis. http://www.who.int/leishmaniasis/burden/en/ (accessed Jan 2017).

2. Pathak, R.; Batra, S. Malaria and leishmaniasis: Current status of chemotherapy, new leads and targets for drug discovery. *Anti-Infective Agents in Medicinal Chemistry.* **2009**, *8* (3), 226-267.

3. Centers for Disease Control and Prevention. Disease. https://www.cdc.gov/parasites/leishmaniasis/disease.html (accessed Jan 2017).

4. Centers for Disease Control and Prevention. Resources for Health Professionals. https://www.cdc.gov/parasites/leishmaniasis/health_professionals/ (accessed Jan 2017).

5. Taylor, V. M.; Munoz, D. L.; Cedeno, D. L.; Jones, M. A.; Robledo, S. M. Leishmania tarentolae: utility as an in vitro model for screening of antileishmanial agents. *Experimental Parasitology.* **2010**, *126* (4), 471-475.

6. Centers for Disease Control and Prevention. Biology. https://www.cdc.gov/parasites/leishmaniasis/biology.html (accessed Jan 2017).

7. Peixoto, M. P.; Beverley, S. M. In vitro activity of sulfonamides and sulfones against leishmaniasis major promastigotes. *Antimicrobial Agents and Chemotherapy.* **1987**, *31* (10), 1575-1578.

8. Allen, S.; Neal, R. A. The in vitro susceptibility of macrophages infected with amastigotes of Leishmania spp. to pentavalent anitmonial drugs and other compounds with special relevance to cutaneous isolates. In *Leishmaniasis;* Hart, D. T., Ed.; Plenum Press: New York, New York, 1998; pp 711-720.

9. Chakravarty, J.; Sundar, S. Drug Resistance in Leishmaniasis. *Journal of Global Infectious Diseases.* **2010**, *2* (2), 167-176.

10. Bristol-Myers Squibb Company. Fungizone - amphotericin B for injection, USP. https://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=10618 (accessed February 2017).

11. Mbongo, N.; Loiseau, P. M.; Billion, M. A.; Robert-Gero, M. Mechanism of ampohtericin B resistance in Leishmania donovani promastigotes. *Antimicrobial Agents Chemotherapy.* **1998**, *42* (2), 352-357.

12. Sundar, S.; Olliaro, P. L. Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. *Journal of Therapeutics and Clinical Risk Management.* **2007**, *3* (5), 733-740.

13. Bryceson, A. A policy for leishmaniasis with respect to the prevention and control of drug resistance. *Tropical Medicine and International Health.* **2001**, *6* (11), 928-934.

14. Escobar, P.; Marques, C.; Croft, S. L. Sensitivites of Leishmania species to hexadecylphosphocholine (miltefosine), ET-18-OCH(3) (edelfosine) and amphotericin B. *Acta Tropica.* **2002**, *81* (2), 151-157.

15. World Health Organization. *Control of leishmaniasis: report of a meeting of the WHO Expert Committee on the control of leishmaniasis, Geneva;* World Health Organization: Geneva, 2010.

16. Stork, W. Prontosil. *Chemical and Engineering News.* **2005**, *83* (25).

17. Otten, H. Domagk and the development of the sulphonamides. *Journal of Antimicrobial Chemotherapy.* **1986**, *17* (6), 689-690.

18. Kolaczek, A.; Fusiarz, I.; Lawecka, J.; Branowska, D. Biological activity and synthesis of sulfonamide derivatives: a brief overview. *Chemik.* **2014**, *68* (7), 620-628.

19. Kanda, Y.; Kawanishi, Y.; Oda, K.; Sakata, T.; Mihara, S.; Asakura, K.; Kanemasa, T.; Ninomiya, M.; Fujimoto, M.; Kanoike, T. Synthesis and structure-activity relationships of potent and orally active sulfonamide ETB selective antagonists. *Bioorganic and Medicinal Chemistry.* **2001**, *9* (4), 897-907.

20. Stokes, S. S.; Albert, R.; Buurman, E. T.; Andrews, B.; Shapiro, A. B.; Green, O. M.; McKenzie, A. R.; Otterbein, L. R. Inhibitors of acetyltransferase domain of N-acetylglucosamine-1-phosphate-uridylyltransferase/glucosamine-1-phosphate acetyltransferase (GlmU). Part 2: Optimization of physical properties leading to antibacterial aryl sulfonamides. *Bioorganic and Medicinal Chemistry.* **2012**, *22* (23), 7019-7023.

21. Chibale, K.; Haupt, H.; Kendrick, H.; Yardley, V.; Saravanamuthu, A.; Fairlamb, A. H.; Croft, S. L. Antiprotozoal and cytotoxicity evaluation of sulfonamide and urea analogues of quinacrine. *Bioorganic and Medicinal Chemistry.* **2001**, *11* (19), 2655-2657.

22. Rahavi, E. I.; Camoutsis, C.; Zoumpoulakis, P.; Geronikaki, A.; Sokovic, M.; Glamocilija, J.; Ciric, A. Sulfonamide-1,2,4-triazole derivatives as antifungal and antibacterial agents: Synthesis, biological evaluation, lipophilicity, and conformational studies. *Bioorganic and Medicinal Chemistry.* **2008**, *16* (3), 1150-1161.

23. Wilson, C. O.; Gisvold, O.; Block, J. H. *Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry, 11th ed;* Lippincott Williams and Wilkins: Philadelphia, 2004.

24. Levin, J. I.; Chen, J. M.; Du, M. T.; Nelson, F. C.; Killar, L. M.; Skala, S.; Sung, A.; Jin, G.; Cowling, R.; Barone, D.; March, C. J.; Mohler, K. M.; Black, R. A.; Skotnicki, J. S. Anthranilate sulfonamide hydroxamate TACE inhibitors. Part 2: SAR of the acetylenic PI' group. *Bioorganic and Medicinal Chemistry Letter.* **2002**, *12* (8), 1199-1202.

25. Ma, T.; Fuld, A. D.; Rigas, J. R.; Hagey, A. E.; Gordon, G. B.; Dmitrovsky, E.; Dragnev, K. H. A phase I trial and in vitro studies combining ABT-751 with carboplatin in previously treated non-small cell lung cancer patients. *Chemotherapy.* **2012**, *58* (4), 321-329.

26. Roush, W. R.; Gwaltney, S. L.; Cheng, J.; Scheidt, K. A.; McKerrow, J. H.; Hansell, E. Vinyl sulfonate esters and vinyl sulfonamides: Potent, irreversible inhibitors of cysteine proteases. *Journal of American Chemical Society.* **1998**, *120* (42), 10994-10995.

27. Dekker, M. *In Protease Inhibitors in AIDS Therapy;* Basel: New York, 2001.

28. Marks, L. What Are Sulfonamides? http://www.everydayhealth.com/sulfonamides/guide/ (accessed February 2017).

29. Moore, E. M.; Lockwood, D. N. Treatment of visceral leishmaniasis. *Journal of Global Infectious Diseases.* **2010**, *2* (2), 151-158.

30. Naughton, C. A. Drug-induced nephrotoxicity. *American Family Physician.* **2008**, *78* (6), 743-750.

31. Henry, R. J. The mode of action of sulfonamides. *Bacteriol Reviews.* **1943**, *7* (4), 175-262.

32. Schweitzer, B. I.; Dicker, A. P.; Bertino, J. R. Dihydrofolate reductase as a therapeutic target. *Federation of American Societies for Experimental Biology.* **1990**, *4* (8), 2441-2452.

33. Liu, Z.; Tian, W.; Wang, S.; Luo, X.; Yu, Q. A novel sulfonamide agent, MPSP-001, exhibits potent activity against human cancer cells in vitro through disruption of microtubule. *Acta Pharmacologica Sinica.* **2012**, *33* (2), 261-270.

34. Ramos-Montoya, A.; Lee, W. P.; Bassilian, S.; Lim, S.; Trebukhina, R. V.; Kazhyna, M. V.; Cuidad, C. J.; Noe, V.; Centelles, J. J.; Cascante, M. Pentose phosphate cycle oxidative and nonoxidative balance: A new vulnerable target for overcoming drug resistance in cancer. *International Journal of Cancer.* **2006**, *119* (12), 2733-2741.

35. Miles, B. Pentose Phosphate Pathway AKA the Hexose Monophosphate Pathway. https://www.tamu.edu/faculty/bmiles/lectures/Pentose%20Phosphate%20Pathway.pdf (accessed February 2017).

36. JMC Corp. *Standard Specification - OPTSA (30/70);* JMC Corp, 2005.

37. Hartley, M. Sulfa Drugs and the Skin. http://www.dermnetnz.org/topics/sulfa-drugs-and-the-skin/ (accessed Feb 2017).

38. Crider, K. S.; Yang, T. P.; Berry, R. J.; Bailey, L. B. Folate and DNA methylation: a review of molecular mechansims and the evidence for folate's role. *Advances in Nutrition.* **2012**, *3* (1), 21-38.

39. Scala, I.; Granese, B.; Sellitto, M.; Salome, S.; Sammartino, A.; Pepe, A.; Mastroiacovo, P.; Sebastio, G.; Andria, G. Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring. *Genetics in Medicine.* **2006**, *8* (7), 409-416.

40. Schnell, J. R.; Dyons, H. J.; Wright, P. E. Structure, dynamics, and catalytic function of dihydrofolate reductase. *Annual Review of Biophysics and Biomolecular Structures.* **2004**, *33,* 119-140.

41. Czekster, C. M.; Vandemeulebroucke, A.; Blanchard, J. S. Kinetic and chemical mechanism of the dihydrofolate reductase from Mycobacterium tuberculosis. *Biochemistry.* **2011**, *50* (3), 367-375.

42. Carreras, C. W.; Santi, D. V. The catalytic mechanism and structure of thymidylate synthase. *Annual Review of Biochemistry.* **1995**, *64* (1), 721-762.

43. Vickers, T.; Beverley, S. M. Folate metabolic pathways in leishmania. *Essays in Biochemistry.* **2011,** *51,* 63-80.

44. Nare, B.; Hardy, L. W.; Beverley, S. M. The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite Leishmania major. *Journal of Biological Chemistry.* **1997**, *272* (21), 13883-13891.

45. Bello, A. R.; Nare, B.; Freedman, D.; Hardy, L.; Beverley, S. M. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite Leishmania major. *Proceedings of the National Academy of Sciences of the United States of America.* **1994**, *91* (24), 11442-11446.

46. Ong, H. B.; Sienkiewicz, N.; Wyllie, S.; Fairlamb, A. H. Dissecting the metabolic roles of pteridine reductase 1 in Trypanosoma brucei and Leishmania major. *Journal of Biological Chemistry.* **2011**, *286* (12), 10429-10438.

47. Kovacs, J. A.; Allegra, C. J.; Masur, H. Characterization of dihydrofolate reductase of Pneumocystis carinii and Toxoplasma gondii. *Experimental Parasitology.* **1990**, *71* (1), 60-68.

48. Morgenthaler, J. B.; Peters, S. J.; Cedeno, D. L.; Constantino, M. H.; Edwards, K. A.; Kamowski, E. M.; Passini, J. C.; Butkus, B. E.; Young, A. M.; Lash, T. D.; Jones, M. A. Carbaporphyrin ketals as potential agents for a new photodynamic therapy treatment of leishmaniasis. *Biorganic and Medicinal Chemistry.* **2008**, *16* (14), 7033-7038.

49. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods.* **1983**, *65* (1-2), 55-63.

50. Katinas, J.; Epplin, R.; Hamaker, C.; Jones, M. A. Sulfonamides as inhibitors of Leishmania - Potential new treatments for leishmaniasis. *Anti-Infective Agents.* **2017.**

51. Beverley, S. M.; Ellenberger, T. E.; Cordingley, J. S. Primary structure of the gene encoding the bifunctional dihydrofolate reductase-thymidylate synthase of Leishmania major. *Proceedings of the National Academy of Sciences of the United States of America.* **1986**, *83* (8), 2584-2588.

52. Sambrook, J.; Russell, D. *Molecular Cloning - A Laboratory Manual,* 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001; Vol. 2, 3 vols..

53. Promega. DNA Ladders. https://www.promega.com/products/cloning-and-dna-markers/molecular-weight-markers/dna-ladders/?activeTab=2 (accessed February 2017).

54. Sigma-Aldrich. P2714 Sigma Protease Inhibitor Cocktail powder - Product Information, 2010. Sigma-Aldrich. http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/5/p2714dat.pdf (accessed February 2017).

55. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* **1976**, *7* (72), 248-254.

56. Sigma-Aldrich. Product Information - Bradford Reagent, 2011. Sigma-Aldrich. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/b6916bul.pdf (accessed February 2017).

57. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227,* 680-685.

58. Bio-Rad. Precision plus protein Kaleidoscope prestain protein standards. http://www.bio-rad.com/en-us/sku/1610375-precision-plus-protein-kaleidoscope-prestained-protein-standards (accessed March 2017).

59. Stone, S. R.; Morrison, J. F. Kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from Escherichia coli. *Biochemistry.* **1982**, *21* (16), 3757-3765.

60. U.S. National Library of Medicine. Basic local alignment search tool. https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed February 2017).

61. Swiss Institute of Bioinformatics. Translate. http://web.expasy.org/translate/ (accessed Mar 2017).

62. European Molecular Biology Laboratory. Multiple Sequence Alignment. http://www.ebi.ac.uk/Tools/msa/kalign/ (accessed March 2017).

63. Swiss Institute of Bioinformatics. BoxShade. http://www.ch.embnet.org/software/BOX_form.html (accessed March 2017).

64. Schormann, N.; Senkovich, O.; Chattopadhyay, D. 3HBB. http://www.rcsb.org/pdb/explore/explore.do?structureId=3HBB (accessed March 2017).

65. Gibson, M. W.; Dewar, S.; Ong, H. B.; Sienkiewicz, N.; Fairlamb, A. H. Trypanosoma brucei DHFR-TS revisited: Characterization of a bifunctional and higly unstable recombinant dihydrofolate reductase-thymidylate synthase. *PLOS One Neglected Tropical Diseases.* **2016**, *10* (5), 1-20.

66. Garrett, R. H.; Grisham, C. M. *Biochemistry 3rd Edition;* Thomson Books/Cole: Belmont, CA, 2005; pp 1005-1006.

67. Hartl, F. U.; Hayer-Hartl, M. Converging concepts of protein folding in vitro and in vivo. *Nature Structural and Molecular Biology.* **2009**, *16,* 574-581.

68. Agilent Technologies. ArcticeExpress competent cells, 2017. Agilent Technologies - Genomics. http://www.genomics.agilent.com/article.jsp?pageId=468 (accessed March 2017).

69. Saha, A. K.; Mukherjee, T.; Bhaduri, A. Mechanism of action of amphotericin B on Leishmania donovani promastigotes. *Molecular Biochemical Parasitology.* **1986**, *19* (3), 195-200.

70. Sundar, S.; Chakravarty, J. Liposomal amphotericin B and leishmaniasis: Dose and response. *Journal of Global Infective Disease.* **2010**, *2* (2), 159-166.

71. Rakotomanga, M.; Blanc, S.; Gaudin, K.; Chaminade, P.; Loiseau, P. M. Miltefosine affects lipid metabolism in Leishmania donovani promastigotes. *Antimicrobial Agents and Chemotherapy.* **2007**, *51* (4), 1425-1430.

72. Moreira, E. S.; Petrillo-Peixoto, M. L. In vitro activity of meglumine antimoniate, a pentavelent antimonial drug, on Leishmania promastigotes. *Brazialian Journal of Medical and Biological Research.* **1991**, *24* (5), 459-469.

73. Haldar, A. K.; Sen, P.; Roy, S. Use of antimony in the treatment of leishmaniasis current status and future directions. *Molecular Biology International.* **2011**, *2011* (571242), 1-23.

74. Supuran, C. T.; Briganti, F.; Tilli, S.; Chegwidden, W. R.; Scozzafava, A. Carbonic anhydrase inhibitors: sulfonamides as antitumor agents? *Bioorganic Medicinal Chemistry.* **2001**, *9* (3), 703-714.

75. Center for Disease Control and Prevention. Centers for Disease Control and Prevention. http://www.cdc.gov/parasites/leishmaniasis/biology.html (accessed Jan. 2017).

76. Schlecht, H. P.; Bruno, C. Sulfonamides. http://www.merckmanuals.com/professional/infectious-diseases/bacteria-and-antibacterial-drugs/sulfonamides (accessed Jan 2017).

77. Sigma-Aldrich. Folic acid metabolism: A role in cancer's cause and cure. http://www.sigmaaldrich.com/life-science/learning-center/biofiles/biofiles-5-6/folic-acid-metabolism.html (accessed February 2017).

78. Brenneman, A. R.; Kaufman, S. The role of tetrahydropteridines in the enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine. *Biochemical and Biophysical Research Communications.* **1964**, *17* (2), 177-183.

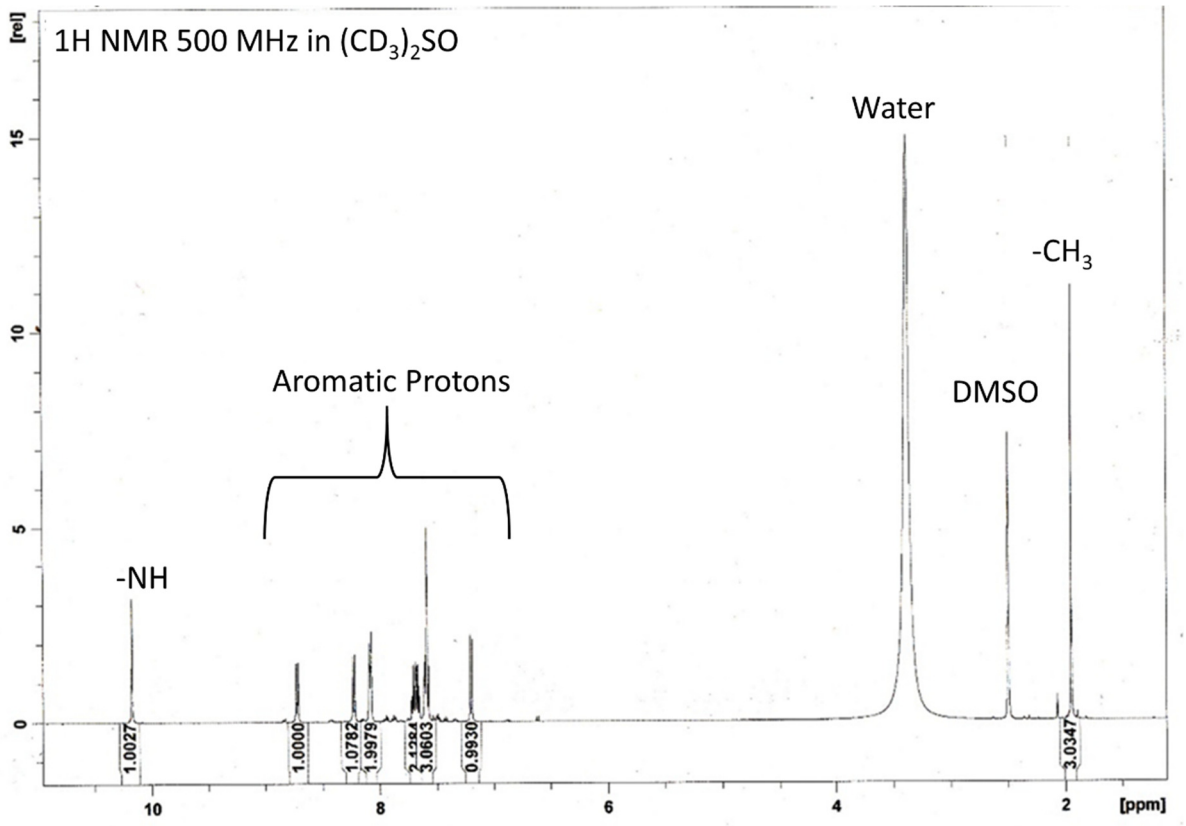
79. Tayeh, M. A.; Marletta, M. A. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. *Journal of Biological Chemistry.* **1989**, *264* (33), 19654-19658.

80. Tietz, A.; Lindberg, M.; Kennedy, E. P. A new pteridine-requiring enzyme system for oxidation of glyceryl ethers. *Journal of Biological Chemistry.* **1964**, *239* (12), 4081-4090.

81. Senkovich, O.; Schormann, N.; Chattopadhyay, D. Structures of dihydrofolate reductase-thymidylate synthase of Trypanosoma cruzi in the folate-free state and in complex with two antifolate drugs, trimetrexate and methotrexate. *Acta Crystallographica - Structural Biology.* **2009**, *65,* 704-716.

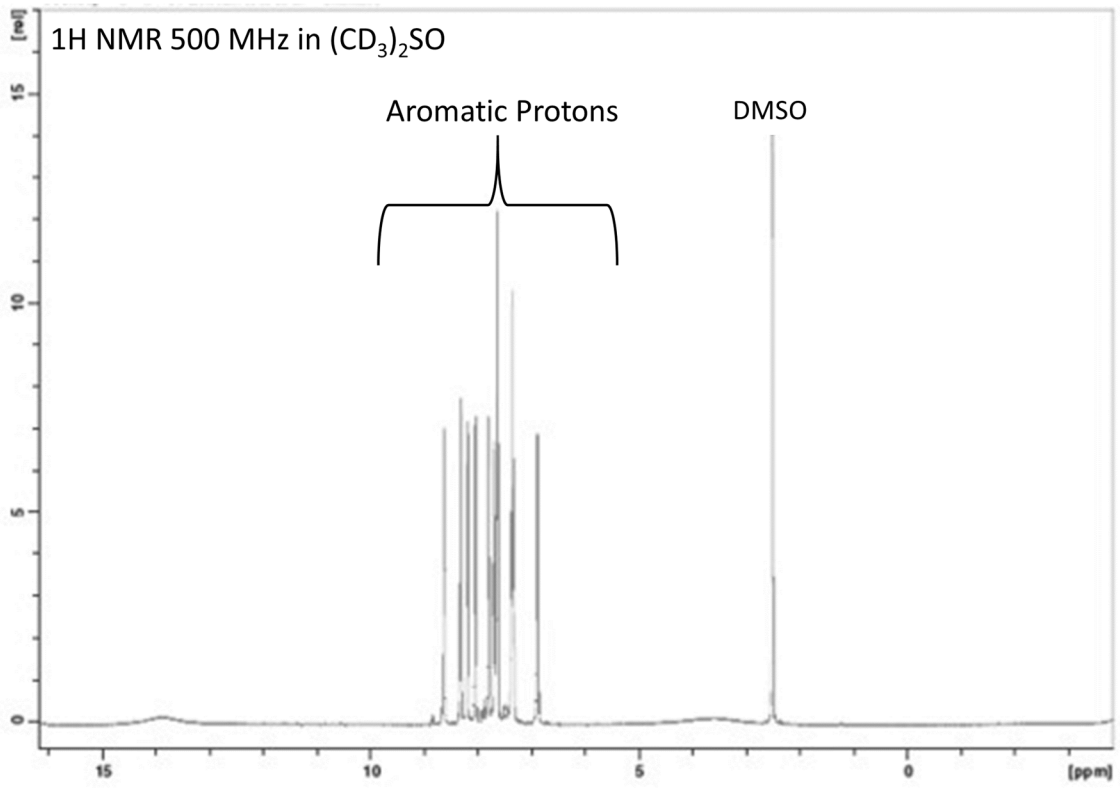
APPENDIX A

^{1}H-NMR OF *N*-(2'-METHYLPHENYL)-(4-CARBOXYL)-1-NAPTHALENE SULFONAMIDE (COMPOUND D)



APPENDIX B

^{1}H-NMR OF *N*-(4-CARBOXYL)-1-NAPTHALENE SULFONAMIDE (COMPOUND E)



APPENDIX C

RAW SEQUENCING DATA FROM UNIVERSITY OF ILLINOIS, URBANA-CHAMPAIGN (UIUC)

Promoter Primer –

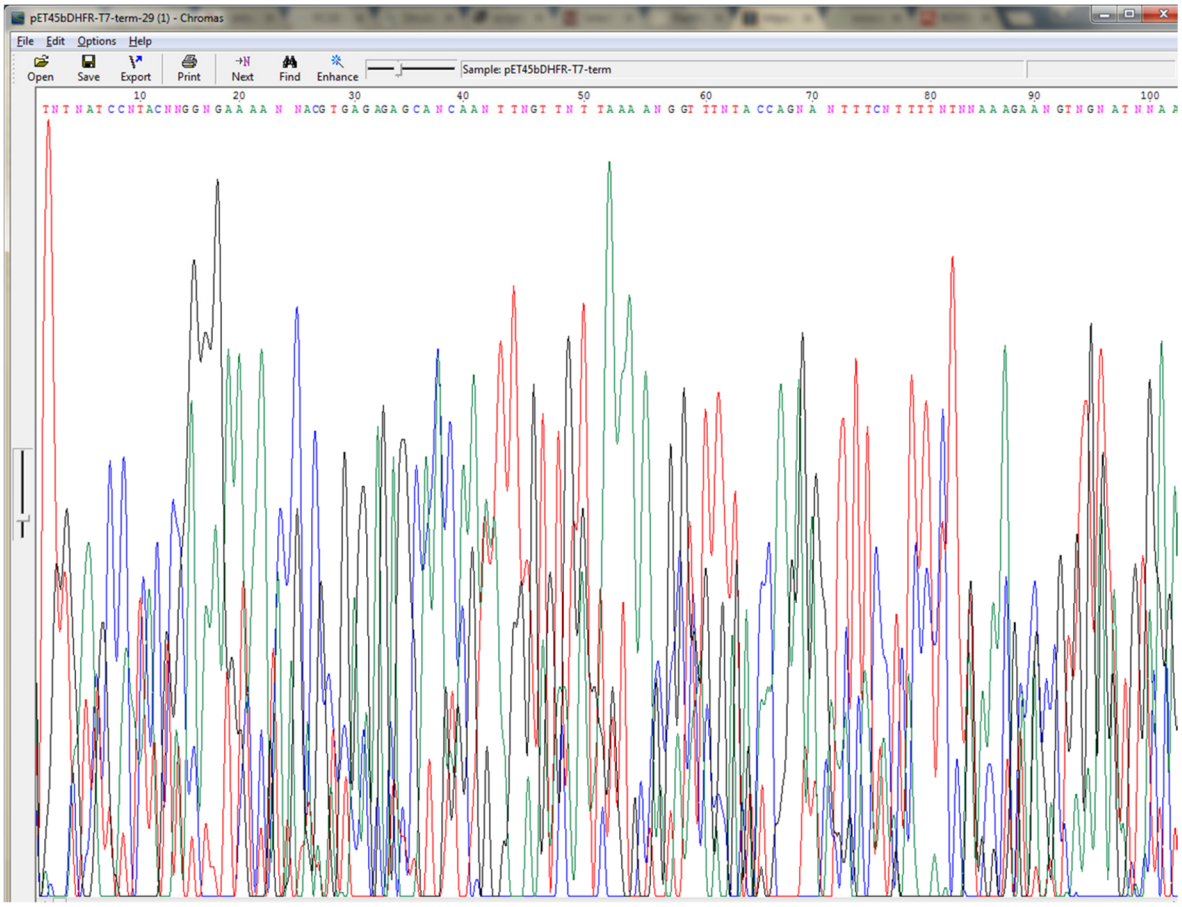
NNNNGNTGNNNNNTNNNNNNTNNNNNGNNNCNNTNGGGNNNNNNNNTGNNNNNNNNNNNNNNNNNNNNNNAGGGGGGA
ANTCCTNCACAAAATTTTGTTTACTTAAGAAGGAGATATACCANGGNACATCCCTCTTNAAAGGNCGCTGGGTACCG
GTTCGAATGATGACGACGACTAGAGNNCGGATCCCATGTCCAGGGCAGCTGCGAGGTTTAAGATTCCGATGCCGGAG
ACGAAGGCAGACTTTGCTTTCCCCTCCCTGCGCGCCTTCTCCATCGTCGTGGCCCTCGATATGCAGCACGGCATCGG
CGACGGCGAGTCGATCCCGTGGCGGGTGCCGGAGGACATGACGTTCTTCAAGAACCAGACGACGCTGCTGCGCAACA
AGAAGCCGCCGACGGAGAAGAAGCGCAACGCCGTCGTGATGGGCCGCAAGACTTGGGAGAGCGTCCCGGTAAAGTTC
CGACCACTCAAGGGACNNCTGAACATCGTGTTATCCTCGAAGGCCACCGTCGAGGAGCTTCTGGCGCCGCTGCCGGA
GGGACAGCGCGCGGCGGCGGCGCAGGATGTGGTGGTGGTGAACGGCGGTCTGGCCGAGGCGCTCCGCCTCCTCGCAC
GCCCGCTGTACTGCAGCTCCATCGAGACAGCGTATTGCGTCCGGTGGTGCGCAGGTTTACGCGGACGCCATGCTGTC
GCCGTGCATTCGAGNAAACTGCAAGGAAAGTGTACCTGGACCCGCATCTTACNCCGACGGCNGCCTGCGTNGTACGC
GCCTTCTTTTNCCGTTTTCCCGCCCCGAATAACGGCGGGCCNACGGGCNGNNGGGNACCTTGGNNGNTCGTTCNTCN
ANGGGNANGNCCGCAAANAANNCGAAGGCGGTAAAGGGGNCCCTCCAAAGTTTNCAGAGGAATCTGGNAAAAGNNAC
GGTGGCCNNGNGGNAAANCCNACNAAAGNAAGCCGGGNNAAGTTACCCCTTNAAGNCCNGGAANTNGNNACCCGCCT
CNTCCNATGNAANAAAANCGGGGGGAANTCCGNNGGNAAAAGGGANNGGAACCCNGCTCACCCGGGGGCCNAGNGGG
GGCGACCCCNTTTCANGNCCTNCTNCTTTGNGGGGANNCCCCCGNAAAATGGNNGGCTTTTNNTGNCCCCCCNAANG
GCGCNGAAAAAACACCNCGGCCTTNTGGCCCGCCTTTGNGTTNGNATCNNAAACCAANAAAGCCGNTGTGGTG

Terminal Primer –

NNNNNNNNNNNNNNNNNNNNNNNNNNNGGCNGTNTTAGCANCGCNGTAGACGAGTCATGTGCTGGCGTTCAAATTTC
GCAGCAGCGGTTTCCTTTANTAGACTCGAGTGCGGCCGCAAGCTTCTATACGGCCATCTCCATCTTGATCGCCGGGT
GTGGAACGTAGTCGATCACCTCCATGTCCGTCAACTCGTAGTCCTCGAGGTACTGCCGCTCCTCCTTGAAGATGAGG
GTCGGGAACGCGTGCGGGACTCGCTCGAGCTGCGCCTTGAGGGCATCAACGTGGTTGCGGTAGACGTGGGCGTCGCC
GAGGGTGTGCACAAGCTCACCAGGCCGCAGACCCGTCGCCTTGGCAATGAGGATGGTGAGCAGCGCGTAGGAGGCAA
TGTTGAAGGGGACGCCAAGACCCATGTCACACGAGCGCTGGTACAACATGCAGGATAGCTCGCTCGTGTCTGTGTTC
ACGTAGAACTGAGCAAGCAAGTGGCACGGCGGCAGCGCCATCTTTTGCAGCGCGCACGGGTTCCAGGCAGTGACTAG
GAGGCGGCGGTCGTTCGGGTTCGTCTTGATGGTCTCCACGATGAGCTTGATCTGGTCCACCCCTTCGCCGTCGTAGT
TCGCTTCAAACCCCTTGTAATCTGCCCCGAAGTGGCGCCACTGGAAGCCGTAGACAGGGCCGAGGTCCATCTCCTTA
TTCTCTGTCAAGCCGCGGCTGTCGAGAAACTCGCGCGAACCGTTGCCGTCCCAGATGTGAATGTCCTTGTCTGCCAG
CAGGNTGCGCACTCGTCTCCCCGCGCAGGAACCACAGNAGCTCCTCGCAACACGCCGNGCCAAGAAAANACGCTTCG
TCGTTCAGCAGCNGCNAGGCGGTTGTCGCGTAAGGGAGAAGCGCATCTTGGGGCGCCNAAAGAGGCTGATGGTGCCC
CACGCCCGGTGCGGTCCCTCCTTNCACGATNCCCGTCTTTCTTNATGCGGTTNAAANCAGCCTCAAGGGTAANTGCC
CGCTNCNNCNNGGGTTGCNCCGGCANCTTANCTTNCAGAATNTTCGAAAATNNAANGCCCCNCCNCCCTCCCTTTTT
TGGGGGGTNCCNTAAAAAAAAACCCNNNGTTCCCNCCCCCNNGTCCNCNTTTTTNCGGGNGGAAAANAGAAAANAAN
AGGGNGTAANNCTAGGTNCCNTNTNGAAANATNCNGGTNNCAGNAANCTTCCNNCNNTTTTNCNAATACCNGGNAAA
AACTGNCNTCCNCNNAAAANCTGNGNAAANCCNAAAAAAAAAACTNTTTTCNGNACTTNNATNCNACNTTCTTTNNA
NAAAANGAAANTNCTGGTANAAACCNTTTTAANAACNAANTTGNTGCTCTCTCACGTNNTTTTCNCCNNGTANGGAT
NANA

APPENDIX D

EXAMPLE TRACE FILE FROM UIUC



APPENDIX E

NUCLEOTIDE SEQUENCE ALIGNMENT

```
DHFRTS          1 ATGTCCAGGGCAGCTGCGAGGTTTAAGATTCCGATGCCGGAGACGAAGGCAGACTTTGCT
Pro             1 ATGTCCAGGGCAGCTGCGAGGTTTAAGATTCCGATGCCGGAGACGAAGGCAGACTTTGCT
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term      1 ------------------------------------------------------------
RC-Term         1 ------------------------------------------------------------

DHFRTS         61 TTCCCCTCCCTGCGCGCCTTCTCCATCGTCGTGGCCCTCGATATGCAGCACGGCATCGGC
Pro            61 TTCCCCTCCCTGCGCGCCTTCTCCATCGTCGTGGCCCTCGATATGCAGCACGGCATCGGC
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term      1 ------------------------------------------------------------
RC-Term         1 ------------------------------------------------------------

DHFRTS        121 GACGGCGAGTCGATCCCGTGGCGGGTGCCGGAGGACATGACGTTTTTCAAGAACCAGACG
Pro           121 GACGGCGAGTCGATCCCGTGGCGGGTGCCGGAGGACATGACGTTTTTCAAGAACCAGACG
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term      1 ------------------------------------------------------------
RC-Term         1 ------------------------------------------------------------

DHFRTS        181 ACGCTGCTGCGCAACAAGAAGCCGCCGACGGAGAAGAAGCGCAACGCCGTCGTGATGGGC
Pro           181 ACGCTGCTGCGCAACAAGAAGCCGCCGACGGAGAAGAAGCGCAACGCCGTCGTGATGGGC
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term      1 ------------------------------------------------------------
RC-Term         1 ------------------------------------------------------------

DHFRTS        241 CGCAAGACTTGGGAGAGCGTCCCGGTAAAGTTCCGACCACTCAAGGGACGGCTGAACATC
Pro           241 CGCAAGACTTGGGAGAGCGTCCCGGTAAAGTTCCGACCACTCAAGGGACGGCTGAACATC
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term      1 ----------------------------------------------CGGGCTGAACATC
RC-Term         1 ------------------------------------------------------------

DHFRTS        301 GTGTTATCCTCGAAGGCCACCGTCGAGGAGCTTCTGGCGCCGCTGCCGGAGGGACAGCGC
Pro           301 GTGTTATCCTCGAAGGCCACCGTCGAGGAGCTTCTGGCGCCGCTGCCGGAGGGACAGCGC
Mid-Pro         1 ------------------------------------------------------------
RCMid-Term     14 GTGTTATCCTCGAAGGCCACCGTCGAGGAGCTTCTGGCGCCGCTGCCGGAGGGACAGCGC
RC-Term         1 ------------------------------------------------------------

DHFRTS        361 GCGGCGGCGGCGCAGGATGTGGTGGTGGTGAACGGCGGTCTGGCCGAGG-CGCTCCGCCT
Pro           361 GCGGCGGCGGCGCAGGATGTGGTGGTGGTGAACGGCGGTCTGGCCGAGG-CGCTCCGCCT
Mid-Pro         1 --------------------TGGTGGTGGTGAACGGCGGTCTGGCCGAGGCTCCTCCACCT
RCMid-Term     74 GCGGCGGCGGCGCAGGATGTGGTGGTGGTGAACGGCGGTCTGGCCGAGG-CGCTCCGCCT
RC-Term         1 ------------------------------------------------------------

DHFRTS        420 CCTCGCACGCCCGCTGTACTGCAGCTCCATCGAGACAGCGTATTGCGTC-GGTGGTGCGC
Pro           420 CCTCGCACGCCCGCTGTACTGCAGCTCCATCGAGACAGCGTATTGCGTC-GGTGGTGCGC
Mid-Pro        46 CCTCGCACGCCCGCTGTACTGCAGCTCCATCGAGACAGCGTATTGCGTC-GGTGGTGCGC
RCMid-Term    134 CCTCGCACGCCCGCTGTACTGCAGCTCCATCGAGACAGCGTATTGCGTC-GGTGGTGCGC
RC-Term         1 ------------------------------------------------------------

DHFRTS        479 AGGTTTACGCGGACGCCATGCTGTCGCCGTGCATCGAGAAACTGCAGGAAGTGTACCTGA
Pro           480 A-----------------------------------------------------------
```

```
Mid-Pro      105 AGGTTTACGCGGACGCCATGCTGTCGCCGTGCATCGAGAAACTGCAGGAAGTGTACCTGA
RCMid-Term   193 AGGTTTACGCGGACGCCATGCTGTCGCCGTGCATCGAGAAACTGCAGGAAGTGTACCTGA
RC-Term        1 ------------------------------------------------------------

DHFRTS       539 CCCGCATCTACGCGACGGCGCCTGCGTGTACGCGCTTCTTTCCGTTTCCGCCCGAGAACG
Pro                ------------------------------------------------------------
Mid-Pro      165 CCCGCATCTACGCGACGGCGCCTGCATGTACGCGCTTCTTTCCGTTTCCGCCCGAGAACG
RCMid-Term   253 CCCGCATCTACGCGACGGCGCCTGCGTGTACGCGCTTCTTTCCGTTTCCGCCCGAGAACG
RC-Term        1 ------------------------------------------------------------

DHFRTS       599 CGGCCACGGCGTGGGACCTGGCGTCGTCTCAGGGACGCCGCAAGAGCGAGGCGGAGGGCC
Pro                ------------------------------------------------------------
Mid-Pro      225 CGGCCACGGCGTGTGACCTGGCGTCGTCTCAGGGACGCCGCAAGAGCGAGGCGGAGGGCC
RCMid-Term   313 CGGCCACGGCGTGGGACCTGGCGTCGTCTCAGGGACGCCGCAAGAGCGAGGCGGAGGGCC
RC-Term        1 ------------------------------------------------------------

DHFRTS       659 TCGAGTTCGAGATCTGCAAGTACGTGCCGCGCAACCACGAGGAGCGGCAGTACCTTGAGC
Pro              ------------------------------------------------------------
Mid-Pro      285 TCGAGTTCGAGATCTGCAAGTACGTGCCGCGCAACCACGAGGAGCGGCAGTACCTTGAGC
RCMid-Term   373 TCGAGTTCGAGATCTGCAAGTACGTGCCGCGCAACCACGAGGAGCGGCAGTACCTTGAGC
RC-Term        1 ------------------------------------------------------------

DHFRTS       719 TGATTGACCGCATCATGAAGACGGGGATCGTGAAGGAGGACCGCACCGGCGTGGGCACCA
Pro                ------------------------------------------------------------
Mid-Pro      345 TGATTGACCGCATCATGAAGACGGGGATCGTGAAGGAGGACCGCACCGGCGTGGGCACCA
RCMid-Term   433 TGATTGACCGCATCATGAAGACGGGGATCGTGAAGGAGGACCGCACCGGCGTGGGCACCA
RC-Term        1 ------------------------------------------------------------

DHFRTS       779 TCAGCCTCTTCGGCGCCCAGATGCGCTTCTCCCTACGCGACAACCGCCTGCCGCTGCTGA
Pro                ------------------------------------------------------------
Mid-Pro      405 TCAGCCTCTTCGGCGCCCAGATGCGCTTCTCCCTACGCGACAACCGCCTGCCGCTGCTGA
RCMid-Term   493 TCAGCCTCTTCGGCGCCCAGATGCGCTTCTCCCTACGCGACAACCGCCTGCCGCTGCTGA
RC-Term        1 ------------------------------------------------------------

DHFRTS       839 CGACGAAGCGTGTCTTCTGGCGCGGCGTGTGCGAGGAGCTGCTGTGGTTCCTGCGCGGG
Pro                ------------------------------------------------------------
Mid-Pro      465 CGACCAAGCGTGTCTTCTGGCGCGGCGTGTGCGAGG----------------------
RCMid-Term   553 CGACGAAGCGTGTCTTCTGGCGCGGCGTGTGCGAGGAGCTGCTGTGGTTCCTGCGCGGG
RC-Term        1 ----------------------------------AGCTGCTGTGGTTCCTGCGCGGG

DHFRTS       898 GAGACGAGTGCGCAGCTGCTGGCAGACAAGGACATTCACATCTGGGACGGCAACGGTTC
Pro                ------------------------------------------------------------
Mid-Pro            ------------------------------------------------------------
RCMid-Term   612 GAGACGAGTGCGCAGCTGCTGGCAGACAAGGACATTCACATGNTGGGANCGGGCA
RC-Term       24 GAGACGAGTGCGCAGCTGCTGGCAGACAAGGACATTCACATCTGGGACGGCAACGGTTC

DHFRTS       957 GCGCGAGTTTCTCGACAGCCGCGGCTTGACAGAGAATAAGGAGATGGACCTCGGCCCTGT
Pro                ------------------------------------------------------------
Mid-Pro            ------------------------------------------------------------
RCMid-Term   672 ANCGGTTTCGTC------------------------------------------------
RC-Term       83 GCGCGAGTTTCTCGACAGCCGCGGCTTGACAGAGAATAAGGAGATGGACCTCGGCCCTGT

DHFRTS      1017 CTACGGCTTCCAGTGGCGCCACTTCGGGGCAGATTACAAGGGGTTTGAAGCGAACTACGA
Pro                ------------------------------------------------------------
Mid-Pro            ------------------------------------------------------------
RCMid-Term         ------------------------------------------------------------
RC-Term      143 CTACGGCTTCCAGTGGCGCCACTTCGGGGCAGATTACAAGGGGTTTGAAGCGAACTACGA
```

```
DHFRTS      1077 CGGCGAAGGGGTGGACCAGATCAAGCTCATCGTGGAGACCATCAAGACGAACCCGAACGA
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      203 CGGCGAAGGGGTGGACCAGATCAAGCTCATCGTGGAGACCATCAAGACGAACCCGAACGA

DHFRTS      1137 CCGCCGCCTCCTAGTCACTGCCTGGAACCCGTGCGCGCTGCAAAAGATGGCGCTGCCGCC
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      263 CCGCCGCCTCCTAGTCACTGCCTGGAACCCGTGCGCGCTGCAAAAGATGGCGCTGCCGCC

DHFRTS      1197 GTGCCACTTGCTTGCTCAGTTCTACGTGAACACAGACACGAGCGAGCTATCCTGCATGTT
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      323 GTGCCACTTGCTTGCTCAGTTCTACGTGAACACAGACACGAGCGAGCTATCCTGCATGTT

DHFRTS      1257 GTACCAGCGCTCGTGTGACATGGGTCTTGGCGTCCCCTTCAACATTGCCTCCTACGCGCT
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      383 GTACCAGCGCTCGTGTGACATGGGTCTTGGCGTCCCCTTCAACATTGCCTCCTACGCGCT

DHFRTS      1317 GCTCACCATCCTCATTGCCAAGGCGACGGGTCTGCGGCCTGGTGAGCTTGTGCACACCCT
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      443 GCTCACCATCCTCATTGCCAAGGCGACGGGTCTGCGGCCTGGTGAGCTTGTGCACACCCT

DHFRTS      1377 CGGCGACGCCCACGTCTACCGCAACCACGTTGATGCCCTCAAGGCGCAGCTCGAGCGAGT
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      503 CGGCGACGCCCACGTCTACCGCAACCACGTTGATGCCCTCAAGGCGCAGCTCGAGCGAGT

DHFRTS      1437 CCCGCACGCGTTCCCGACCCTCATCTTCAAGGAGGAGCGGCAGTACCTCGAGGACTACGA
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      563 CCCGCACGCGTTCCCGACCCTCATCTTCAAGGAGGAGCGGCAGTACCTCGAGGACTACGA

DHFRTS      1497 GTTGACGGACATGGAGGTGATCGACTACGTTCCACACCCGGCGATCAAGATGGAGATGGC
Pro              ------------------------------------------------------------
Mid-Pro          ------------------------------------------------------------
RCMid-Term       ------------------------------------------------------------
RC-Term      623 GTTGACGGACATGGAGGTGATCGACTACGTTCCACACCCGGCGATCAAGATGGAGATGGC

DHFRTS      1557 CGTATAG-
Pro              --------
Mid-Pro          --------
RCMid-Term       --------
RC-Term      683 CGTATAG-
```

DHFRTS is the *L. major* gene from the gene data bank, Pro is the sequencing data from the promoter primer of the recombinant *L. major* gene, Mid-Pro is the second primer that was designed to capture the mid-region of the recombinant gene sequence, RCMid-Term is the reverse compliment of the sequence from the mid-region of the recombinant gene sequence, and RC-Term is the reverse compliment of the terminal region of the recombinant gene sequence.