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Identification of Novel Hits Against

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Identification of Novel Hits Against *Leishmania donovani*

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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# Table of Contents

List of Tables ............................................................................................................................. iv  

List of Figures ............................................................................................................................ v  

Abstract ................................................................................................................................... vii  

Chapter One: Introduction ........................................................................................................... 1  
  Abstract ................................................................................................................................. 1  
  Leishmania: Background ................................................................................................. 2  
  Leishmania Life Cycle ..................................................................................................... 5  
  Clinical Manifestations .................................................................................................... 6  
  Diagnosis .......................................................................................................................... 9  
  Epidemiology ................................................................................................................... 10  
  Current Treatments .......................................................................................................... 12  
    Pentavalent Antimonials ............................................................................................ 13  
    Miltefosine ................................................................................................................ 14  
    Amphotericin B ......................................................................................................... 15  
    Paromomycin ............................................................................................................ 16  
    Pentamidine ................................................................................................................ 16  
    Azoles ........................................................................................................................... 17  
    Drug Combination Therapies ................................................................................. 17  
  Vaccine ............................................................................................................................ 18  
  Leishmania Drug Targets ............................................................................................ 19  
  Drug Discovery .............................................................................................................. 20  
  Screening Applications Used in Drug Discovery ....................................................... 21  
  Focus of the Study .......................................................................................................... 24  
  List of References ............................................................................................................ 30  

Chapter Two: Discovery of Novel Antileishmanial Hits from Natural Product ....................... 1  
  Abstract ................................................................................................................................. 1  
  Introduction ......................................................................................................................... 2  
  Materials and Methods ..................................................................................................... 5  
    Mangrove Endophytic Fungal Extracts .................................................................... 5  
    Antarctic Deep sea Coral; Keipukalides and Furanocembrane Diterpenes ............... 6  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  
    Catharanthus roseus Hairy Root Extracts ................................................................. 7  

Results and Discussion ........................................................................................................... 102
List of References .................................................................................................................. 108

Chapter Five: Summary ........................................................................................................... 112
Introduction ............................................................................................................................. 112
Natural Products ..................................................................................................................... 113
Synthetic Compounds ............................................................................................................. 114
Antileishmanial Lead Optimization of Quinazolines (KVH-14) ............................................. 116
List of References .................................................................................................................. 119

Appendices: ............................................................................................................................ 122
Appendix A: Copyright Information .......................................................................................... 123
List of Tables

Table 2.1: Mangrove Endophytic Fungi Pure Compounds Activity (IC$_{50}$ µM) ...............16
Table 2.2: Bioactivity of Plumarella Terpenes (IC$_{50}$ µM) ...........................................18
Table 2.3: Catharanthus roseus Hairy Root Extracts Activity (IC$_{50}$ µg/ml) ......................20
Table 3.1: Leishmaniasis Activity of R1 Positional Scanning Study of Lib1 .....................58
Table 3.2: Leishmaniasis Activity of R2 Positional Scanning Study of Lib1 .....................61
Table 3.3: Leishmaniasis Activity of R3 Positional Scanning Study of Lib1 .....................63
Table 3.4: Individually Synthesized Compound Activity .................................................66
Table 3.5: Analogue of 21 and 26 .................................................................................68
Table 3.6: Analogs of 6−8 .........................................................................................70
Table 3.7: Monoguanidines .......................................................................................74
Table 3.8: Biological and Physicochemical Data For All Analogs ..............................76
Table 3.9: Activity of Mono- amidine Compounds in Multiple Assays (IC50 µM) .........79
Table 3.10: Activity of Quinazolines (IC$_{50}$ <1 µM in Infected Macrophage Model) ......81
Table 3.11: Thiazole Antileishmanial Activity (IC$_{50}$ µM) ...........................................82
Table 3.12: Primaquine and Primaquine-Coumarin Fluorescent Probe IC50 Values in Multiple Assays (IC$_{50}$ µg/ml) .................................................................85
Table 4.1: IC$_{50}$ Values in Drug Sensitivity Assays (µM) .............................................105
Table 4.2: Percentage of Parasite Inhibition in all Tissues in Severe Hamster Model .107
Table 5.1: Summary of Novel Natural Product-based Antileishmanial Hits .............118
Table 5.2: Summary of Novel Synthetic Antileishmanial Hits ..............................119
List of Figures

Figure 1.1: *Leishmania* Life Cycle ........................................................................................................26
Figure 1.2: *Leishmania* Species Promastigotes ..................................................................................27
Figure 1.3: *Leishmania* Species Amastigotes ....................................................................................28
Figure 1.4: Drug Discovery Process and Timeline ................................................................................29
Figure 1.5: Hit to Lead Process in Drug Discovery ..............................................................................30
Figure 2.1: Mangrove Endophytic Fungi Extracts Activity in *L. donovani* Axenic Amastigotes Drug Sensitivity Assay and Cytotoxicity Assay ..................................................14
Figure 2.2: Mangrove Endophytic Fungi Extracts Activity in *L. donovani* Infected Macrophage Assay ..............................................................................................................................15
Figure 2.3: Structure of Phomopsichromin C .......................................................................................17
Figure 2.4: Chemical structures of *Plumarella* Terpenes ....................................................................19
Figure 3.1: Compound 1 ........................................................................................................................52
Figure 3.2: Compound 2 ........................................................................................................................53
Figure 3.3: Preparation of Compounds 9-11 ........................................................................................54
Figure 3.4: Minimized Poses of 11 in Hsp90 Showing The Geminal Methyl Groups Pointing Toward Hydrophobic Pockets ..........................................................................................55
Figure 3.5: Preparation of Compounds 12-17 .......................................................................................56
Figure 3.6: Preparation of Compounds 18-26 .......................................................................................57
Figure 3.7: Similarity of R1 and R3 .........................................................................................................73
Figure 3.8: Currently Used Antileishmaniasis Agents ...........................................................................75
Figure 3.9: Activity of Mono- Amidine Series in Multiple Assays ............................................................77
Figure 3.10: Chemical Structure of DB2381 ................................................................. 78
Figure 3.11: Quinazolines Antileishmanial and Cytotoxicity Activity .......................... 80
Figure 3.12: Active Thiazoles in Multiple Antileishmanial Assays ............................... 83
Figure 3.13: Chemical structure of KBA 16 ................................................................. 84
Figure 4.1: Operetta Pictures of Treated Infected Macrophages ................................. 106
Figure 4.2: Percentage of Parasite Inhibition in all Tissues in Severe Hamster Model 108
Figure 5.1: Current Visceral Leishmaniasis Treatments ............................................. 117
Abstract

Leishmaniasis is a disease caused by obligate intracellular parasites of the genus *Leishmania*, including 20 species that are pathogenic to humans. Female sandfly is the known vector that can transmit the disease. Visceral leishmaniasis is the severe form of the disease that affects internal organs and can be fatal with inappropriate diagnosis or treatment. *Leishmania donovani* is the causative agent of visceral leishmaniasis. Approximately 350 million in 89 countries are at risk of infection. Around 2 million new cases are reported annually with 500,000 of these are visceral leishmaniasis. Current drug therapies are inadequate due to their toxicity, high cost, severe adverse reaction, limited availability, and the emergence of resistance. With all these limitations, the need for new drugs is urgent.

Pentavalent antimonials are the first line of treatment for leishmaniasis since the 1940s. Although amphotericin B, pentamidine and paromomycin are current drugs that treat leishmaniasis, they were discovered initially as a treatment for other pathogens. Furthermore, miltefosine the only available oral drug for leishmaniasis is an anticancer drug that found to be active against *Leishmania*. Therefore, we used our quantitative *Leishmania donovani* axenic amastigote assay and the clinically relevant infected macrophage assay to identify new antileishmanial hits from unstudied or understudied natural product sources such as mangrove endophytic fungi, Antarctic deep-sea coral, and terrestrial plants. We also used the same assays to screen synthetic compounds form multiple chemical scaffolds.
Our well-established assays led to the identification of new antileishmanial hits from unstudied natural products and the discovery of new classes of molecules from synthetic compounds that possess potent activity against *Leishmania donovani*. Finally, we conducted an in vivo hamster study on an active hit that revealed high efficacy against *Leishmania donovani* in this severe model leading to promising antileishmanial drug development.
Chapter One: Introduction

Abstract

Leishmaniasis is a disease caused by obligate intracellular parasites of the genus *Leishmania*. The genus includes 20 species that are pathogenic to humans. Female sand fly is the known vector that can transmit the disease. *Leishmania donovani* is the causative agent of visceral leishmaniasis. Visceral leishmaniasis is the severe form of the disease that affects internal organs and can be fatal with inappropriate diagnosis or treatment. Other forms of the disease include cutaneous and mucocutaneous. Approximately 350 million in 89 countries are at risk of infection. Around 2 million new cases are reported annually, of which 500,000 present in the visceral form. Current drug therapies are inadequate due to their toxicity, high cost, severe adverse reaction, limited availability, and the emergence of resistance. With all these limitations, the need for new drugs is urgent.

Pentavalent antimonials have been the first line of treatment for leishmaniasis since the 1940s. Although amphotericin B, pentamidine and paromomycin are current drugs that treat leishmaniasis, they were discovered initially as a treatment for other pathogens. Miltefosine, the only available oral drug for leishmaniasis, is an anticancer drug that was found to be active against *Leishmania*. This project investigated new antileishmanial hits from unstudied or understudied natural products using quantitative *Leishmania donovani* axenic amastigote assay and the clinically relevant infected
macrophage assay. Natural project sources include mangrove endophytic fungi, Antarctic deep-sea coral, and terrestrial plants. Additionally, the same assays were used to screen synthetic compounds from multiple chemical scaffolds.

Our well-established assays led to the identification of new antileishmanial hits from unstudied natural products and the discovery of new classes of molecules from synthetic compounds that possess potent activity against *Leishmania donovani*. Finally, we conducted an in vivo hamster study on an active hit that revealed high efficacy against *Leishmania donovani* in this severe model leading to promising antileishmanial drug development.

**Leishmania: Background**

Most parasitic diseases are neglected in the developing world, but represent significant burdens in areas of poor health, poor access to healthcare, and overwrought infrastructures due to war or social unrest lead. These combination of factors are favorable to the establishment of recurrent epidemic cycles of transmission. Although leishmaniasis is one of these neglected diseases, vectors responsible for transmitting the disease is not associated with living conditions; thus controlling this disease is a challenge (Beyrer et al., 2007). *Leishmania* is an obligate intracellular protozoan parasite with a divalent life cycle. Its flagellated promastigote stage is found in the sandfly vector, and inside the macrophages of the host (animals-humans) an amastigote stage with a truncated flagellum is found. This devastating parasite belongs to the *Leishmania* genus (Domain Eukaryota, Phylum Euglenozoa, Class Kinetoplastida, Order Trypanosomatida) (Neuber, 2008).
According to estimates by the World Health Organization (WHO), leishmaniasis collectively affects 12 million people around the globe. Approximately 310 million additional people worldwide are at risk of infection (Neuber, 2008). The disease is spread by the bite of female sand flies (definitive host) of the genera *Lutzomyia* (new world) and *Phlebotomus* (old world), although the taxonomic status of some of them is disputed, 20 different species of flagellated unicellular protozoa belonging to the genus *Leishmania* are known to infect these sand flies. In fact, there are approximately 600 sand-fly species, but only 10% of them can act as vectors of leishmaniasis (Sharma & Singh, 2008). The female sand-flies require blood meals to obtain protein for their egg broods and typically will travel several hundred meters from their habitat at night and dusk to feed (Sharma & Singh, 2008).

The parasite affects people of many cultures in the world which has resulted in multitude of names for the disease including: Aleppo boil, Valley sickness, Andean sickness, Leprosy of the mountains, White Leprosy, DumDum fever, Kala-azar (black fever), Grayish, Assam fever, and Jericho buttons (James, Elston, & Berger, 2011). Human and animals have been infected with the *Leishmania* parasite for at least 4000 years as manifest by positive amplification of *Leishmania* kinetoplast DNA in Egyptian and Nubian mummies (Zink et al., 2006). William Boog Leishman discovered the causative agent of leishmaniasis in 1903, when he examined the oval bodies (amastigotes) found in the spleen of a British soldier who died from DumDum fever in India (Leishman, 1903). Independently, Charles Donovan found oval bodies (amastigotes) in patients who died from leishmaniasis. Accordingly, Ronald Ross
suggested that this newly discovered organism be named after these two men who discovered the causative agent of this disease (Ross, 1903).

Since this discovery, over one-hundred-years of research has been devoted to studying *Leishmania* including epidemiology, host-parasite interactions, as well as drug and vaccine discovery. Because it is a neglected tropical disease and a particularly complicated organism, progress has been slow (Zink et al., 2006). The pathology of *Leishmania donovani* was first described in detail by Christophers (1904), the finding showed that parasites proliferate in macrophages and aggregate in the liver, spleen, and bone marrow (Christophers, 1904). Learning how to culture *Leishmania* was the first step to study this parasite in the lab. In 1904, Rogers was the first to grow *Leishmania*, but the cultures could only survive for 2 weeks (Rogers, 1904). In 1909 Ch. Nicolle used Novy-McNeal medium that was used to grow *Leishmania* for a couple of weeks and modified it into Nicolle-Novy- McNeal (NNM) medium which consists of bacto beef, neopeptone, bacto agar, and rabbit blood to subculture *Leishmania* indefinitely (Row, 1912; Wang et al., 2010).

Meleney (1925) infected hamsters from in vitro cultures, and studied the progression of the disease, and many species were capable of being infected with *Leishmania* (Croft, Seifert, & Yardley, 2006). Because hamsters have a similar physiological response to humans in regards to the progression of the disease, they became an important model organism for studying *L. donovani* (Simon L Croft, Shyam Sundar, & Alan H Fairlamb, 2006; Melby, Chandrasekar, Zhao, & Coe, 2001). Stauber in 1958 developed a method to quantify accurately parasite burden in the tissues of these hamsters. He took impression smears of infected organs and determined
Leishman Donovan Units (LDU), which is calculated as organ mass (g) multiplied by the number of amastigotes per one thousand macrophage nuclei. The development of the LDU method established a standard method to evaluate drug effectiveness in vivo that is still in use today (Stauber, 1958).

**Leishmania Life Cycle**

The parasite may follow two different transmission cycles: zoonotic cycle and anthroponotic cycle. In the zoonotic cycle, dogs are a vital animal reservoir in addition to other mammals; while in the strictly anthroponotic cycle, humans are the only host, which is typically observed in densely inhabited urban areas (Lainson & Shaw, 2010).

*Leishmania* life cycle (Figure 1.1) mainly depends on the exchange of two morphological forms; promastigotes (Figure 1.2) in sand fly vector and amastigotes (Figure 1.3) in the mammalian host cells. Promastigotes can differentiate into different forms inside the sand fly. Its growth cycle starts with the dividing procyclic form in the abdominal midgut of the sand fly during a blood meal. Then it transforms into the non-dividing nectomonad form, which is responsible for the migration of the infection and transforms into leptomonad form in the anterior midgut. Leptomonads transforms into the infectious form, metacyclic promastigote (Gossage, Rogers, & Bates, 2003). The infectious metacyclic promastigotes are then injected into the host’s body by the bite of female sand fly during a blood meal, which are then taken up by macrophages where the parasite transforms into the pathogenic tissue stage (amastigote). The latter form then multiplies asexually and infects other macrophages. Finally, the sand fly gets infected during a blood meal by ingesting infected cells with amastigotes, which then transform into promastigotes in the sand fly gut. Until recently, promastigotes were
believed to undergo asexual division inside the sand fly. In 2009 Akopyants et al. discovered the ability of promastigotes to go through sexual division inside the sand fly (Akopyants et al., 2009).

**Clinical Manifestations**

Clinical disease due to *Leishmania* presents in three major forms: visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis.

Visceral leishmaniasis is a systemic severe form of the disease that can be fatal if left untreated (Kumari & Ram, 2002), (Nagle et al., 2014). The main clinical features include enlarged liver and spleen, severe anemia, weight loss, and prolonged fever (Hirve et al., 2017), (Guerin et al., 2002). These symptoms usually appear 3 to 6 months post infection. In some cases, it takes years for the disease to manifest, where asymptomatic patients remain undiagnosed unless they become immunocompromised then the symptoms will appear (Polak et al., 2012). Darkening of the skin is another clinical feature of VL and mainly occurs in South Asia and called Kala-azar, which means black fever in the Hindi language (Dorlo, Balasegaram, Beijnen, & de Vries, 2012) (Sacks et al., 1995). Post Kala-azar dermal leishmaniasis (PKDL) is a dermal form of leishmaniasis and can appear months or years after a successful treatment of visceral leishmaniasis (Redhu, Dey, Balooni, & Singh, 2006), (Burza et al., 2014) (Sundar & Chakravarty, 2013). PKDL is characterized by papule skin lesions on the face that increase in size over time, then spread all over the body as nodules and requires prolonged chemotherapy (R. P. Singh et al., 2012). The clinical feature of PKDL depends on the geographical distribution of the disease. In Indian subcontinent, PKDL is rare and the nodules transform into plaques (Zijlstra, Alves, Rijal, Arana, &
While in East Africa, PKDL is more frequent and the nodules become ulcers. In some cases, PKDL can cause blindness or nerve damage (Rijal, Agrawal, Agarwalla, Agrawal, & Rijal, 2005), (R. Singh et al., 2006), (Zijlstra, Musa, Khalil, el-Hassan, & el-Hassan, 2003), (el Hassan et al., 1992). Furthermore, PKDL patients may act as a reservoir hosts since the nodules contain *Leishmania* parasites and can be picked up by sandflies (Gupta & Nishi, 2011; Roatt et al., 2014; Wang et al., 2010). Patients with visceral leishmaniasis are at high risk of developing bacterial infections such as pneumonia and gastrointestinal infection (Pasyar, Alborzi, & Pouladfar, 2012), (Andrade, Carvalho, & Rocha, 1990). Moreover, HIV patients are more likely to develop visceral leishmaniasis at a rate approximately 2000 times more when compared to non-HIV infected individuals (Pineda et al., 1998), (Lindoso, Cunha, Queiroz, & Moreira, 2016).

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis. CL is caused by both New World and Old World species of *Leishmania* and the symptoms of the disease depend on the *Leishmania* species and the mode of transmission. The disease manifests as ulcerative lesions that develop at the site of the sand fly bite, which can grow progressively larger and fail to heal naturally (Salman, Rubeiz, & Kibbi, 1999). These lesions often appear on exposed areas of the body such as the face, and are associated with significant social stigma and disability. If infected with bacteria, these lesions can be quite painful (Layegh et al., 2015). Lymphadenopathy often precedes appearance of the lesion. Even with this localized form of the disease, often multiple satellite lesions appear, and can persist for months or years (Harms, Fraga, Batroff, Oliveira, & Feldmeier, 2005). Successful treatment does not eliminate the
noticeable scars that remain visible for life (Markle & Makhoul, 2004). Diffuse cutaneous leishmaniasis (DCL) is a rare condition characterized by multi non-ulcerated nodules with high parasite load. It is seen in HIV infected persons and occurs due to immune system deficiency (Sinha, Fernandez, Kapila, Lambert, & Schwartz, 2008).

Mucocutaneous leishmaniasis (MCL) is a severe mutilating rare form of the disease, which typically occurs as a metastatic dissemination of the parasite from the site of initial infection on the skin to mucosal membranes of the body, primarily around the nose and mouth (Markle & Makhoul, 2004), (Kedzierski, 2011). This disease also known as espundia and Breda (Neuber, 2008). Complete ulcerative destruction of the oropharyngeal tissues is often seen in the absence of timely treatment. The first symptoms such as persistent nosebleeds may appear several months or even years after the healing of the primary skin lesion. Other symptoms include; fever, lymphadenopathy, enlarged liver. Severe cases of MCL may experience complications within the nasal cavity including edema, septum perforation in addition to periodontitis and destruction of oronasopharyngeal mucosa and obstruction of the airway. This condition can be life-threatening (Diniz, Costa, & Goncalves, 2011). The disease pathogenesis is poorly understood, and is often associated with lack of appropriate treatment of cutaneous lesions (Moreira et al., 2016). However, a recent study linked the aggressive phenotype of MCL to the presence of Leishmania RNA virus (Zangger et al., 2013). Leishmania RNA virus is a Totiviridae double stranded RNA virus presented in several Leishmania parasites’ species and serve as a natural host for the virus (Hartley, Ronet, Zangger, Beverley, & Fasel, 2012) (Adaui et al., 2016). Furthermore,
host genetics factors may play an important role in the progression of the disease (Mangano & Modiano, 2014).

**Diagnosis**

The management of leishmaniasis is reliant upon a proper diagnosis. However, the diagnosis of the disease is a challenge and misdiagnosis may resulted in an unfavorable outcome (Handler, Patel, Kapila, Al-Qubati, & Schwartz, 2015b).

Microscopic examination is mainly used to diagnose visceral leishmaniasis by visualizing amastigotes in samples collected from the lymph node, spleen aspiration, and bone marrow. This method is used to diagnose the disease and to evaluate the effectiveness of the treatment (Sakkas, Gartzonika, & Levidiotou, 2016). PCR can be used to determine the infecting parasite species and for quantification assessment of parasite burden by amplifying *Leishmania* gene targets such as ß tubulin, kinetoplast DNA, and cytochrome b (Osman et al., 1997). Furthermore, serological tests such as indirect immunofluorescence assay test, direct agglutination test, and fast agglutination screening test are used to monitor specific antileishmanial antibodies (Lockwood & Sundar, 2006). On the other hand, antigen detection tests are used as an alternative to antibody detection, which has high specificity but low sensitivity (Vallur et al., 2015), (Boelaert et al., 2014). The parasite burden has shown to be 10-fold higher in VL/ HIV-positive patients compared to VL/ HIV-negative patients; therefore, direct microscopic examination and quantitative tests are highly sufficient and sensitive for the diagnosis of HIV/VL coinfected patients (van Griensven, Carrillo, Lopez-Velez, Lynen, & Moreno, 2014). In PKDL patients, the sample collection method of tissue biopsy is invasive, and the number of parasites is low in the papulae, which complicate the detection of the
infection. Also, misdiagnosis of leprosy is not uncommon in PKDL patients (el Hassan, Hashim, Abdullah, Zijlstra, & Ghalib, 1993). Therefore, the diagnosis of PKDL mainly relies on the previous history of VL and its serological tests (Salotra & Singh, 2006).

Cutaneous leishmaniasis is diagnosed by visual microscopic examination of the parasites on samples collected from skin scraping of ulcer margins, punch biopsy or needle aspirate. PCR quantification, serological tests and antigenic tests are also used to diagnose CL (de Vries, Reedijk, & Schallig, 2015)

Mucocutaneous leishmaniasis is diagnosed by physical examination and medical history evaluation by a healthcare provider. Serological tests along with PCR and DNA testing can be used to confirm the infection (Handler et al., 2015b).

Improved diagnostic tests with low cost, less invasive sample collection; high sensitivity and specificity are much needed for better diagnosis of the disease (Nagle et al., 2014).

**Epidemiology**

The accurate epidemiological data from many endemic countries is lacking (Jorge Alvar et al., 2012). Thus the actual burden of the disease may be higher than official WHO estimates, especially if the mental health repercussions, in the form of social stigma and ostracization associated with some clinical manifestations, are appropriately factored in (Lainson & Shaw, 2010). Asymptomatic cases may act as significant reservoir for re-infection as it is known to exist in endemic areas (O. P. Singh, Hasker, Sacks, Boelaert, & Sundar, 2014).

Visceral leishmaniasis has two forms differ in the causative *Leishmania* species and the parasite reservoir. *Leishmania infantum* causes the zoonotic form of VL and
occur mainly in the Mediterranean basin and Central and South America. In this form, dogs are the main parasites reservoir (Quinnell & Courtenay, 2009), (Shimozako, Wu, & Massad, 2017). The other form of VL is caused by *Leishmania donovani* and found in India, Nepal, Bangladesh and East Africa (Guerin et al., 2002), (Tchokouaha Yamthe et al., 2017), (Ready, 2014). VL has been reported in 98 countries around the globe with approximately 300,000 new cases occur annually leading to 40,000 deaths, although the real number may be higher due to poor epidemiological surveillance in many areas of active transmission (Leta, Dao, Mesele, & Alemayehu, 2014). 90% of new VL cases are concentrated in 6 countries: Bangladesh, India, Ethiopia, Sudan, South Sudan, and Brazil (Gupta & Nishi, 2011), (de Araujo et al., 2013) and round 60% of reported new cases occur in Bihar state in India (Hasker et al., 2012). The absences of proper control measures make VL a serious public health issue (Nagle et al., 2014).

Cutaneous leishmaniasis is estimated to infect around 1.3 million new patients each year worldwide according to the WHO Global Health Observatory (Vitoriano-Souza et al., 2008). Approximately 70% of the cases occur in ten countries: Sudan, Afghanistan, Ethiopia, Algeria, Iran, Syria, Brazil, Peru, Costa Rica, and Colombia (J. Alvar et al., 2012). There is an increased risk of CL incidents due to military work, travel and immigration from endemic areas (Khan et al., 2016). About 40% of reported CL cases in the USA are from tourists who traveled to Central and South America and military who served in endemic areas (Herwaldt & Berman, 1992). In the New World, around 60% of skin diseases are due to CL (Pavli & Maltezou, 2010). Different *Leishmania* parasite species cause CL and that include: *Leishmania mexicana, Leishmania amazonensis, Leishmania braziliensis, Leishmania panamensis,*
**Leishmania guyanensis**, **Leishmania aethiopica**, **Leishmania venezuelensis**, **Leishmania lainsoni**, **Leishmania shawi**, **Leishmania peruviana**, **Leishmania naiffi**, **Leishmania lindenbergi**, **Leishmania. martiniquensis** and **Leishmania waltoni in the New World and Leishmania tropica, Leishmania aethiopica and Leishmania major in the Old World** (Tchokouaha Yamthe et al., 2017).

Mucocutaneous leishmaniasis mostly occurs in South America, specifically in Brazil, Paraguay, Ecuador, Bolivia, Peru, Colombia and Venezuela. Around 90% of the cases occur in Peru, Brazil and Bolivia (Handler, Patel, Kapila, Al-Qubati, & Schwartz, 2015a), (Grogl, Oduola, Cordero, & Kyle, 1989). MCL is caused by New World species of **Leishmania** belonging to the Viannia subgenus, namely **Leishmania Viannia braziliensis**, **Leishmania Viannia panamensis**, **Leishmania Viannia guyanensis**, and sometimes **L. amazonensis** (Tchokouaha Yamthe et al., 2017), (Bilbao-Ramos et al., 2017).

**Current Treatments**

Due to the absence of a vaccine against **Leishmania**, chemotherapy is the only choice for treatment. Drug development against **Leishmania** is challenging because an active drug needs to have the ability to pass through several membranes to reach the target. It also must maintain the pH changes to get into the parasite. Therefore, there are a limited number of available drugs to treat leishmaniasis. However, these available drugs suffer from limitations. The recommended treatment of visceral leishmaniasis is different between endemic regions (Nagle et al., 2014). In Africa, the WHO recommends a combination of pentavalent antimonials and parmamycin to treat visceral leishmaniasis (Berman, 2006). On the other hand, in South Asia, a combination of a
single dose of liposomal amphotericin B and a short course miltefosine is effective. In rich countries, liposomal amphotericin B is the drug of choice (Moore & Lockwood, 2010). In South America, meglumine antimoniate is the treatment of choice for mild and severe cases of visceral leishmaniasis, while liposomal amphotericin B is used for severe cases and pregnant women (Romero et al., 2017).

**Pentavalent Antimonials:** for decades, Antimony based compounds have been used to treat leishmaniasis. Brahmachari discovered the effectiveness of the pentavalent antimony compound urea stibamine against VL in 1925, which saved millions of infected individuals with VL especially in India (Marsden, 1986). Later on, in 1937 Solustibosan an antimony gluconate compound was synthesized. Besides, in 1945 Pentostam a sodium stibogluconate compound was discovered and used as an antileishmanial drug as well (Haldar, Sen, & Roy, 2011). Sodium stibogluconate (Pentostam) and meglumine antimonite (Glucantime) are the two currently used formulations of pentavalent antimonials that are used as a treatment for leishmaniasis. Both formulations suffer from difficult administration route (intramuscular injection or intravenous infusions) (Kato et al., 2014). Pentavalent antimonials are still used as the first-line drugs in many countries to treat all forms of leishmaniasis. However, they have several limitations such as prolonged treatment time that causes pain in the injection site, severe side effects (toxicity in the heart, liver and pancreas), drug resistance and the requirement of medical supervision. Apparently, VL/HIV positive patients are at higher risk of toxicity and mortality caused by the use of antimonials compared to VL/HIV negative patients treated with antimonials, or VL/HIV positive patients treated with amphotericin B or miltefosine (Ritmeijer et al., 2006). The mechanisms of
pentavalent antimonials are not fully understood and it is not clear if the final active form is Sb(V) or Sb(III). However, there are two main proposed mechanism of actions. The prodrug model where pentavalent antimonials Sb(V) is reduced to trivalent antimonials Sb(III) by macrophages or amastigotes in order to act as an antileishmanial agent (Frezard, Demicheli, Ferreira, & Costa, 2001). On the other hand, according to active pentavalent antimonials model, Sb(V) possess intrinsic antileishmanial activity (Walker & Saravia, 2004). Other studies showed that the level of expression of AQP1 transporter my play a role in parasite resistance against Sb(III) (Gourbal et al., 2004). In the early 2000s, the efficacy of antimonials decreased in India and more than 60% of VL infected patients in Bihar state did not respond to the treatment (Sundar et al., 2000). It is still not clear what causes the emergence of antimony resistant Leishmania donovani in Bihar state. A misuse of the drug might be behind the emergence of antimonials resistance. In another hypothesis, groundwater in Bihar state was contaminated with naturally occurring trivalent arsenic. Therefore, Bihar population was chronically exposed to low concentration of arsenic in drinking water, which may have led to the resistance in the Bihar population (Perry et al., 2015). The resistance to antimonials in India encouraged the development of other drugs. Antimonials are also used to treat cutaneous leishmaniasis and mucocutaneous leishmaniasis as well (Minodier & Parola, 2007).

Miltefosine: Miltefosine is an alkyle-phospholipid that is known as an anticancer drug (Dorlo et al., 2012). Miltefosine was tested against Leishmania and exhibited an exceptional activity, which was then approved to be the first oral VL treatment in India in 2002 (Sundar et al., 2012). The most common side effects of miltefosine are vomiting,
nausea, diarrhea, decreased appetite, headache and liver toxicity. Teratogenicity is another limitation of miltefosine (Tiwari, Gedda, Tiwari, Singh, & Singh, 2018). The mechanism of action is partially known as most studies were conducted on tumor cell lines. It is suggested that miltefosine induces apoptosis (Cell death) (Verma & Dey, 2004), (Paris, Loiseau, Bories, & Breard, 2004) and inhibit the synthesis of phosphatidyl choline (Wright, Howe, & Zaremberg, 2004), (Cui & Houweling, 2002). Recently, a study showed a decline in miltefosine efficacy with a decreased cure rate of around 90% and miltefosine resistance has been reported (Sundar et al., 2012), (Srivastava et al., 2017). There are several reported studies of lab induced miltefosine resistant *Leishmania* strains. One study showed that the transporter LdMT (P-type ATPase) is responsible for the translocation of miltefosine and the mutations in this gene were observed in the resistant strain (Perez-Victoria, Gamarro, Ouellette, & Castanys, 2003). It was also found that the overexpression of LiABCG4 leads to drug efflux resulting in resistance (Castanys-Munoz, Alder-Baerens, Pomorski, Gamarro, & Castanys, 2007).

**Amphotericin B:** In 1955, Amphotericin B was discovered as an antifungal drug. It was isolated from *Streptomyces nodosus* as a polyene antibiotic (Donovick, Gold, Pagano, & Stout, 1955). Then in 1960, the antileishmanial activity was reported, and a successful treatment against visceral leishmaniasis was achieved in 1963 in Brazil (Nagle et al., 2014), (Furtado, Cisalpino, & Santos, 1960), (Adler, 1964). This drug is the first line treatment for visceral leishmaniasis in the Mediterranean region. It is also the drug of choice for HIV/LV co-infection. Amphotericin B can be given as deoxycholate or as various lipid formulations. The main limitations of the deoxycholate form of the drug are infusion reactions (fever, chills, and thrombophlebitis), severe toxicity (myocarditis,
nephrotoxicity, and some times, death). These serious side effects require hospitalization for close monitoring, thus increasing the cost of the treatment and limiting the access for infected individuals. However, Lipid formulation of amphotericin B is less toxic but more expensive (Sundar & Chakravarty, 2010), (Messori, Fadda, Maratea, Trippoli, & Marinai, 2013). Amphotericin B is believed to bind to ergosterol in the cell membrane leading to the loss of membrane integrity resulting in cell death (Matsumori et al., 2009). Resistance to amphotericin B has recently emerged in the field (Purkait et al., 2012). A study showed that the significant sterol in amphotericin B resistant parasite was cholesa-5, 7, 24-trien-3β-ol, while the major sterol in amphotericin B sensitive parasite was ergosterol, which is the drug target (Purkait et al., 2012).

**Paromomycin:** Paromomycin is an aminoglycoside- aminocyclitol antibiotic that was isolated from *Stryptomyces krestomuceticus* in the 1950s. It is believed that paromomycin binds to 16S rRNA inhibiting the proteosynthesis (Vicens & Westhof, 2001). This drug was first used as a treatment for cutaneous leishmaniasis in 1966, and for visceral leishmaniasis since 1990 in Kenya (Nagle et al., 2014). The most significant side effect is pain at the injection site. Ototoxicity and an elevation of liver transaminases were reported (Sundar, Jha, Thakur, Sinha, & Bhattacharya, 2007). A randomized vehicle-control phase 3 trial of topical Paromomycin treatment with and without gentamicin for cutaneous leishmaniasis was conducted in Tunisia. The cure rate of index lesion was 81% and 82% for paromomycin-gentamicin and paromomycin alone respectively (Ben Salah et al., 2013).

**Pentamidine:** Pentamidine is being has been used for treatment of sleeping sickness since the 1940s (Rollo, Williamson, & Lourie, 1949) (Nagle et al., 2014). It was
first used as a treatment of VL in India in 1949 (Hazarika, 1949), and in Spain in 1950 (Martinez Garcia, Guasch, & Llaurado, 1950), (Nagle et al., 2014). The most serious and irreversible side effect of pentamidine is hypoglycemia. Therefore, it has to be given with extra care, particularly with diabetic patients (Bouchard et al., 1982). Other adverse effects include renal toxicity, hypotension and fever (Lai, Vrede, Soetosenojo, & Lai, 2002). Pentamidine is the drug of choice in French Guiana, and in Suriname (Roussel et al., 2006). Until recently, pentamidine was used as a second option drug to treat VL cases that did not respond to antimony treatment in India. However, the use of this drug was discontinued due to its toxicity and resistance and was replaced with amphotericin B (Sundar et al., 2001).

**Azoles:** Azoles are oral drugs that treat fungal infections. The main mechanism of action is inhibition of ergosterol biosynthesis that leads to the accumulation of 14 α-methyl sterols. Since *Leishmania* parasites share the biosynthesis of ergosterol with fungi, azoles antileishmanial activity was evaluated. Ketoconazole and itraconazole are being used as drugs against leishmaniasis with high efficacy (S. L. Croft, S. Sundar, & A. H. Fairlamb, 2006).

**Drug Combination Therapies:** Due to the few options of currently available antileishmanial drugs and their limitations, drug combination therapy with available treatments are being used as a fast approach to overcome these issues. A combination of sodium stibogluconate and paromomycin treatment was used in Bihar state and East Africa, and exhibited a high cure rate (Musa et al., 2012), (Thakur et al., 1992). Sequential use of different drugs was also used as another fast and safe approach of combination therapies. A study in India showed that a single infusion of AmBisome (5
mg/kg) followed by miltefosine (50 mg/kg/day) for 7 days or paromomycin (11 mg/kg/day) for 10 days displayed a 97.5% cure rate 6 months post treatment (Sundar et al., 2012).

**Vaccine**

There are various reasons that make the idea of producing an effective vaccine against *Leishmania* possible. The parasite does not undergo genetic variations, macrophages are the single host cell, and amastigotes are the only pathogenic stage of the disease (Reed, Coler, Mondal, Kamhawi, & Valenzuela, 2016), (Vijayakumar & Das, 2018). People in endemic areas have demonstrated acquisition of long-life immunity to the disease by injecting themselves with *Leishmania* lesion exudate (Khamesipour, Rafati, Davoudi, Maboudi, & Modabber, 2006). The historical practice of “leishmanization,” whereby live parasites that cause CL are inoculated in the skin in a cosmetically acceptable part of the body, is the only fully effective way to gain immunity to CL. The fact that individuals who fully recover from VL are then resistant to re-infection suggests that immunity to symptomatic visceral disease can be achieved via vaccination (Costa et al., 2011). Despite the tremendous work in the field to generate an effective vaccine against *Leishmania*, to date, there is no active vaccine for humans in the market yet. LEISH-F1 was the first vaccine that made it to phase I and II clinical trials and it has shown its effectiveness and safety. In addition, LEISH-F2 is a redesign to LEISH-F1 to overcome regulatory concern and enhances the manufacture process. This later vaccine has been evaluated in phase I and II clinical trials and was safe. Recently, LEISH-F3 was developed and successfully evaluated in phase I showing high
immunogenicity and safety. In addition, third generation vaccine for VL and PKDL was reported recently (Vijayakumar & Das, 2018).

**Leishmania Drug Targets**

Identifying a suitable drug target from a biological pathway is one of the major first steps in drug discovery projects. A molecular target can be enzymes, receptors, RNA, transcription factors and ion channels that present in the pathogen with biological activity linked to the disease (Hosen et al., 2014). The availability of the genome sequences of both *Leishmania major* and *Leishmania infantum* has disclosed information that can help in the identification of distinctive novel drug targets to these parasites. Fortunately, trypanosomatids are branched out quite early from the higher eukaryotes in the genetic phylogram. In fact, there are several cellular processes and metabolic pathways that are unique to the Leishmanial parasites that do not exist in the mammalian host. Thus, these pathways can be used as potential drug targets against *Leishmania* (Chawla & Madhubala, 2010). There are several important criteria to follow when selecting a target in a pathogen for drug development. A target must either not be present in the host or entirely divergent from the host homolog. It also should be essential for the survival of the pathogen and should be demonstrated in the host stage rather than the vector stage. Furthermore, the target gene should be expressed in the stage of the life cycle that is pathogenic to the host. Besides, it is crucial that the selected target has high assay ability in order to evaluate its efficacy (Frearson, Wyatt, Gilbert, & Fairlamb, 2007), (Vijayakumar & Das, 2018).

There are several unique metabolic pathways for trypanosomes that are absent in humans, making them good target for drug development against leishmaniasis.
Those include, SMT enzyme in the sterol (ergosterol) pathway, Glycolytic pathway, and enzymes involved in the trypathione synthesis pathway (Vijayakumar & Das, 2018).

**Drug Discovery**

Drug discovery is a process where potential new medicines are identified. Collaboration between academia and industry is required for a successful process. This process can take up to 15 years and cost over $1 billion (Hughes, Rees, Kalindjian, & Philpott, 2011), (Figure 1.4). Hit identification and lead discovery are one of the first steps in drug discovery after target identification and validation. In these steps, screening assays are applied (Figure 1.5). A hit molecule is defined as a compound with a desired activity when screened against the pathogen of interest and its activity is confirmed by retesting. There are a wide variety of screening strategies that are used to identify hit molecules and that include high throughput screening (large number of compounds screened in 384-well plates by complex laboratory automation), focused screen (compounds with similar structures or previously identified as hitting specific classes of targets), Fragment screen (obtain compounds with small mM activity), structural aided drug design, virtual screen, physiological screen, NMR screen (screen small compounds by soaking into protein targets of known crystal), and phenotypic screen. After hit confirmation by retesting, several compounds will be chosen for further exploration such as testing analogues to define Quantitative structure-activity relationship (QSAR). Up on QSAR confirmation, selected compounds will undergo lead optimization. In this phase, lead compounds are synthesized. A lead compound is defined as a chemical compound with a desirable pharmacological or biological activity to become a useful therapy. Lead compound structure may require chemical
modification to become a better fit to the target such as improve selectivity, potency and pharmacokinetic parameters (Frearson & Collie, 2009). The desirable characteristics for an ideal compound candidate in drug discovery are: show high affinity to the target (< 1 µM), has small molecular weight and lipophilicity, does not interfere with P450 enzyme or with P-glycoprotiens, chemically tractable, does not bind to human serum albumin, stable, drugable (druglikness), water soluble, rapidly metabolized, highly selective, demonstrate no cytotoxicity, and show cell membrane permeability (Hughes et al., 2011).

Leishmaniasis treatment relies mainly on therapeutic drugs. Currently available drugs suffer from various limitations such as toxicity, questionable efficacy, resistance, and high cost. Hence, new effective and affordable drugs are urgently needed (Giarolla & Ferreira, 2015). Regardless of the significant patient's population, there is limited available funding that supports the development of new antileishmanial treatments. Therefore, there have been tremendous efforts to repurpose drugs that treat different diseases to reduce the cost of developing a new effective drug against leishmaniasis (Handler, Patel et al. 2015). This strategy proved to be successful as several currently available antileishmanial medicines such as miltefosine, amphotericin B, and pentamidine were previously designed and approved to treat other diseases (Ashburn & Thor, 2004), (Novac, 2013). Identification of drug target is another significant approach in Leishmania drug discovery as it produces more specific results (Chawla & Madhubala, 2010). Moreover, screening chemical libraries is the most used and affordable approach when searching for novel antileishmanial hits.

**Screening Applications Used in Drug Discovery**
The identification of novel hits to undergo further development is a significant challenge in drug discovery project. Hit identification, lead optimization, suitable physiochemical properties and pharmacokinetic profile are initial steps in the drug discovery process and are required to increase the chances of developing compounds with high potency and selectivity to the target of interest (Andricopulo, Salum, & Abraham, 2009). There are several medicinal chemistry approaches that can be used to identify hits and generate leads such as structure based drug design (SBDD), receptor based pharmacophore model, molecular docking methods and structure based virtual screening. Other experimental methods include high throughput screening, structure activity relationship studies of biologically interested molecules, and fragment-based screening. The application of computational methods can improve the process of drug discovery with reduced cost and time efficient manner (Heifetz, Southey, Morao, Townsend-Nicholson, & Bodkin, 2018), (Njogu, Guantai, Pavadai, & Chibale, 2016). Moreover, bioinformatics is being used as an approach to accelerate the process of drug development. Besides, microarrays and proteome analysis of the complete genome, whenever available, provide valuable information in drug development by selecting novel enzymes and receptors that are specific for the parasites (Vijayakumar & Das, 2018). Most novel compounds against infectious diseases were identified by phenotypic drug discovery screening assays, but until recently, it was not the case when searching for promising hits against *Leishmania* due to the complexity of the biology of the parasite. However, new and improved technologies made it possible to identify novel agents against *Leishmania*. 
Improved in vitro and in vivo systems are being used for hit identification and lead optimization. The improved technologies allowed mammalian stage amastigotes and the sand fly stage promastigotes to be grown in axenic conditions. Therefore, direct drug screening methodologies against these stages can be applied. In vitro testing is considered quick, relatively cheap, and the results are consistent. However, the results generated from the in vitro system always need to be confirmed in animal studies (in vivo).

The intracellular amastigotes model is the most clinically relevant in vitro testing for screening compounds against *Leishmania* parasites to identify hits. It reflects the drug efficacy in the parasite natural environment since it can evaluate the drug ability to pass through the host cell membrane and test its effects on the parasites. In this model, murine peritoneal macrophages or human monocyte transformed macrophages (THP-1) are used as host cells. The drug activity can be then assessed by automated image analysis that counts the number of amastigotes per macrophage and generates IC$_{50}$ (Siqueira-Neto et al., 2012), (Gupta & Nishi, 2011).

The axenic amastigotes model is also used in screening compounds against *Leishmania*. This model tests the direct effect of compounds on the parasites but does not test its ability to penetrate the host cell or the effect of macrophage environment on the activity of tested compounds. Moreover, axenic amastigotes may express different metabolic process than intracellular amastigotes (Gupta & Nishi, 2011). There are several methods used to assess the activity of screened compounds against axenic amastigotes: viability of cells (cellTiter 96 non-radioactive cell proliferation based method), (Sereno et al., 1998), (Ganguly, Bandyopadhyay, Sarkar, & Chatterjee, 2006),
determination of ornithine decarboxylase activity (Callahan, Portal, Devereaux, & Grogl, 1997), fluorescent dye based methods such as propidium iodide and fluorescence activated cell sorter (Gazanion et al., 2011), (Sereno, Alegre, Silvestre, Vergnes, & Ouaissi, 2005).

Until recently, axenic promastigotes model was used to screen potential antileishmanial agents. However, promastigotes (sand fly stage) is widely different from the relevant stage amastigotes (host stage) in terms of ecology and metabolism of the parasite’s stages. In addition, promastigotes culture is maintained at 24-27 °C, this temperature is opposed to the in vivo temperature (37 °C) hence; the activity of the compounds may have different effects. Therefore, data obtained from screened compounds against axenic promastigotes may not be reflected in axenic amastigotes, infected macrophage model nor animal studies (Peters, Evans, & Lanham, 1983), (Simon L Croft, Karin Seifert, et al., 2006).

In vivo model can assess not only the activity of the compounds against Leishmania but also the rout of administration (absorption) and the ability of the compounds to reach the site of infection (distribution). Furthermore, determine if the compound acts as a pro-drug or as an immune-modulator (metabolism). It can also monitor the excretion of the compounds from the host body as well as giving an early indication of any toxicity.

**Focus of the Study**

This study aims to identify hit compounds from unstudied or understudied natural product and synthetic sources, with a focus on improved efficacy, selectivity, and oral bioavailability. We used our well-established axenic amastigote and infected
macrophage assays to screen thousands of natural product and synthetic compounds against *Leishmania donovani*. We also evaluated active hits against the mammalian cell line J774A.1 to ensure parasite selectivity. We were able to identify more than 2000 compounds with IC$_{50}$ values of $\leq 1$ $\mu$M against *Leishmania donovani* in multiple assays. Potent hits can be used as starting points for drug development efforts for the treatment of *Leishmania donovani*. 
Figure 1.1: *Leishmania* Life Cycle

(CDC, 2018).
Figure 1.2: *Leishmania* Species Promastigotes

Promastigotes are motile thin elongated cells with anterior kinetoplast and posterior free flagellum. They range in size from 5-14µm in length by 1.5-3.5µm in width (CDC, 2018).
Figure 1.3: *Leishmania* Species Amastigotes

Amastigotes are small round non-motile cells, measures around 2-4µm in diameter and has a nucleus and kinetoplast (CDC, 2018).
Figure 1.4: Drug Discovery Process and Timeline

Drug discovery process from basic research to FDA approved drug and the approximate timeline for the process.
Figure 1.5: Hit to Lead Process in Drug Discovery

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Chapter Two: Discovery of Novel Antileishmanial Hits from Natural Product

Note to reader

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Abstract

Leishmania is the causative agent of the neglected tropical disease leishmaniasis, which is transmitted by a bite of sand fly. Current treatments suffer from limitations such as toxicity, severe side effects, parasite resistance and high cost. Considering the current problems related to antileishmanial drugs, and the urgent need for alternatives, we investigated the leishmanicidal activity of unstudied natural product-based extracts such as mangrove endophytic fungal extracts, Antarctic deep-sea coral, and hairy root extracts of Catharanthus roseus. The investigation resulted in the identification of potent natural product–based compounds with submicromolar activity against Leishmania donovani.
Introduction

Leishmaniasis is a devastating parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania* (Wang et al., 2010). Sand fly (Phlebotomine) is the only known vector that transmits the disease (Bates et al., 2015). An estimation of 900,000 to 1 million people are infected with leishmaniasis per year and more than 300,000 deaths are reported annually (Kamhawi, 2017). Visceral leishmaniasis is the most fatal form of the disease if left untreated. Around 20 species of *leishmania* are pathogenic to humans.

*Leishmania donovani* is the causative agent for visceral leishmaniasis on the Indian subcontinent and Africa, while *Leishmania infantum* causes the same form of disease in the Middle East, Central and South America (Sundar & Singh, 2016). Pentavalent antimony is the drug of choice in treating leishmaniasis. However, resistance to pentavalent antimony is increasing, particularly in India. Amphotericin B is the second choice drug used primarily when pentavalent antimonials fail to treat leishmaniasis in addition to Miltefosine, Pentamidine and Paromomycin (Ponte-Sucre et al., 2017). The mentioned drugs suffer from limitations such as; high cost, severe side effects, difficult administration route and emergence of resistance (Vijayakumar & Das, 2018). Although, there are several ongoing vaccines projects against visceral leishmaniasis, they probably will not be available in the near future (Duthie et al., 2017). Therefore, new drugs are urgently needed to treat visceral leishmaniasis.

Natural products have proven to be particularly effective in treatment of parasitic diseases (Annang et al., 2015). There are numerous natural products with potent leishmanicidal activities in the drug discovery pipeline (Singh, Mishra, Bajpai, Singh, &
Tiwari, 2014). Approximately, 60% of most drugs are natural product derivatives (Newman & Cragg, 2016). Similarly, anticancer drugs such as vinblastine and vincristine are isolated from the plant Catharanthus roseus, camptothecin is isolated from Camptotheca acuminata and paclitaxel is isolated from Taxus brevifolia. Also, the antimalarial drug such as Artemisinin is isolated from the plant Artemisia annua (Gutierrez-Rebolledo, Drier-Jonas, & Jimenez-Arellanes, 2017). Furthermore, new potential microbial extracts has been identified from the MEDINA natural product library and showed activity against kinetoplastid parasites, including Leishmania (Annang et al., 2015). There are few existing natural-product based drugs used to that treat leishmaniasis. Miltefosine, the only available oral drug to treat visceral leishmaniasis, is an alkylphospholipid. Parmomycin is an aminoglycoside molecule and sitamaquine is an 8-aminoquinoline. Berberine also is active and is isolated from Berberis vulgaris (Wink, 2012). In addition, Gutierrez et al. have reported some reported plants extracts that exhibited activity against Leishmania major; these include dihydrocorynantheine, corynantheine, corynantheidine and other extracts against Leishmania donovani. Also, in Mexico, around 20 medicinal plants derivatives have been reported to have leishmanicidal activity. Pentalinonsterol was the only extract among the 20 to undergo in vivo evaluation (Gutierrez-Rebolledo et al., 2017).

Octocorals belong to the Cnidarian Order Alcyonacea are prolific sources of bioactive natural products, (Blunt, Copp, Keyzers, Munro, & Prinsep, 2017) including neurotoxic venoms used to paralyze vertebrate prey (Jouiaei et al., 2015) and potent small molecule toxins that are most often terpenoids (Roethle & Trauner, 2008). Octocoral diterpenes exhibit potent environmental toxicity, including the protection of
coral eggs during annual spawning events, inhibit fouling by microbes and algae, ichthyotoxicity, and contribute to the destruction of coral reefs (Coll, 1992). Biomedical studies have followed such ecological observations and found many of the same diterpenes cytotoxic to mammalian cells, though the most notable biomedical advancement was the introduction of the diterpene pseudopterosin for commercial use in anti-inflammatory and wound healing applications. (Martins, Vieira, Gaspar, & Santos, 2014)

Octocorals range in distribution from shallow water coral reefs to the depths of the abyssal plane. Shallow water species dominate the alcyonaceans with nearly 4000 species described in the World Record of Marine Species (WoRMS) database ("WoRMS, World Register of Marine Species," 2017) compared to just over 600 (15%) species that are recorded in the Deep Sea Octocorals Online database (Mainieri et al., 2015). Natural product reports from the two groups indicates the deep water species are less studied: roughly 750 alcyonacean compounds are cataloged in MarinLit, (Bernasconi et al., 2015) but only 28 (3.5%) compounds have been described from 12 deep water species (Iken & Baker, 2003; Skropeta, 2008; Skropeta & Wei, 2014; von Salm et al., 2014). The difficulty of accessing deep sea habitats has clearly hampered research of species hiding there, yet bioactivity profiles of, for example, shagenes (von Salm et al., 2014) and cristaxenicin A (Ishigami et al., 2012) suggest the deep water species are an understudied drug discovery resource (Thomas et al., 2017).

Although most drug discovery studies have focused on terrestrial plants as a natural product source when searching for active molecules to treat diseases, the search for novel bioactive agents from marine organisms has increased in recent years.
In 2004, Ziconotide was the first marine based drug to be approved in the United States for the treatment of back injuries and chronic pain (Molinski, Dalisay, Lievens, & Saludes, 2009). Over the years, marine algae have been used as anti-inflammatory, analgesic, antibacterial, antiviral, anti protozoans and anticancer drugs. In recent studies, marine algae showed antileishmanial activity, which provided potential lead for antileishmanial drug discovery (Tchokouaha Yamthe et al., 2017). However, the translation of potential drug candidates into usable leishmaniasis treatment has been a challenge in drug discovery projects (Zulfiqar et al., 2017). Also, finding active compounds from natural product crude extracts is time consuming and expensive. Therefore, it is very important to use an effective high throughput screening assays to ensure the selection of extracts with desirable pharmacological properties to undergo further development (Annang et al., 2015).

In this study we assessed natural product extracts and pure compounds from a variety of sources to identify new starting points for antileishmanial drug discovery.

Materials and Methods

Mangrove Endophytic Fungal Extracts

As part of research collaboration with Dr. Bill Baker, his team at the University of South Florida has collected, cultured and extracted mangrove endophytic fungi. Of these, 5,664 extracts have been generated for this study and were prepared in 96- well plates. These extracts have been tested in Leishmania donovani axenic amastigotes drug sensitivity assay, cytotoxicity assay and infected macrophage drug sensitivity assay. A top 100 list was generated according to the activity of these extracts in the infected macrophage drug sensitivity assay (< 1 µg/ml) and their selectivity over J774
macrophages (selectivity index > 50). A selectivity index is calculated by dividing the cytotoxicity IC\(_{50}\) by the infected macrophage IC\(_{50}\). The top 100 extracts were then retested in the infected macrophage model to confirm their activity. From these data a top 40 list was generated from the retested extracts results according to the same criteria used in selecting the top 100 extracts. Then the top 10 extracts from the list were scaled up and tested in the infected macrophage model. From these data one fungal isolate was selected and scaled up and produced pure compounds that were tested in the infected macrophage model and cytotoxicity model as well.

**Antarctic Deep sea Coral; Keikipukalides and Furanocembrane Diterpenes**

The Baker group collected the deep water alcyonacean *Plumarella delicatissima* from the ‘Plateau of Fascination’, approximately 240 km NE of Stanley, Falkland Islands (Islas Malvinas), in the Southern Ocean. While *Plumarella* sp. from the northwest Pacific Ocean near the Kuril Islands was reported to produce the diterpene plumarellide, (Stonik, Kapustina, Kalinovsky, Dmitrenok, & Grebnev, 2002) a rearranged furanocembranoid, the Southern Ocean species *P. delicatissima* has yielded five new furanocembranoid diterpenes, keikipukalide A-E. The pukalides were originally isolated from a Hawaiian soft coral collected near the popular tourist attraction known as the Blow Hole (Missakian et al.,*Tetrahedron* 1975, 31, 2513). Puka is the Hawaiian word for coral. We use the Hawaiian word for child, keiki, to distinguish our pukalide derivatives. (1-5), along with the known diterpene pukalide aldehyde (Abramson et al., 1991) (6) and norditerpenoid ineleganolide (Duh, Wang, Chia, & Chiang, 1999) (7) (Figure 2.2). All seven compounds have been tested in *Leishmania donovani* axenic
amastigotes drug sensitivity assay, cytotoxicity assay and infected macrophage drug sensitivity assay (Thomas et al., 2017).

**Catharanthus roseus Hairy Root Extracts**

Seven C. roseus hairy root extracts have been tested against *Leishmania donovani* and cytotoxicity as part of collaboration with Dr. Carolyn W.T. Lee-Parsons at Northeastern University. Her group prepared the extracts using different growth cycle and conditions to provide crude extracts to be tested in *Leishmania* bioassays.

**Macrophage Media Preparation and Culture**

J774A.1 macrophages (ATCC, Manassas, VA) were cultured in a vented 75 cm² tissue culture flask using RPMI 1640 (Gibco), 1% Penicillin-Streptomycin (Gibco, 10,000 u/ml), 10% 24 hour heat inactivated fetal bovine serum (Gibco) at a pH of 7.2. Twenty five ml of fresh media were added to the flasks 3 times per week and cells were serially passaged biweekly.

**Axenic Amastigotes Media Preparation and Culture**

*L. donovani* axenic amastigotes MHOM/SD/75/1246/130/ Khartoum (WR378) laboratory cell lines were cultured in a non-vented 25 cm² tissue culture flask (Coning, Lowell, MA) using RPMI 1640 (Gibco) with 5.86 g/L MES (Sigma-Aldrich, St Louis, MO), 7.5 g/L Hepes (Invitrogen Corp., Carlsbad, CA), 2 g/L sodium bicarbonate (Fisher Scientific, Pittsburg, PA), 100 µM Xanthine (Sigma-Aldrich, St Louis, MO), 10 mg/L Hemin (Sigma-Aldrich, St Louis, MO), 40 mg/L Tween-80 (Sigma-Aldrich, St Louis, MO), 5 g/L Trypton-Peptone (BD Bioscience, Sparks, MD), 1 % Penicillin- Streptomycin (Gibco, 10,000 u/ml), and 20 % 24 hours heat-inactivated FBS (Gibco). Media was set at a pH of 5.5.
L. donovani axenic amastigotes were cultured 1:5 three times per week and incubated at 37°C.

**Leishmania donovani Axenic Amastigotes Drug Sensitivity Assay**

Compounds were tested against *Leishmania donovani* axenic amastigotes (MHOM/SD/75/1246/130) using the CellTiter 96 AQueous one-solution cell proliferation assay (Promega, Madison, WI). In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 200µg/ml to 6.25µg/ml. Ten µL from each well was transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, #3628) and then 90 µL of parasites in media in a 66,000 cell per well concentration was added to produce a final concentration range from 20µg/ml to 625ng/ml. The plates were then incubated at 37°C for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium},(Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e (Molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC₅₀ values.

**Cytotoxicity Assay**

Compounds were tested against J774.A1 macrophages using the CellTiter 96 AQueous one-solution cell proliferation assay (Promega, Madison, WI). In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 500µg/ml to 15.625µg/ml. Ten µL from each well was
transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, # 3628) and 90µL of macrophages in media in a 50,000 cell per well concentration was added to produce a final concentration range from 50µg/ml to 1.562 µg/ml. The plates were then incubated at 37°C, 5% CO₂ for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium} (Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e (molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC₅₀ values.

**Infected Macrophage Drug Sensitivity Assay**

In a 384 well assay plate (CellCarrier-384 Black, Optically Clear Bottom, Tissue Culture Treated, Sterile, #6007550), 2000 J774A.1 macrophages were seeded and incubated for at least an hour to allow adherence. The plate was then washed with pre-warmed media to remove the unadhered cells. *L. donovani* axenic amastigotes were added to the plate in a ratio of 10:1 and incubated at 37 °C, 5% CO₂ for 24 hours. The excess extracellular amastigotes were then washed away using pre-warmed media. Compounds were prepared in a 384-drug plate (Thermo Scientific™ Nunc™ 384-Well Clear Polystyrene Plates with Non /treated Surfaces, #242757) with a starting concentration of 10 µg/ml and serially diluted in a 1:2 ratio. Drugs were then added to the assay plate and incubated at 37 °C, 5% CO₂ for 72 hours. After that, drugs were removed from the plate and the adhered cells were fixed with 2% paraformaldehyde (Alfa Aesar™ Paraformaldehyde, 16% w/v aq. soln., methanol free, #30525-89-4) and incubated for 15 minutes at room temperature. Fixative was then removed and cells
were stained with 5 µM Draq5 (Thermo Scientific DRAQ5 fluorescent probe, DNA stain, #62251) and incubated for 5 minutes at room temperature. Stain was removed and fresh media was added to the plate. High content fluorescent imaging (Perkin Elmer Operetta) was used to capture images used to perform cell counts. Harmony software calculated the number of amastigotes per 500 macrophages in each well and used these counts to generate IC$_{50}$ values.

In all three assays, Miltefosine was used as a negative growth control drug and culture media was used as a positive growth control.

**Results and Discussion**

Natural products are well known as a rich source for lead compounds in drug development (Kobayashi, 2016). Not long ago, most drug discovery projects that relied on natural products resources focused on terrestrial plants when searching for antiparasitic agents with potent activity (Carvalho et al., 2017). Apparently, the interest in studying marine organisms as potential source for lead bioactive materials has elevated recently (Molinski et al., 2009). Additionally, *Catharanthus roseus* plant is one of the most broadly studied plants for medicinal use (Zhu, Zeng, Sun, & Chen, 2014). *C. roseus* leaves extracts have been reported to show activity against *Plasmodium falciparum* (Ponarulselvam et al., 2012).

In an effort to discover new antileishmanial compounds from natural products, several bioactive molecules were isolated from mangrove endophytic fungal extracts and Antarctic deep-sea coral (keikipukalides and furanocembrane diterpenes) with submicromolar activity against *Leishmania donovani* infected macrophage assay. We
also investigated the antileishmanial activity in some terrestrial plant extracts such as *Catharanthus roseus*

**Mangrove Endophytic Fungal Extracts**

A total of 5,664 extracts were screened in *L. donovani* axenic amastigote drug sensitivity assay and cytotoxicity assay; of these, 1193 showed activity with an IC$_{50}$ less than 20 µg/ml. From this group 797 of 1193 were non toxic with an IC$_{50}$ greater than 50 µg/ml against J774 macrophages (Figure 2.1). A total of 2,140 extracts have been tested in infected macrophage drug sensitivity assay and 1564 extracts showed activity with an IC$_{50}$ less than 10 µg/ml (Figure 2.2).

A list of top 100 extracts was generated according to the activity of the infected macrophage drug sensitivity assay results with an IC$_{50}$ of <0.5 µg/ml and a selectivity index greater than 50. The top 100 active extracts were retested in the infected macrophage drug sensitivity assay and showed a 90% hit confirmation rate. A top 40 list was then generated from the reconfirmation list according to the IC$_{50}$ value < 0.5 µg/ml and a selectivity index >50. The top 10 extracts were selected, scaled up and retested in the infected macrophage model, which demonstrated a 60% re-hit rate. From these top 10 list, one fungal Isolate was selected, scaled up and produced a number of pure compounds, Phomopsichromin A, Phomopsichromin B, Phomopsichromin C, Phomopsichromin D, Phomopsichromin E. These pure compounds were tested in the infected macrophage model and cytotoxicity model. Phomopsichromin C was the most active compounds with an IC$_{50}$ = 0.67 µM and a selectivity index = 74.6 (Table 2.1), (Figure 2.3).
Antarctic Deep sea Coral; Keikipukalides and Furanocembrane Diterpenes

The seven terpenoids isolated from *Plumarella delicatissima* were evaluated against a number of infectious disease targets. *Leishmania donovani* was quite sensitive, displaying an IC$_{50}$ of 1.9-11.9 µM for compounds 2-7 (Table 2.2), (Figure 2.4) in the infected macrophage assay, compared to the IC$_{50}$ of 6.2 µM for miltefosine, a drug currently used for the treatment of leishmaniasis. No mammalian cytotoxicity was detected in the compounds below 50 µM. However, testing in a number of other infectious diseases, including, *Naegleria fowleri*, the ESKAPE (Rice, 2008) panel of drug resistant bacteria and *Clostridium difficile*, found none of these pathogens susceptible to the compounds (data not shown). Although this seeming specificity for *L. donovani* is promising for this scaffold, most of these compounds contain the neurotoxic pharmacophore of lophotoxin,(Abramson et al., 1991) which may prove to be a liability. *Plumarella* belongs in the family Primnoidae (suborder Calcaxonia), while nearly all similar furanocembranoid scaffolds have been reported from the Alcyoniidae or Gorgoniidae (suborders Alcyoniina or Holaxonia, respectively). However, briarane diterpenes have been isolated from Ellisellidae, which, like the Primnoidae, is in the suborder Calcaxonia. Octocoral phylogeny is in a state of flux after the integration of molecular data, and further investigations into the origin of these metabolites to possibly define a pattern between this and other coral genera could give chemotaxonomic or evolutionary insight into octocoral species around the globe (Thomas et al., 2018).

*Catharanthus roseus* Hairy Root Extracts

Seven crude extracts of *Catharanthus roseus* Hairy Root have been tested in *Leishmania donovani* axenic amastigotes drug sensitivity assay and cytotoxicity assay.
All seven extracts showed no activity when tested at the highest concentration in the axenic amastigote model with an IC₅₀ of >20 µg/ml and no sign of cytotoxicity with an IC₅₀ of >50 µg/ml. Interestingly, all 7 extracts displayed activity in the infected macrophage model with an IC₅₀ ranged between 2.42 µg/ml to 5.32 µg/ml (Table 2.3). To our knowledge, no leishmanicidal activity of *Catharanthus roseus* hairy root extracts has been reported previously.

By screening these natural products against *Leishmania donovani*, we were able to identify a number of novel hits with excellent antileishmanial activity. Further investigation and lead optimization are needed to determine if lead compounds have chemical and physical properties that would make them orally active drugs. Therefore, increase their possibility to undergo clinical trials with a good chance of reaching the market as a drug for leishmaniasis.
Figure 2.1: Mangrove Endophytic Fungi Extracts Activity in L. donovani Axenic Amastigotes Drug Sensitivity Assay and Cytotoxicity Assay

Total number of tested mangrove endophytic fungi extracts in L. donovani axenic amastigotes drug sensitivity assay and cytotoxicity assay, number of active extracts with an IC$_{50}$ less than 20 µg/ml and the number active not toxic extracts with an IC$_{50}$ >50 µg/ml in the cytotoxicity assay.
Figure 2.2: Mangrove Endophytic Fungi Extracts Activity in L. donovani Infected Macrophage Assay

Total number of tested mangrove endophytic fungi extracts in the infected macrophage drug sensitivity assay and the number of active extracts with an IC<sub>50</sub> < 10 µg/ml.
Table 2.1: Mangrove Endophytic Fungi Pure Compounds Activity (IC$_{50}$ µM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Infected Macrophage</th>
<th>Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phomopsichromin A</td>
<td>3</td>
<td>&gt;50</td>
<td>16.6</td>
</tr>
<tr>
<td>Phomopsichromin B</td>
<td>1.9</td>
<td>&gt;50</td>
<td>26.3</td>
</tr>
<tr>
<td>Phomopsichromin C</td>
<td>0.67</td>
<td>&gt;50</td>
<td>74.6</td>
</tr>
<tr>
<td>Phomopsichromin D</td>
<td>&gt;10</td>
<td>&gt;50</td>
<td>5</td>
</tr>
<tr>
<td>Phomopsichromin E</td>
<td>0.80</td>
<td>&gt;50</td>
<td>62.5</td>
</tr>
</tbody>
</table>

The > symbol indicates the sample was inactive at the highest concentration tested.
Figure 2.3: Structure of Phomopsichromin C

Infected macrophage IC$_{50}$ = 0.67 μM and a selectivity index = 74.6.
Table 2.2: Bioactivity of Plumarella Terpenes (IC$_{50}$ µM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Infected Macrophage</th>
<th>A549 Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keikipukalide -A</td>
<td>&gt;28.1</td>
<td>&gt;50</td>
<td>5</td>
</tr>
<tr>
<td>Keikipukalide -B</td>
<td>8.5</td>
<td>&gt;50</td>
<td>5.88</td>
</tr>
<tr>
<td>-Keikipukalide -C</td>
<td>8.8</td>
<td>&gt;50</td>
<td>5.68</td>
</tr>
<tr>
<td>Keikipukalide -D</td>
<td>11.9</td>
<td>&gt;50</td>
<td>5</td>
</tr>
<tr>
<td>Keikipukalide -E</td>
<td>8.8</td>
<td>&gt;50</td>
<td>5.68</td>
</tr>
<tr>
<td>Pukalide aldehyde</td>
<td>1.9</td>
<td>&gt;50</td>
<td>26.3</td>
</tr>
<tr>
<td>Ineleganolide</td>
<td>4.4</td>
<td>&gt;50</td>
<td>11.3</td>
</tr>
<tr>
<td>Miltefosine (control)</td>
<td>6.2</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

The > symbol indicates the sample was inactive at the highest concentration tested (Thomas et al., 2018).
Figure 2.4: Chemical structures of Plumarella Terpenes

Five new furanocembranoid diterpenes, keikipukalide A-E (1-5), known diterpene pukalide aldehyde (6) and norditerpenoid ineleganolide (7), (Thomas et al., 2018).
Table 2.3: *Catharanthus roseus* Hairy Root Extracts Activity (IC$_{50}$ µg/ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Axenic Amastigotes</th>
<th>J774 Cytotoxicity</th>
<th>Infected Macrophage</th>
<th>Infected Macrophage Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;20</td>
<td>&gt;50</td>
<td>2.53</td>
<td>19</td>
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<td>2</td>
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<td>&gt;50</td>
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<tr>
<td>6</td>
<td>&gt;20</td>
<td>&gt;50</td>
<td>5.13</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>&gt;20</td>
<td>&gt;50</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>1.5</td>
<td>&gt;50</td>
<td>2.56</td>
<td>20</td>
</tr>
</tbody>
</table>

The > symbol indicates the sample was inactive at the highest concentration tested.

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Chapter Three: Discovery of Potential Antileishmanial Synthetic Compounds

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Abstract

Leishmaniasis is a major neglected tropical disease caused by *Leishmania* species parasites. Visceral leishmaniasis is the most devastating form of the disease and can be fatal if left untreated. Limitations to current treatments such as the development of resistance by the parasite against drugs, high toxicity, difficult administration route and limited availability in some endemic areas are major concerns. Therefore, the search for new drugs is urgently needed to treat this disease. Thus, a total of 46,011 synthetic compounds with different classes of molecules were...
synthesized by different collaborators and screened in multiple *Leishmania donovani* assays in our lab. In this project, we were able to identify new hits from several different classes of compounds by using phenotypic screening assays and high content imaging screening assay. The following represent the most potent antileishmanial compound in each class in the infected macrophage model exhibiting limited toxicity toward the host macrophages; Monoamidines DB2381 (IC50= 0.15 µM, SI= 181), Thiazoles KP-16 (IC50= 0.19 µg/ml, SI= 247), Quinazolines QUI1209 (IC50= 0.27 µM, SI= 85), Pyrazolo[3,4-c]pyrimidines SNX2112 compound 18 (IC50= 1.06 µM, SI= 47), Monoguanidines 64 (IC50= 1.13 µM, SI= 23). These potent compounds were significantly more active than the currently used oral drug, miltefosine.

**Introduction**

Visceral leishmaniasis is the severe form of the neglected tropical disease leishmaniasis caused by *Leishmania donovani* parasite (Ozgilgin et al., 2017). Transmission of the disease can occur by a bite of the Phlebotomine sand fly. The infective stage promastigote is injected into the host body during a blood meal and transforms into the intracellular pathogenic tissue stage amastigotes (Tomassone et al., 2018).

The treatment of visceral leishmaniasis is very challenging due to limited chemotherapy options. Furthermore, these drugs suffered from severe side effects, toxicity, high cost, limited availability, difficult administration route, long treatment duration and elevated parasite resistance to administered drugs (Ortiz et al., 2017). Although there are several reports and clinical trials for potential vaccines against visceral leishmaniasis, an effective vaccine is not available to date (Kusakisako et al.,
These issues increased the complexity of the treatment of this disease. Therefore, additional new drugs with improved properties to overcome the current problems are vitally needed. Several reported studies addressed the urgent need for new chemotherapies and identified novel antileishmanial hits.

Wang et al. identified a new class of molecules (AIAs, arylimidamides containing 2 pyridylimidamide terminal groups) with submicromolar activity against *Leishmania donovani* axenic amastigote assay and infected macrophage assay (Wang et al., 2010). The two novel orally bioavailable AIA’s molecules DB745 and DB766 were exceptionally active in the *Leishmania donovani* infected macrophage assay (IC₅₀ less than 0.12 µM). Both compounds were also evaluated in vivo for efficacy and inhibited parasitemia level in *Leishmania donovani* infected hamsters and mice (Wang et al., 2010). Mono-AIA (containing a single pyridylimidamide terminal group) was also identified as a new class of lead compounds for drug development against *Leishmania donovani* (Zhu et al., 2016).

On the other hand, quinazoline derivatives were also reported for their promising antileishmanial activity. Quinazoline analogs are characterized by their ease of synthesis and suitable physiochemical properties (Sahu, Kumar, & Agrawal, 2017). These features made quinazoline an attractive class of candidate molecules for antileishmanial drug development. Agarwal et al. designed and synthesized 4- (Substituted-benzylidine)-2-substituted-5,6-dihydrobenzo[h]quinazoline and 4- (substituted-benzylidine)-2-substituted-3, 4, 5, 6-tetrahydrobenzo[h]quinazoline from 2- (substituted-benzylidine)tetralone-1 and several substituted guanidine sulfates. The 2 compounds profound promising antileishmanial hit against *Leishmania donovani*.
In addition, a series of N2,N4-disubstituted quinazoline-2,4-diamines are considered promising lead for the development of antileishmanial agent due to their ease of synthesis and desirable physiochemical properties. The potent compound (23) from this series demonstrated efficacy in the murine model of visceral leishmaniasis with 37% inhibition of liver parasitemia when given at 15 mg/kg for 5 consecutive days (Van Horn et al., 2014). It is believed that quinazolines inhibit dihydrofolate reductase (Gilbert, 2002). Van Horn et al investigated the possibility of this mechanism of action and found that quinazolines were less active in the presence of folic acid, which supported the hypothesis of previous studies (Van Horn et al., 2014).

Moreover, Hsp90 inhibitors displayed activity against some protozoan parasites including Leishmania (Varela, Mollinedo-Gajate, Muro, & Mollinedo, 2014). Hsp90 is a protein that overexpressed in stressed cells (Taipale, Jarosz, & Lindquist, 2010).

Another class of compounds such as azoles was also investigated as an antileishmanial agent. Fexinidazole (nitro-imidazole) and PA-824 (4-nitroimidazo-oxazine) are in phase II clinical trial with promising antileishmanial results. Fexinidazole is a treatment for human African Trypanosome and believed to act as a prodrug that releases cytotoxic metabolites damaging DNA, lipid, and protein. In contrast, PA-824 has antibacterial properties against M. tuberculosis. PA-824 displayed high efficacy when tested in a murine model of visceral leishmaniasis (Sundar & Singh, 2016).

In this study we evaluated synthetic compounds from a variety of different chemical scaffolds to identify new hits and to begin structure activity studies to optimize
the hits into lead compounds suitable for further development as new drugs for visceral leishmaniasis.

**Materials and Methods**

**Macrophage Media Preparation and Culture**

J774A.1 macrophages (ATCC, Manassas, VA) were cultured in a vented 75 cm² tissue culture flask using RPMI 1640 (Gibco), 1% Penicillin-Streptomycin (Gibco, 10,000 u/ml), 10% 24 hour heat inactivated fetal bovine serum (Gibco) at a pH of 7.2. Twenty-five ml of fresh media were added to the flasks 3 times per week and cells were serially passaged biweekly.

**Axenic Amastigotes Media Preparation and Culture**

*L. donovani* axenic amastigotes MHOM/SD/75/1246/130/ Khartoum (WR378) laboratory cell lines were cultured in a non-vented 25 cm² tissue culture flask (Coning, Lowell, MA) using RPMI 1640 (Gibco) with 5.86 g/L MES (Sigma-Aldrich, St Louis, MO), 7.5 g/L Hepes (Invitrogen Corp., Carlsbad, CA), 2 g/L sodium bicarbonate (Fisher Scientific, Pittsburg, PA), 100 µM Xanthine (Sigma-Aldrich, St Louis, MO), 10 mg/L Hemin (Sigma-Aldrich, St Louis, MO), 40 mg/L Tween-80 (Sigma-Aldrich, St Louis, MO), 5 g/L Trypton-Peptone (BD Bioscience, Sparks, MD), 1 % Penicillin- Streptomycin (Gibco, 10,000 u/ml), and 20 % 24 hours heat-inactivated FBS (Gibco). Media was set at a pH of 5.5. *L. donovani* axenic amastigotes were cultured 1:5 three times per week and incubated at 37°C.

**Leishmania donovani Axenic Amastigotes Drug Sensitivity Assay**

Compounds were tested against Leishmania donovani axenic amastigotes (MHOM/SD/75/1246/130) using the CellTiter 96 AQueous one-solution cell proliferation
In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 200µg/ml to 6.25µg/ml. Ten µL from each well was transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, #3628) and then 90µL of parasites in media in a 66,000 cell per well concentration was added to produce a final concentration range from 20µg/ml to 625ng/ml. The plates were then incubated at 37°C for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium}, (Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e ( Molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC50 values.

**Cytotoxicity Assay**

Compounds were tested against J774.A1 macrophages using the CellTiter 96 AQueous one-solution cell proliferation assay (Promega, Madison, WI). In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 500µg/ml to 15.625µg/ml. Ten µL from each well was transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, #3628) and 90µL of macrophages in media in a 50,000 cell per well concentration was added to produce a final concentration range from 50µg/ml to 1.562 µg/ml. The plates were then incubated at 37°C, 5% CO2 for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-
carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium} (Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e (molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC\textsubscript{50} values.

**Infected Macrophage Drug Sensitivity Assay**

In a 384 well assay plate (CellCarrier-384 Black, Optically Clear Bottom, Tissue Culture Treated, Sterile, #6007550), 2000 J774A.1 macrophages were seeded and incubated for at least an hour to allow adherence. The plate was then washed with pre-warmed media to remove the unadhered cells. *L. donovani* axenic amastigotes were added to the plate in a ratio of 10:1 and incubated at 37 °C, 5% CO\textsubscript{2} for 24 hours. The excess extracellular amastigotes were then washed away using pre-warmed media. Compounds were prepared in a 384-drug plate (Thermo Scientific™ Nunc™ 384-Well Clear Polystyrene Plates with Non-treated Surfaces, #242757) with a starting concentration of 10 µg/ml and serially diluted in a 1:2 ratio. Drugs were then added to the assay plate and incubated at 37 °C, 5% CO\textsubscript{2} for 72 hours. After that, drugs were removed from the plate and the adhered cells were fixed with 2% paraformaldehyde (Alfa Aesar™ Paraformaldehyde, 16% w/v aq. soln., methanol free, #30525-89-4) and incubated for 15 minutes at room temperature. Fixative was then removed and cells were stained with 5 µM Draq5 (Thermo Scientific DRAQ5 fluorescent probe, DNA stain, #62251) and incubated for 5 minutes at room temperature. Stain was removed and fresh media was added to the plate. High content fluorescent imaging (Perkin Elmer Operetta) was used to capture images used to perform cell counts. Harmony software
calculated the number of amastigotes per 500 macrophages in each well and used these counts to generate IC$_{50}$ values.

In all three assays, Miltefosine was used as a negative growth control drug and culture media was used as a positive growth control.

**Guanidines**

Giulianotti et al have previously reported on the use of mixture-based libraries to rapidly identify and prioritize active scaffolds (Rideout et al., 2011; Wu et al., 2013). Using this approach, we evaluated a scaffold-ranking library containing >6,000,000 compounds in a *L. donovani* axenic amastigote assay as well as a high-content imaging screen utilizing macrophages infected with *L. donovani* amastigotes. As previously described in detail (Rideout et al., 2011; Wu et al., 2013) a scaffold-ranking library is a collection of mixture samples where each mixture sample contains only compounds with the same core scaffold, and therefore all the compounds in a given mixture are close structural analogs. Additionally, each compound in a specific mixture sample is represented in approximately equal molar concentration. From this initial screen, the bis-cyclic guanidine scaffold-ranking sample Lib1 demonstrated potency in both of the aforementioned leishmaniasis assays with no sign of cytotoxicity in an uninfected macrophage counter screen. A positional scanning library was then screened in order to develop an SAR profile around the core bis-cyclic guanidine scaffold. From this analysis, 27 individual compounds were selected as good candidates to validate the profile.

Upon confirmation of the SAR trends with the individual compounds, a lead optimization campaign was undertaken to further explore the SAR profile around the
scaffold. These efforts resulted in the discovery of several new compounds possessing sub-micromolar IC$_{50}$s in the *L. donovani* infected macrophage assay. Among the hits is a monocyclic guanidine compound, 64 (Giulianotti et al., 2017).

**SNX2112 Analogues**

We recently found that SNX2112, 1 (Figure 3.1), (Chandarlapaty et al., 2008) showed potent activity in a high throughput axenic *L. donovani* amastigote assay (Varela et al., 2014). This compound was previously known to have potent antitumor activity, and a prodrug analog SNX5422, 2 (Figure 3.2) is currently in multiple clinical trials for oncology indications (Reddy et al., 2013), (Infante et al., 2014). The biochemical target of 1 is Hsp90, a ubiquitous chaperone protein known to facilitate protein folding and one that is often overexpressed in stressed cells (Taipale et al., 2010). It is the putative target of known bioactive natural products such as radicicol (Sharma, Agatsuma, & Nakano, 1998), and has proven to be a viable focus for oncology drug discovery. A number of Hsp90 inhibitors have been investigated in human clinical trials such as breast cancer, colorectal cancer and hepatic cancer (Trepel, Mollapour, Giaccone, & Neckers, 2010). Given that Hsp90 or related orthologs have been identified not just in mammals but in organisms from bacteria to yeast, it seemed reasonable that it could therefore serve as a viable target for the development of antiprotozoal agents. Indeed, there have been a number of reports of Hsp90 inhibitors that also possess activity against protozoan parasites (Woodford et al., 2016), including *Trypanosoma brucei* (African sleeping sickness), (Meyer & Shapiro, 2013), (Giannini & Battistuzzi, 2015), *Plasmodium falciparum* (malaria) (Giannini & Battistuzzi, 2015) (Shahinas, Folefoc, & Pillai, 2013) and *Leishmania* spp. (leishmaniasis), (Varela et al., 2014). We therefore
sought to explore the prospects of optimizing this initial hit as a potential antileishmanial agent. Critical to these efforts would be to ensure that any analogs would demonstrate this activity without demonstrating significant cytotoxicity, which would mitigate the potential usefulness of these agents as selective antileishmanial agents.

The initial discovery of 1 focused primarily on modifications to the anthranilamide portion in an attempt to optimize for pharmacokinetic parameters (Huang et al., 2009). This decision was based in part on docking studies that suggested the anthranilamide contributed to two of the three critical hydrogen bonds with the target and the apparent solvent exposure of the amine substituent. Notably, little attention was paid to the geminal methyl substituents other than to note that their presence was essential for activity. Given that their presence was primarily a result of the use of dimedone as the starting material for the synthesis of the initial lead series, we felt that this portion warranted additional consideration.

In order to confirm the initial hit compound, we prepared compounds 9 – 11 (Figure 3.3). We eschewed the CF₃ substituent in the hopes of restricting the cytotoxicity in this series (Huang et al., 2009). We also prepared quinazoline 11, which restricted the majority of the rotatable bonds to facilitate docking studies. Since this compound also displayed activity in our initial assay, we felt that it was an excellent candidate for docking into the active site of Hsp90. Using publicly available coordinates (3D0B) (Barta et al., 2008) for a co-crystal in AutoDock 1.5.6 (Trott & Olson, 2010) suggested that 11 would fit well into the active site, and that the geminal methyl substituents would access a hydrophobic pocket that might be exploited for additional activity or selectivity (Figure 3.4). We therefore set out to prepare analogs that would
allow us to introduce diversity late in the synthesis in order to evaluate their activity. We also considered the typical Lipinski/physicochemical parameters for each of these analogs in order to maximize the likelihood that any active compounds would have a reasonable chance at achieving acceptable oral bioavailability (Veber et al., 2002).

We envisioned that an appropriately adorned pyrazolo[3,4-c]pyridine core would serve as a suitable replacement for the initial scaffold such that the amine would provide a versatile handle for the installation of functionality that could probe this pocket. Toward that end, alkylation of the ethyl ester of N-benzylglycine with chloroacetone followed by tert-butoxide promoted cyclization gave cyclic β-diketone 13 (Figure 3.5) (Ziegler & Bennett, 1973). C-Acylation of this material proved more difficult than we anticipated, but the initial mixture of C- and O-acylated compounds could be converted to the desired 14 by cyanide equilibration. Condensation of this material with the arylhydrazine gave the requisite bromonitrile 15, which was able to undergo Buchwald-Hartwig amination and nitrile hydration to afford tertiary amine 16. The benzyl group could be reductively removed under acidic conditions to give 17, which served as a viable intermediate for the generation of substituted analogs. Acylation of 17 was able to be conducted with a series of readily available acid chlorides (Figure 3.5). Similarly, sulfonylation was easily achieved. Urea formation was conducted through the auspices of a benzyle carbamate, which could be debenzylated to give the parent 26.

In addition to evaluating these analogs in the initial high throughput axenic amastigote assay, we also investigated their activity in a high content screening (HCS) infected macrophage assay using transformed J774 murine macrophages that also allows us to evaluate cytotoxicity and ensure that the activity we see is not a general
cytotoxic phenomenon (Siqueira-Neto et al., 2012). Since the ultimate goal of this work is to identify a compound that not only demonstrates antileishmanial activity but also has the potential to be orally bioavailable, we considered the typical Lipinski “rule of 5” parameters for each of the compounds evaluated. Furthermore, we also considered other factors such as the number of rotatable bonds and the total polar surface area (tPSA) (Veber et al., 2002). These evaluations were completed using the DruLiTo drug likeness tool open source calculator (Kanwar et al., 2017).

**Mono-amidine Analogues**

Dr. David Boykin research group at Georgia State University focuses on the synthesis and development of molecules based on its ability to selectively bind to the minor groove of AT regions of DNA in order to inhibit one or more DNA dependent enzymes. The Boykin group has found that certain linear diamidines can attach to the minor groove with high affinity; therefore, they extended the design strategy of these molecules and evaluated their activity in multiple protozoan diseases and AIDS-related opportunistic diseases. Their focus was also on developing not only active antimicrobial agents but also has the potential to be orally bioavailable. As of that, 38 mono-amidine samples were selected and evaluated in the antileishmanial axenic amastigotes and infected macrophage assays in our lab.

**Quinazolines**

Quinazolines have been reported to have a significant antileishmanial activity. In previous work, Van Horn et al. showed a series of quinazolines as promising antileishmanial compounds. The efficacy of these compounds was also evaluated in a murine visceral leishmaniasis model (Van Horn et al., 2014).
Dr. Roman Manetsch research group at Northeastern University has synthesized a series of new quinazoline analogues. All 76 compounds were examined in the axenic amastigote and infected macrophage assays. Their toxicity was also assessed as described above. The IC\(_{50}\) values of all compounds were calculated and, some of them exhibited a submicromolar number in the infected macrophage assay with high selectivity index.

**Thiazole**

Thiazole based compounds showed potent activity against *Leishmania donovani* in many reported studies. Among these reports, a series of aminothiazoles were tested in in vitro and showed a potent antileishmanial activity as low as IC\(_{50}\) = 0.003 µM but had some issues with metabolic stability (Bhuniya et al., 2015). A series of phthalimido-thiazole derivatives was also reported to have activity against *Leishmania infantum* (Alianca et al., 2017).

Dr. Kirpal Bisht research group at USF synthesized and developed 19 thiazole analogues. Their antileishmanial activity and mammalian cytotoxicity were tested and the IC\(_{50}\) values were calculated as mentioned previously. Most of these analogues showed very potent submicromolar activity in both the axenic amastigotes and infected macrophage assays with excellent selectivity index comparable to the reference drug miltefosine.

**Primaquine-coumarin fluorescent probe (PQCP)**

Dr. Adonis McQueen (a former PhD student in the Kyle Lab) developed Primaquine-coumarin fluorescent probe (PQCP, a fluorescent-tagged primaquine) to discover cellular localization in *Plasmodium falciparum*, a human malaria parasite.
Primaquine (PQ) is the only known antimalarial drugs that clears dormant parasites in the liver and stop the transmission cycle of the parasites. We evaluated the antileishmanial activity of both PQCP and PQ in the *Leishmania donovani* axenic amastigote assay and assessed their cytotoxicity using J774A.1 macrophages (McQueen et al., 2017).

**Results and Discussion**

We have been actively engaged in drug discovery projects and collaborating with universities and institutes aiming for the discovery of novel antileishmanial compounds. Drug discovery for protozoan diseases has been improved by the use of improved screening assays. We used our well-established screening assays and screened millions of compounds against *Leishmania donovani* axenic amastigotes and infected macrophage. These compounds were provided by different drug discovery expertise in the field and demonstrated exceptional antileishmanial activity in multiple assays.

**Guanidines**

We initiated our study by screening 28 of our scaffold-ranking libraries at 4 doses (50, 25, 5, and 2.5 µM) in an assay using axenic *L. donovani* amastigote strains, CR6 and CS1 as described in Wang et al (Wang et al., 2010). Only scaffold-ranking sample **Lib1** showed substantial activity at any of the doses tested, producing >90% inhibition at the 25 and 50 µM doses.

While the axenic amastigote assay allows for a quick assessment of a sample’s intrinsic anti-leishmanial activity, it does have the weakness in that the effect of the host mammalian cell cannot be taken into account. In order to assess the anti-leishmanial efficacy of the scaffold-ranking samples against intracellular parasites, a high content
screening (HCS) assay with *L. donovani* infected J774 macrophages was utilized (Siqueira-Neto et al., 2012). In addition, a counter screen assay utilizing non-infected J774 macrophages was performed on the scaffold-ranking samples to assess their potential toxicity to the host mammalian cell. From these two assays, a selectivity index (SI) was calculated by dividing the IC\textsubscript{50} obtained from the non-infected macrophage by the IC\textsubscript{50} from the infected macrophage assay. From this infected macrophage screen, we found several scaffolds with attractive activity (sub-micromolar IC\textsubscript{50}) and selectivity (> 50 SI) profiles, one of which was **Lib1**. We thus turned our attention to exploring a positional scanning study on this scaffold.

Using our previously described synthetic methods (Wang et al., 2010), (Reed, Coler, Mondal, Kamhawi, & Valenzuela, 2016), we screened a 110 sample array around **Lib1** (42 carboxylic acids x 42 carboxylic acids x 26 amino acids). In this manner, we were able to evaluate a total of 45,864 different bis-cyclic guanidine analogs, where each compound was replicated in 3 different samples. The results of these screens are shown in (Table 3.1-3.3). Although the libraries were evaluated at several concentrations, the best range of results was apparent at 5 µM, and the percent inhibition at this concentration is shown (Santos et al., 2013). This data allowed us to rank order the relative likelihood that active individual compounds contain certain functionalities at a given position and to begin to augur certain trends. For example, based on the 5 µM data, it is clear that the mixture defined with cyclohexylbutyl at the R\textsubscript{1} position (Table 3.1 sample **Lib1.031**), is more active (100%) than the one defined with isobutyl (0%) at the same R\textsubscript{1} position (Table 3.1 sample **Lib1.022**). This implies that it is more likely to find active individual compounds with cyclohexylbutyl defined at R\textsubscript{1} than
with isobutyl at R₁. Across all three of these positions, oxygenated substituents dramatically reduced activity at the 5 µM dose. Also, there appears to be little preference for stereochemical specificity at the R₂ position (Table 3.2), where diastereomeric mixtures (e.g. Lib1.046/Lib1.054 and Lib1.049/Lib1.057) demonstrated nearly identical activities. In addition to the axenic amastigote data in (Table 3.1-3.3), the IC₅₀ data from the HCS infected macrophage assay is also provided. The infected macrophage data does not correlate perfectly with the axenic amastigote data, which is consistent with what we observed in our initial scaffold-ranking studies. While both assays measure the ability of a sample to inhibit the growth of the amastigotes at a given dose, the infected macrophage assay requires the samples to penetrate the macrophage first in order to get to the amastigote. Regardless, a number of the samples that produced sub 2.5 µM IC₅₀ in the infected macrophage assay also produced a >80% response at the 5 µM dose in the axenic amastigotes assay, demonstrating these samples’ ability to inhibit the growth of the amastigotes in both assay formats. In order to confirm some of the SAR trends that were observed in the positional scanning library data as well as to identify individual hit compounds with antileishmanial activity, a set of previously synthesized bis-cyclic guanidines were selected for testing (Fleeman et al., 2015). The compounds were synthesized as previously described incorporating 3 different functionalities in each of the R₁, R₂ and R₃ positions resulting in 27 structural analogs (Table 4). These different functionalities, when incorporated into the positional scanning library, demonstrated a range of activities in the anti-Leishmania proliferation assays (Table 3.1-3.3) therefore making them a good set to test the SAR, which is shown in (Table 3.4). In general, when
cyclohexylbutyl was fixed in the R₁ position (Compounds 15 to 23), the compounds showed an increased response in the axenic amastigote assay (6 out of 9 showed > 65% inhibition at 2.5 µM) relative to the other compounds (2 out of 18). These results are consistent with the data generated from the positional scanning library data (Table 3.1) where Lib1.031 showed greater activity than Lib1.041 or Lib1.007. Of those 9 individual compounds (15-23), the compounds where R₂ was R-2-naphthylmethyl were the inactive analogs (18-20). Again, this is consistent with the positional scanning data (Table 3.2) showing that Lib1.066 was much less active than Lib1.063 or Lib1.067. Using these trends as well as the range of activity in the infected macrophage and cytotoxicity assays, we initiated lead optimization activities. To facilitate screening, each sample was run as single data points, with miltefosine as a control on each plate. The IC₅₀ for this standard is 1.43 ± 0.20 µM, and no plates contained significant outliers for the control.

The first two candidates we selected for further exploration were 21 and 26, both of which showed submicromolar activity in the infected macrophage assay with dramatically different activities in the axenic amastigote assay. In both cases, we prepared a series of deletion analogs (in hopes of decreasing their lipophilicity) as well as inverting the stereocenter built into the initial scaffold (33 and 40, (Table 3.5). In both cases, this scaffold change resulted in a significant decrease in the activity. For the series around 21, most of the deletion compounds resulted in a significant deleterious effect on the infected macrophage activity, with the sole exception being 34. However, the good activity and SI profiles for 42 and 43 suggested that we might be able to focus our attention on monosubstituted analogs. Toward this end, while biphenyl compound
was not particularly active, the truncated phenethyl compound 47 showed excellent activity and selectivity.

We also pursued additional studies around compounds 6 and 8, which differed only about the R₃ position, but showed considerable activity in the infected macrophage assay (Table 3.6). While we again observed some compounds with acceptable activity and SI profiles, we were pleased to note that monosubstituted analog 50 was quite potent. We therefore synthesized and analyzed some analogs around this compound. Desfluoro analog 55 gave an excellent profile and indicated a preference for meta-substitution (see 54, 56 and 57). Saturation of the ring revealed that 59 was another analog with an excellent profile.

Encouraged by the identification of multiple monosubstituted analogs with submicromolar activity and low cytotoxicity, we wanted to determine the necessity of having two guanidine units. We were further struck by the prospect that the butyl substitution at R₂ could serve as a surrogate for the scaffold linker (Figure 3.7). In this regard, we could consider the R₁ and R₃ substituents together. Toward this end, we synthesized a series of monoguanidine analogs with and without the butyl substituent (Table 3.7), which clearly showed that the butyl group improved the activity. Furthermore, the optimal activity of 47 was closely reflected in analog 64, which showed an excellent SI and was more potent in our infected macrophage assay as miltefosine, a clinically-used antileishmanial agent that is used as our standard. By using Torry Pines scaffold ranking and positional scanning libraries, we were able to identify a series of guanidine-based compounds that possessed potent antileishmanial activity in multiple assays as well as selectivity over human macrophages, demonstrating that activity is
unlikely to arise through simple promiscuous interactions. These compounds are structurally distinct from any of the currently used antileishmaniasis agents as shown in (Figure 3.8), and thus represent a novel opportunity for lead optimization. We were subsequently able to use these libraries to focus in on compounds such as 64 that retain not only excellent activity (an IC\textsubscript{50} of 1.13 µM in the infected macrophage assay when run in quadruplicate with a standard deviation of 0.81 µM) and selectivity (an SI of 23 over J774 macrophages) but a reasonable molecular weight (245 g/mol) and physicochemical properties (e.g. cLogP = 3.26, tPSA = 39). Work toward the further optimization of these compounds with optimal pharmacokinetic and pharmacodynamics properties as well as attempts to ascertain their target or mechanism of action is underway and will be reported in due course (Giulianotti et al., 2017).

**SNX2112 Analogs**

The activity of all analogs in a high content screening infected macrophage assay using transformed J774 murine macrophages is displayed in (Table 3.8). As expected, analog 9 displayed potent and selective activity in the axenic amastigote assay, while the desmethylated 10 showed no activity at 25 µM. The HCS data indicates that the secondary and tertiary amines (17 and 16) are not particularly active, though it is noteworthy that the more substituted benzyl amine is the more active of the two which suggests a preference for greater substitution. This diminished activity pre-sumably arises from the physiological protonation of the amine under the assay conditions. More significantly, all of the amides showed reasonable HCS activity, and acetamide 18 was nearly equipotent with the parent (9). By contrast, sulfonamide 24 was considerably less active, and neither of the ureas showed any activity. It should also be noted that none of
the compounds prepared demonstrated any significant cytotoxicity, suggesting that the activity was specific to the Leishmania amastigotes. Notably, some analogs such as 18 and 24 show considerably more activity in the infected macrophage assay than in our initial axenic amastigote assay. This type of behavior has previously been observed with opioid receptor antagonists (De Muylder et al., 2011), suggesting more than one target could be involved in the observed macrophage activity. With the exception of the number of rotatable bonds for 25, each of the compounds fell within acceptable parameters for oral bioavailability. We tested our most active N-linked analog 18 in a kinetic solubility assay to confirm that these modifications did not result in a deleterious effect on the physicochemical properties of the original lead, and were pleased to see that it demonstrates excellent solubility (> 490 µM) in PBS buffer (pH 7.4). We also sought to determine whether the antileishmanial activity could be tied to Hsp90 inhibition. However, we were surprised to find that none of the new analogs prepared displayed significant inhibitory activity against human Hsp90 (Kim et al., 2004). In fact, unsubstituted analog 10 was more potent against Hsp90 than any of the compounds that were active in the antileishmanial assays. Among the possible explanations for this are these compounds are selective for the protozoan ortholog for Hsp90 (Hsp83) over human Hsp90 or that they are exerting their antileishmanial activity through a different mechanism (Kanwar et al., 2017).

In summary, we have found a series of novel pyrazolo[3,4-c]pyrimidines based on the known Hsp90 agent SNX-2112 that show activity against *Leishmania donovani* in multiple assays. Significantly, several of these new compounds demonstrate more potent activity than the clinically used miltefosine in both of these assays, and excellent
selectivity over any observed cytotoxicity against human J774 macrophage cells. Work toward a better understanding of this activity, including the biochemical target, is currently underway. Although the starting point for this project was a compound that demonstrated an excellent ADME/PK profile, this is an early drug discovery project and we will continue to assess these parameters as we seek a lead compound with optimal properties (Kanwar et al., 2017).

**Mono-amidine Analogs**

A library of aromatic diamidines and their analogues have been previously screened against *Leishmania donovani*, and novel DB745 and DB766 were discovered. Both compounds showed in vivo and in vitro exceptional antileishmanial activities. They also showed excellent activity against antimony-resistant Leishmania parasites. Despite that AIAs are structural analogs of cationic amidines (e.g., Pentamidine), they have different physiochemical properties and bioavailability (Wang et al., 2010). In an effort to discover more leishmanicidal analogs with improved properties, monoamidines compounds were synthesized, and their antileishmanial activity were evaluated against *Leishmania donovani* axenic amastigote assay and infected macrophage assay. Forty percent of the tested monoamidines analogs were potent leishmanicidal with submicromolar IC\textsubscript{50} values in the infected macrophage model (Figure 3.9). In particular, DB2381 IC\textsubscript{50} was among the most potent, with an IC\textsubscript{50} of 0.15 µM and a selectivity of 181 (Figure 3.10), which is substantially more active than miltefosine (Table 3.9). The data suggested that DB2381 is a promising hit candidate for drug development. Further work on DB2381 such as, in vivo antileishmanial activity and pharmacokinetics properties is needed in order to confirm its novel activity, and increase its chances to
undergo preclinical development and hope to become a treatment for visceral
leishmaniasis.

**Quinazoline**

Quinazolines have also been reported to have antileishmanial activity (Agarwal et al., 2009). As part of our effort to discover new classes of drugs to treat visceral leishmaniasis, in vitro activities of 76 quinazoline analogs were evaluated using axenic *Leishmania donovani* amastigote assay and infected macrophage assay (Table 3.10). Sixty percent of the tested analogues revealed activity in the infected macrophage assay, and only 20% were active in the axenic amastigote assay (Figure 3.11). Notably, QUI-1209 had an IC$_{50}$ of 0.27 µM with selectivity index equals to 85. The lack of activity seen in some quinazolines in the axenic amastigotes assay and their high potency in the infected macrophage assay suggest that these compounds’ action could be cell-mediated (Vermeersch et al., 2009). In vivo evaluation of QUI-1209 and pharmacokinetics need to be addressed to ensure its activity and physiochemical properties. The ease of synthesis and suitable physiochemical properties are features that make quinazolines an attractive class of candidate molecules for antileishmanial drug development.

**Thiazole**

Thiazoles based compounds have shown antileishmanial activity (Ardestani et al., 2012). Bhuniya et al. have synthesized a series of aminothiazoles and were screened for in vitro efficacy, microsomal stability, and solubility. Their results showed that this class of compounds has a promising submicromolar activity against visceral leishmaniasis (Bhuniya et al., 2015). In contrast, Thompson et al. screened
nitroimidazothiazoles and revealed no activity against *Leishmania infantum* (visceral leishmaniasis). Interestingly, it possessed potent activity against *Trypanosoma cruzi* (Chagas disease), (Thompson et al., 2017). The Bisht research group at USF synthesized a new series of thiazoles compounds. These compounds were screened against *Leishmania donovani* axenic amastigote model and infected macrophage model in our lab. Interestingly, 95% of all tested samples displayed activity against *Leishmania donovani* infected macrophage assay (Figure 3.12), with KBA-16 being among the most potent compound with an IC\textsubscript{50} of 0.75 µM and a selectivity index of 237 (Figure 3.13), significantly more potent than miltefosine, which demonstrated an IC\textsubscript{50} of 1.75 µM and a selectivity index of 28. Apparently, compound 16 exhibited no activity when tested against *Leishmania donovani* axenic amastigotes (Table 3.11). There are more than one possible explanation for this phenomenon. This compound might act as a prodrug where it gains its activity after entering the host cell. Or, it might be targeting the macrophage survival therefore leads to the death of the parasite.

**Primaquine-coumarin fluorescent probe (PQCP)**

As reported by McQueen et al. the developed primaquine coumarin- fluorescent probe (PQCP) demonstrated antimalarial potency similar to primaquine (PQ) and the probe was not toxic against HepG2 carcinoma cells. Interestingly, the developed probe decreased the toxicity of primaquine to the mammalian J774 cells. However, PQ was more active against *Leishmania donovani* axenic amastigote compared to PQCP (Table 3.12), suggesting that the demonstrated activity of PQ against *Leishmania donovani* axenic amastigote might be due to its toxicity (McQueen et al., 2017).
Our drug discovery effort led to the recognition of potent new classes and series of antileishmanial compounds with an exceptional submicromolar activity in the infected macrophage assay. Significantly more potent compared to miltefosine, the only available oral antileishmanial treatment. The identified chemical classes haven’t been reported as leishmanicidal in the field yet. However, further studies on these compounds such as in vivo evaluation, structure activity relationship (SAR), and pharmacokinetics are essential to develop new drugs that fit the need of leishmaniasis treatments.
Figure 3.1: Compound 1

(Kanwar et al., 2017).
Figure 3.2: Compound 2

(Kanwar et al., 2017).
Figure 3.3: Preparation of Compounds 9-11

(Kanwar et al., 2017).
Figure 3.4: Minimized Poses of 11 in Hsp90 Showing The Geminal Methyl Groups Pointing Toward Hydrophobic Pockets

(Kanwar et al., 2017).
Figure 3.5: Preparation of Compounds 12-17

(Kanwar et al., 2017).
Figure 3.6: Preparation of Compounds 18-26

(Kanwar et al., 2017).
Table 3.1: Leishmaniasis Activity of R1 Positional Scanning Study of Lib1

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<td>14%</td>
<td>3.5</td>
<td>Lib1.020</td>
<td><img src="image4" alt="Image" /></td>
<td>4%</td>
<td>5.3</td>
</tr>
<tr>
<td>Lib1.010</td>
<td><img src="image5" alt="Image" /></td>
<td>0%</td>
<td>&gt;10</td>
<td>Lib1.021</td>
<td><img src="image6" alt="Image" /></td>
<td>100%</td>
<td>1.3</td>
</tr>
<tr>
<td>Lib1.011</td>
<td><img src="image7" alt="Image" /></td>
<td>34%</td>
<td>&gt;10</td>
<td>Lib1.022</td>
<td><img src="image8" alt="Image" /></td>
<td>0%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.023</td>
<td><img src="image9" alt="Image" /></td>
<td>10%</td>
<td>5.2</td>
<td>Lib1.033</td>
<td><img src="image10" alt="Image" /></td>
<td>0%</td>
<td>4.6</td>
</tr>
<tr>
<td>Lib1.024</td>
<td><img src="image11" alt="Image" /></td>
<td>31%</td>
<td>&gt;10</td>
<td>Lib1.034</td>
<td><img src="image12" alt="Image" /></td>
<td>61%</td>
<td>1.2</td>
</tr>
<tr>
<td>Lib1.025</td>
<td><img src="image13" alt="Image" /></td>
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<td>&gt;10</td>
<td>Lib1.035</td>
<td><img src="image14" alt="Image" /></td>
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<td>1.2</td>
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Table 3.1: (Continued)

<table>
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<th>ID#</th>
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<th>IM</th>
<th>ID#</th>
<th>R₁</th>
<th>AA</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lib1.026</td>
<td><img src="image" alt="Structure" /></td>
<td>93%</td>
<td>0.9</td>
<td>Lib1.036</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.027</td>
<td><img src="image" alt="Structure" /></td>
<td>0%</td>
<td>2.5</td>
<td>Lib1.037</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>2.4</td>
</tr>
<tr>
<td>Lib1.028</td>
<td><img src="image" alt="Structure" /></td>
<td>14%</td>
<td>1.7</td>
<td>Lib1.038</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>1.6</td>
</tr>
<tr>
<td>Lib1.029</td>
<td><img src="image" alt="Structure" /></td>
<td>61%</td>
<td>1.9</td>
<td>Lib1.039</td>
<td><img src="image" alt="Structure" /></td>
<td>98%</td>
<td>1.2</td>
</tr>
<tr>
<td>Lib1.030</td>
<td><img src="image" alt="Structure" /></td>
<td>99%</td>
<td>3.6</td>
<td>Lib1.040</td>
<td><img src="image" alt="Structure" /></td>
<td>63%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.031</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>0.6</td>
<td>Lib1.041</td>
<td><img src="image" alt="Structure" /></td>
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<td>1.2</td>
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<tr>
<td>Lib1.032</td>
<td><img src="image" alt="Structure" /></td>
<td>63%</td>
<td>2.9</td>
<td>Lib1.042</td>
<td><img src="image" alt="Structure" /></td>
<td>99%</td>
<td>0.5</td>
</tr>
</tbody>
</table>

AA is the % inhibition at 5 µM in the axenic amastigotes assay. IM is the IC50 in µM of the infected macrophage assay (Giulianotti et al., 2017).
Table 3.2: Leishmaniasis Activity of R2 Positional Scanning Study of Lib1

<table>
<thead>
<tr>
<th>ID#</th>
<th>R₂</th>
<th>AA</th>
<th>IM</th>
<th>ID#</th>
<th>R₂</th>
<th>AA</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lib1.043</td>
<td>Me</td>
<td>91%</td>
<td>&gt;10</td>
<td>Lib1.056</td>
<td>OH</td>
<td>0%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.044</td>
<td>Ph</td>
<td>99%</td>
<td>&gt;10</td>
<td>Lib1.057</td>
<td></td>
<td>3%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.045</td>
<td>H</td>
<td>84%</td>
<td>&gt;10</td>
<td>Lib1.058</td>
<td>Me</td>
<td>23%</td>
<td>5.3</td>
</tr>
<tr>
<td>Lib1.046</td>
<td>Me-Me</td>
<td>100%</td>
<td>1.2</td>
<td>Lib1.059</td>
<td>OH-Ph</td>
<td>0%</td>
<td>5.4</td>
</tr>
<tr>
<td>Lib1.047</td>
<td>Me-Me</td>
<td>100%</td>
<td>5.1</td>
<td>Lib1.060</td>
<td></td>
<td>29%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.048</td>
<td>OH</td>
<td>0%</td>
<td>5.8</td>
<td>Lib1.061</td>
<td>Me</td>
<td>56%</td>
<td>5.2</td>
</tr>
<tr>
<td>Lib1.049</td>
<td>OH-Me</td>
<td>7%</td>
<td>&gt;10</td>
<td>Lib1.062</td>
<td></td>
<td>98%</td>
<td>5.3</td>
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Table 3.2: (Continued)

<table>
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<th>IM</th>
<th>ID#</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>AA</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lib1.050</td>
<td>Me</td>
<td>65%</td>
<td>&gt;10</td>
<td>Lib1.063</td>
<td>Me</td>
<td>99%</td>
<td>4.5</td>
</tr>
<tr>
<td>Lib1.051</td>
<td>Me</td>
<td>0%</td>
<td>&gt;10</td>
<td>Lib1.064</td>
<td>Me</td>
<td>100%</td>
<td>5.1</td>
</tr>
<tr>
<td>Lib1.052</td>
<td>Me</td>
<td>79%</td>
<td>&gt;10</td>
<td>Lib1.065</td>
<td>Me</td>
<td>95%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.053</td>
<td>Me</td>
<td>96%</td>
<td>&gt;10</td>
<td>Lib1.066</td>
<td>Me</td>
<td>26%</td>
<td>3.8</td>
</tr>
<tr>
<td>Lib1.054</td>
<td>Me</td>
<td>99%</td>
<td>&gt;10</td>
<td>Lib1.067</td>
<td>Me</td>
<td>99%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.055</td>
<td>Me</td>
<td>97%</td>
<td>&gt;10</td>
<td>Lib1.068</td>
<td>Me</td>
<td>100%</td>
<td>0.6</td>
</tr>
</tbody>
</table>

AA is the % inhibition at 5 µM in the axenic amastigotes assay. IM is the IC50 in µM of the infected macrophage assay (Giulianotti et al., 2017).
Table 3.3: Leishmaniasis Activity of R3 Positional Scanning Study of Lib1

<table>
<thead>
<tr>
<th>ID#</th>
<th>R₃</th>
<th>AA</th>
<th>IM</th>
<th>ID#</th>
<th>R₃</th>
<th>AA</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lib1.069</td>
<td>H</td>
<td>0%</td>
<td>5.4</td>
<td>Lib1.080</td>
<td>benzene</td>
<td>13%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.070</td>
<td>Ph-Me</td>
<td>38%</td>
<td>&gt;10</td>
<td>Lib1.081</td>
<td>ethylphenyl</td>
<td>21%</td>
<td>1.3</td>
</tr>
<tr>
<td>Lib1.071</td>
<td>Ph-Me</td>
<td>63%</td>
<td>3.6</td>
<td>Lib1.082</td>
<td>2-methylphenyl</td>
<td>86%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.072</td>
<td>Ph-Me</td>
<td>88%</td>
<td>0.6</td>
<td>Lib1.083</td>
<td>4-chlorophenyl</td>
<td>3%</td>
<td>1.2</td>
</tr>
<tr>
<td>Lib1.073</td>
<td>Ph-F</td>
<td>11%</td>
<td>1.3</td>
<td>Lib1.084</td>
<td>2,4-difluorophenyl</td>
<td>19%</td>
<td>1.3</td>
</tr>
<tr>
<td>Lib1.074</td>
<td>Ph-Br</td>
<td>11%</td>
<td>1.3</td>
<td>Lib1.085</td>
<td>2,4-dimethoxyphenyl</td>
<td>1%</td>
<td>1.8</td>
</tr>
<tr>
<td>Lib1.075</td>
<td>CF₃</td>
<td>25%</td>
<td>1.4</td>
<td>Lib1.086</td>
<td>phenyl</td>
<td>13%</td>
<td>4.8</td>
</tr>
</tbody>
</table>
Table 3.3: (Continued)

<table>
<thead>
<tr>
<th>ID#</th>
<th>R₃</th>
<th>AA</th>
<th>IM</th>
<th>ID#</th>
<th>R₃</th>
<th>AA</th>
<th>IM</th>
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<tbody>
<tr>
<td>Lib1.076</td>
<td><img src="image1.jpg" alt="Image" /></td>
<td>57%</td>
<td>0.4</td>
<td>Lib1.087</td>
<td><img src="image2.jpg" alt="Image" /></td>
<td>2%</td>
<td>1.3</td>
</tr>
<tr>
<td>Lib1.077</td>
<td><img src="image3.jpg" alt="Image" /></td>
<td>11%</td>
<td>6.1</td>
<td>Lib1.088</td>
<td><img src="image4.jpg" alt="Image" /></td>
<td>12%</td>
<td>0.7</td>
</tr>
<tr>
<td>Lib1.078</td>
<td><img src="image5.jpg" alt="Image" /></td>
<td>9%</td>
<td>7</td>
<td>Lib1.089</td>
<td><img src="image6.jpg" alt="Image" /></td>
<td>99%</td>
<td>5.1</td>
</tr>
<tr>
<td>Lib1.079</td>
<td><img src="image7.jpg" alt="Image" /></td>
<td>12%</td>
<td>&gt;10</td>
<td>Lib1.090</td>
<td><img src="image8.jpg" alt="Image" /></td>
<td>0%</td>
<td>0.7</td>
</tr>
<tr>
<td>Lib1.091</td>
<td><img src="image9.jpg" alt="Image" /></td>
<td>7%</td>
<td>0.9</td>
<td>Lib1.101</td>
<td><img src="image10.jpg" alt="Image" /></td>
<td>0%</td>
<td>1.4</td>
</tr>
<tr>
<td>Lib1.092</td>
<td><img src="image11.jpg" alt="Image" /></td>
<td>7%</td>
<td>5.1</td>
<td>Lib1.102</td>
<td><img src="image12.jpg" alt="Image" /></td>
<td>9%</td>
<td>1.2</td>
</tr>
<tr>
<td>Lib1.093</td>
<td><img src="image13.jpg" alt="Image" /></td>
<td>58%</td>
<td>1.3</td>
<td>Lib1.103</td>
<td><img src="image14.jpg" alt="Image" /></td>
<td>91%</td>
<td>1.3</td>
</tr>
<tr>
<td>Lib1.094</td>
<td><img src="image15.jpg" alt="Image" /></td>
<td>79%</td>
<td>1.8</td>
<td>Lib1.104</td>
<td><img src="image16.jpg" alt="Image" /></td>
<td>94%</td>
<td>1.3</td>
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Table 3.3: (Continued)

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<th>IM</th>
<th>ID#</th>
<th>$R_3$</th>
<th>AA</th>
<th>IM</th>
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<td>Lib1.105</td>
<td><img src="image2" alt="Molecule" /></td>
<td>11%</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lib1.096</td>
<td><img src="image3" alt="Molecule" /></td>
<td>21%</td>
<td>1.7</td>
<td>Lib1.106</td>
<td><img src="image4" alt="Molecule" /></td>
<td>97%</td>
<td>3.5</td>
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<tr>
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<td>15%</td>
<td>1.4</td>
<td>Lib1.107</td>
<td><img src="image6" alt="Molecule" /></td>
<td>42%</td>
<td>&gt;10</td>
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<tr>
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<td>92%</td>
<td>&gt;10</td>
<td>Lib1.108</td>
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<td>11%</td>
<td>&gt;10</td>
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<tr>
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<td>4.9</td>
<td>Lib1.109</td>
<td><img src="image10" alt="Molecule" /></td>
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<td>0.7</td>
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<tr>
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<td>1.2</td>
<td>Lib1.110</td>
<td><img src="image12" alt="Molecule" /></td>
<td>62%</td>
<td>2.7</td>
</tr>
</tbody>
</table>

AA is the % inhibition at 5 $\mu$M in the axenic amastigotes assay. IM is the IC50 in $\mu$M of the infected macrophage assay (Giulianotti et al., 2017).
Table 3.4: Individually Synthesized Compound Activity

<table>
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<th>#</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>AA (5 mM)</th>
<th>AA (2.5 mM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mM)</th>
<th>Cytotoxicity IC&lt;sub&gt;50&lt;/sub&gt; (mM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>A</td>
<td>D</td>
<td>G</td>
<td>26%</td>
<td>9%</td>
<td>0.6</td>
<td>48</td>
<td>81</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>D</td>
<td>B</td>
<td>43%</td>
<td>0%</td>
<td>&gt;5</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>D</td>
<td>H</td>
<td>100%</td>
<td>47%</td>
<td>0.3</td>
<td>28</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>E</td>
<td>G</td>
<td>100%</td>
<td>37%</td>
<td>3.5</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>A</td>
<td>E</td>
<td>B</td>
<td>75%</td>
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<td>1.8</td>
<td>50</td>
<td>28</td>
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<td>A</td>
<td>E</td>
<td>H</td>
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<td>0%</td>
<td>1.3</td>
<td>50</td>
<td>39</td>
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<tr>
<td>12</td>
<td>A</td>
<td>F</td>
<td>G</td>
<td>6%</td>
<td>0%</td>
<td>&gt;5</td>
<td>50</td>
<td>10</td>
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<tr>
<td>13</td>
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<td>F</td>
<td>B</td>
<td>94%</td>
<td>0%</td>
<td>1.8</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
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<td>A</td>
<td>F</td>
<td>H</td>
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<td>&gt;5</td>
<td>46</td>
<td>9</td>
</tr>
<tr>
<td>15</td>
<td>B</td>
<td>D</td>
<td>G</td>
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<td>D</td>
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<td>3</td>
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<td>H</td>
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<td>12</td>
<td>2</td>
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<tr>
<td>18</td>
<td>B</td>
<td>E</td>
<td>G</td>
<td>97%</td>
<td>6%</td>
<td>1.3</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td>19</td>
<td>B</td>
<td>E</td>
<td>B</td>
<td>0%</td>
<td>0%</td>
<td>0.9</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>20</td>
<td>B</td>
<td>E</td>
<td>H</td>
<td>0%</td>
<td>15%</td>
<td>&gt;5</td>
<td>34</td>
<td>7</td>
</tr>
<tr>
<td>21</td>
<td>B</td>
<td>F</td>
<td>G</td>
<td>95%</td>
<td>97%</td>
<td>0.7</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>22</td>
<td>B</td>
<td>F</td>
<td>B</td>
<td>95%</td>
<td>77%</td>
<td>&gt;5</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>B</td>
<td>F</td>
<td>H</td>
<td>95%</td>
<td>70%</td>
<td>0.3</td>
<td>20</td>
<td>69</td>
</tr>
<tr>
<td>24</td>
<td>C</td>
<td>D</td>
<td>G</td>
<td>94%</td>
<td>96%</td>
<td>&gt;5</td>
<td>32</td>
<td>6</td>
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Table 3.4: (Continued)

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<th>R₂</th>
<th>R₃</th>
<th>AA (5 mM)</th>
<th>AA (2.5 mM)</th>
<th>IM IC₅₀ (mM)</th>
<th>Cytotoxicity IC₅₀ (mM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>C</td>
<td>D</td>
<td>B</td>
<td>8%</td>
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<td>0.9</td>
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AA is the % inhibition in the axenic amastigotes assay. IM is the infected macrophage assay. Cytotoxicity is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotoxicity/IM) (Giulianotti et al., 2017).
Table 3.5: Analogues of 21 and 26

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IM is the infected macrophage assay. Cytotoxicity is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotoxicity/IM) (Giulianotti et al., 2017).
Table 3.6: Analogs of 6–8

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<th>Cytotoxicity IC&lt;sub&gt;50&lt;/sub&gt; (mM)</th>
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<td>H</td>
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IM is the infected macrophage assay. Cytotoxicity is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotoxicity/IM) (Giulianotti et al., 2017).
Figure 3.7: Similarity of R1 and R3

(Giulianotti et al., 2017).
### Table 3.7: Monoguanidines

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IM is the infected macrophage assay. Cytotoxicity is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotoxicity/IM) (Giulianotti et al., 2017).
Figure 3.8: Currently Used Antileishmaniasis Agents

Table 3.8: Biological and Physicochemical Data For All Analogs

<table>
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<th>Compound</th>
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AA is for Axenic Amastigote, IM is for Infected Macrophage, Cytotox is for Cytotoxicity, and MW is for Molecular Weight (Kanwar et al., 2017).
Figure 3.9: Activity of Mono-Amidine Series in Multiple Assays

40% were active in the infected macrophage assay, 40% were active in the axenic amastigotes assay and 21% were not toxic in the cytotoxicity assay.
Figure 3.10: Chemical Structure of DB2381

Infected macrophage IC$_{50}$ = 0.15µM with SI=181.
Table 3.9: Activity of Mono-amidine Compounds in Multiple Assays (IC50 µM)

<table>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2506</td>
<td>4.4</td>
<td>1.14</td>
<td>11.2</td>
<td>9.8</td>
</tr>
<tr>
<td>DB2507</td>
<td>14.1</td>
<td>0.84</td>
<td>33.7</td>
<td>40.1</td>
</tr>
<tr>
<td>DB2524</td>
<td>11.6</td>
<td>0.62</td>
<td>33.6</td>
<td>54.2</td>
</tr>
<tr>
<td>DB2525</td>
<td>20.0</td>
<td>10</td>
<td>50.0</td>
<td>5.0</td>
</tr>
<tr>
<td>DB2551</td>
<td>12.5</td>
<td>10</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2570</td>
<td>5.8</td>
<td>0.23</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2579</td>
<td>12.5</td>
<td>10</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2585</td>
<td>12.3</td>
<td>0.64</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2593</td>
<td>20.0</td>
<td>0.3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>DB2598</td>
<td>20.0</td>
<td>3.82</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Sixty percent of the tested Quinazolines compounds showed activity in the infected macrophage model, while 20% of them were active in the axenic amastigotes model and 75% of them expressed no sign of cytotoxicity.
Table 3.10: Activity of Quinazolines (IC$_{50}$ <1 µM in Infected Macrophage Model)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Axenic Amastigotes</th>
<th>Cytotoxicity</th>
<th>Infected Macrophage</th>
<th>IM SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>QUI-1209</td>
<td>5.2</td>
<td>23.1</td>
<td>0.27</td>
<td>85.3</td>
</tr>
<tr>
<td>QUI-992</td>
<td>10.0</td>
<td>50.0</td>
<td>0.30</td>
<td>169.5</td>
</tr>
<tr>
<td>QUI-923</td>
<td>10.0</td>
<td>50.0</td>
<td>0.30</td>
<td>166.7</td>
</tr>
<tr>
<td>QUI-898</td>
<td>1.4</td>
<td>50.0</td>
<td>0.30</td>
<td>166.2</td>
</tr>
<tr>
<td>QUI-1279</td>
<td>2.7</td>
<td>30.0</td>
<td>0.32</td>
<td>94.1</td>
</tr>
<tr>
<td>QUI-776</td>
<td>10.0</td>
<td>50.0</td>
<td>0.56</td>
<td>90.1</td>
</tr>
<tr>
<td>QUI-991</td>
<td>10.0</td>
<td>50.0</td>
<td>0.57</td>
<td>87.1</td>
</tr>
<tr>
<td>QUI-882</td>
<td>10.0</td>
<td>50.0</td>
<td>0.62</td>
<td>81.0</td>
</tr>
<tr>
<td>QUI-939</td>
<td>10.0</td>
<td>50.0</td>
<td>0.62</td>
<td>80.5</td>
</tr>
<tr>
<td>QUI-1011</td>
<td>10.0</td>
<td>50.0</td>
<td>0.62</td>
<td>80.1</td>
</tr>
<tr>
<td>QUI-773</td>
<td>10.0</td>
<td>44.5</td>
<td>0.66</td>
<td>67.9</td>
</tr>
<tr>
<td>QUI-1210</td>
<td>10.0</td>
<td>25.0</td>
<td>0.67</td>
<td>37.3</td>
</tr>
<tr>
<td>QUI-812</td>
<td>10.0</td>
<td>50.0</td>
<td>0.68</td>
<td>73.5</td>
</tr>
<tr>
<td>QUI-1280</td>
<td>20.0</td>
<td>44.8</td>
<td>0.87</td>
<td>51.7</td>
</tr>
<tr>
<td>QUI-998</td>
<td>10.0</td>
<td>50.0</td>
<td>0.87</td>
<td>57.5</td>
</tr>
<tr>
<td>QUI 1251</td>
<td>N/A</td>
<td>N/A</td>
<td>0.88</td>
<td>N/A</td>
</tr>
<tr>
<td>QUI-1012</td>
<td>10.0</td>
<td>50.0</td>
<td>0.88</td>
<td>56.7</td>
</tr>
<tr>
<td>QUI-960</td>
<td>8.4</td>
<td>30.2</td>
<td>0.88</td>
<td>34.2</td>
</tr>
<tr>
<td>QUI-880</td>
<td>10.0</td>
<td>50.0</td>
<td>0.88</td>
<td>56.6</td>
</tr>
<tr>
<td>QUI-1277</td>
<td>20.0</td>
<td>28.0</td>
<td>0.89</td>
<td>31.3</td>
</tr>
<tr>
<td>QUI-1214</td>
<td>10.0</td>
<td>50.0</td>
<td>0.89</td>
<td>56.0</td>
</tr>
</tbody>
</table>
### Table 3.11: Thiazole Antileishmanial Activity (IC<sub>50</sub> µM)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Axenic Amastigote</th>
<th>Cytotoxicity (J774)</th>
<th>Infected Macrophage</th>
<th>Infected Macrophage Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBA 1</td>
<td>75.2</td>
<td>96.3</td>
<td>13.52</td>
<td>7.12</td>
</tr>
<tr>
<td>KBA 2</td>
<td>31.7</td>
<td>37.6</td>
<td>4.32</td>
<td>8.70</td>
</tr>
<tr>
<td>KBA 3</td>
<td>47.8</td>
<td>87.1</td>
<td>6.26</td>
<td>13.93</td>
</tr>
<tr>
<td>KBA 4</td>
<td>18.6</td>
<td>62.1</td>
<td>12.52</td>
<td>4.96</td>
</tr>
<tr>
<td>KBA 5</td>
<td>43.1</td>
<td>126.2</td>
<td>33.78</td>
<td>3.74</td>
</tr>
<tr>
<td>KBA 6</td>
<td>37.1</td>
<td>148.4</td>
<td>0.99</td>
<td>150.48</td>
</tr>
<tr>
<td>KBA 7</td>
<td>67.6</td>
<td>168.9</td>
<td>8.32</td>
<td>20.31</td>
</tr>
<tr>
<td>KBA 8</td>
<td>8.1</td>
<td>178.6</td>
<td>4.78</td>
<td>37.37</td>
</tr>
<tr>
<td>KBA 9</td>
<td>4.5</td>
<td>119.0</td>
<td>12.75</td>
<td>9.33</td>
</tr>
<tr>
<td>KBA 10</td>
<td>3.3</td>
<td>176.1</td>
<td>17.37</td>
<td>10.14</td>
</tr>
<tr>
<td>KBA 11</td>
<td>18.3</td>
<td>144.9</td>
<td>1.80</td>
<td>80.74</td>
</tr>
<tr>
<td>KBA 12</td>
<td>13.6</td>
<td>130.0</td>
<td>7.00</td>
<td>18.57</td>
</tr>
<tr>
<td>KBA 13</td>
<td>18.0</td>
<td>84.9</td>
<td>6.54</td>
<td>12.98</td>
</tr>
<tr>
<td>KBA 14</td>
<td>16.6</td>
<td>79.3</td>
<td>2.49</td>
<td>31.90</td>
</tr>
<tr>
<td>KBA 15</td>
<td>74.6</td>
<td>12.8</td>
<td>13.37</td>
<td>0.95</td>
</tr>
<tr>
<td>KBA 16</td>
<td>68.2</td>
<td>177.0</td>
<td>0.75</td>
<td>237.52</td>
</tr>
<tr>
<td>KBA 17</td>
<td>34.9</td>
<td>188.0</td>
<td>3.34</td>
<td>56.31</td>
</tr>
<tr>
<td>KBA 18</td>
<td>49.9</td>
<td>185.2</td>
<td>2.24</td>
<td>82.78</td>
</tr>
<tr>
<td>KBA 19</td>
<td>74.1</td>
<td>185.2</td>
<td>3.58</td>
<td>51.71</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>75.2</td>
<td>96.3</td>
<td>13.52</td>
<td>7.12</td>
</tr>
</tbody>
</table>
Figure 3.12: Active Thiazoles in Multiple Antileishmanial Assays

All Samples were tested in infected macrophage (95% active), axenic amastigotes (79% active), and cytotoxicity assays (68% active not toxic).
Figure 3.13: Chemical structure of KBA 16

Infected macrophage IC$_{50}$= 0.75 µM and a selectivity index= 237.
Table 3.12: Primaquine and Primaquine-Coumarin Fluorescent Probe IC50 Values in Multiple Assays (IC_{50} µg/ml)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Axenic Amastigote</th>
<th>Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQ</td>
<td>4.62</td>
<td>2.28</td>
<td>0.49</td>
</tr>
<tr>
<td>PQCP</td>
<td>11.4</td>
<td>25.2</td>
<td>2.21</td>
</tr>
</tbody>
</table>

PQ and PQCP stand for primaquine and primaquine-coumarin fluorescent probe respectively (McQueen et al., 2017).

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Chapter Four: Antileishmanial Lead Optimization of Quinazolines

(KVH-14)

Abstract

Leishmaniasis is a disease caused by the parasite *Leishmania*. About 1.3 million new cases of leishmaniasis are reported annually causing approximately 30,000 deaths per year. Due to toxicity, high cost and resistance issues of available drugs, new and improved drugs are urgently needed.

Quinazolines are a class of compounds that have been tested against some *Leishmania* species. Some of these compounds exhibited promising activity against *Leishmania donovani*. Compound 23 (KVH-14 in this paper) has been evaluated in severe hamster visceral leishmaniasis model after demonstrating high in vivo efficacy in a previous conducted mouse study where it exhibited a 37% inhibition of parasite burden in the liver when given at 15 mg/kg for 5 consecutive days. In this study, KVH-14 (23) was given orally at 30 mg/kg for 5 consecutive days and showed 89.9% reduction in liver parasitemia. The reference drug, miltefosine, reduced liver parasitemia by 95.6% when given at the same dose.

Introduction

Leishmaniasis is a parasitic disease transmitted by sand fly. The disease is endemic in approximately 98 countries worldwide with 1.3 million new cases reported annually (Lacerda, Pelegrini, de Oliveira, Vasconcelos, & Grossi-de-Sa, 2016).
Leishmaniasis is believed to be the 3rd most common parasitic disease after schistosomiasis and malaria in terms of morbidity with approximately 30,000 deaths per year (Zulfiqar, Shelper, & Avery, 2017). There are 29 species of Leishmania classified by their genetic and biochemical characterizations (Bethony et al., 2011). *Leishmania donovani* and *Leishmania infantum* are known to cause the fatal form visceral leishmaniasis. Drug resistance, toxicity and high cost of currently available treatment options create an urgent need for new drugs.

Quinazolines are a class of compounds that have been reported to have activity against *Leishmania* (Bhattacharjee et al., 2002), (Agarwal, Sharma, Shakya, & Gupta, 2009), (Kumar, Shakya, Gupta, Sarkar, & Sahu, 2009). A class of 2,4-diaminoquinazolines showed an EC$_{50}$ of 0.4 μM in *Leishmania major* amastigotes but high cytotoxicity poses significant issues in clinical use (Berman, King, & Edwards, 1989). It is believed that quinazolines inhibit dihydrofolate reductase (Gilbert, 2002). Van Horn et al investigated the possibility of this mechanism of action and found that quinazolines were less active in the presence of folic acid, which supported the hypothesis of previous studies (Van Horn et al., 2014).

Van Horn et al have tested N2, N4-disubstituted quinazolines 1 and 2 that showed some activity against *Leishmania mexicana* axenic amastigotes (Van Horn et al., 2014). These findings encouraged them to develop compounds structurally related to compounds 1 and 2 as described in the paper, which have been tested against *Leishmania donovani* and *Leishmania amazonensis* intracellular amastigote assays to select candidate molecules. Compounds that showed promising results were then tested for efficacy in a *Leishmania donovani* murine model to ensure their ability to
penetrate the host cell and inhibit the parasite growth without harming the macrophages. Two subseries compounds were prepared and tested to validate and optimize the activity of N2, N4-disubstituted quinazoline-2,4-diamines against *Leishmania*. The structure-activity relationship (SAR) study for the first subseries was focusing on N-2 and N-4 positions, while the second subseries focused on the benzenoid ring of the quinazoline scaffold. The purpose of the second series was to see the effect of the substituted benzenoid ring on the antileishmanial activity of these analogues. The substituted benzenoid ring compounds showed a range of single digit micro molar EC\textsubscript{50} values against *Leishmania donovani* where some of them exhibited no sign of toxicity against J774A.1 with a selectivity index greater than 10. Compounds 15, 16 and 23 were the best candidate for an in vivo study due to their EC\textsubscript{50} value against *Leishmania donovani* and showed low toxicity when tested against J774A.1 with a selectivity index greater than 40, (Van Horn et al., 2014).

**Previous In vivo Study (Acute Murine Visceral Leishmaniasis Model)**

To assess a tolerated dose for in vivo efficacy studies, the three compounds were given to uninfected mice at 30 mg/kg intraperitoneally. Since compounds 16 and 23 were toxic at 30 mg/kg, a lower concentration of 10 mg/kg and 15 mg/kg was administered intraperitoneally for 5 consecutive days in a murine visceral leishmaniasis model respectively. Compounds 15 and 16 did not show any inhibition in the parasites growth in treated mice compared to the vehicle control group, while compound 23 showed a 37% inhibition in parasite burden in the liver when given at 15 mg/kg for 5 consecutive days (Van Horn et al., 2014).

The pharmacokinetics studies and the appropriate physiochemical properties of
compound 23 made it a suitable candidate for further investigation to develop a drug against *Leishmania donovani* (Van Horn et al., 2014).

**Materials and Methods**

**Axenic Amastigotes Media Preparation and Culture**

*L. donovani* axenic amastigotes MHOM/SD/75/1246/130/ Khartoum (WR378) laboratory cell lines were cultured in a non-vented 25 cm² tissue culture flask (Coning, Lowell, MA) using RPMI 1640 (Gibco) with 5.86 g/L MES (Sigma-Aldrich, St Louis, MO), 7.5 g/L Hepes (Invitrogen Corp., Carlsbad, CA), 2 g/L sodium bicarbonate (Fisher Scientific, Pittsburg, PA), 100 µM Xanthine (Sigma-Aldrich, St Louis, MO), 10 mg/L Hemin (Sigma-Aldrich, St Louis, MO), 40 mg/L Tween-80 (Sigma-Aldrich, St Louis, MO), 5 g/L Tryptone-Peptone (BD Bioscience, Sparks, MD), 1 % Penicillin- Streptomycin (Gibco, 10,000 u/ml), and 20 % 24 hours heat-inactivated FBS (Gibco). Media was set at a pH of 5.5. *L. donovani* axenic amastigotes were cultured 1:5 three times per week and incubated at 37°C.

**Macrophage Media Preparation and Culture**

J774A.1 macrophages (ATCC, Manassas, VA) were cultured in a vented 75 cm² tissue culture flask using RPMI 1640 (Gibco), 1% Penicillin-Streptomycin (Gibco, 10,000 u/ml), 10% 24 hour heat inactivated fetal bovine serum (Gibco) at a pH of 7.2. Twenty-five ml of fresh media were added to culture flasks 3 times per week and cells were serially passaged biweekly.

**Leishmania donovani Axenic Amastigotes Drug Sensitivity Assay**

KVH-14 and control drug were tested against *Leishmania* donovani axenic amastigotes (MHOM/SD/75/1246/130) using the CellTiter 96 AQueous one-solution cell
proliferation assay (Promega, Madison, WI). In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 200µg/ml to 6.25µg/ml. Ten µL from each well was transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, #3628) and then 90µL of parasites in media in a 66,000 cell per well concentration was added to produce a final concentration range from 20µg/ml to 625ng/ml. The plates were then incubated at 37°C for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium}, (Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e (Molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC₅₀ values.

Cytotoxicity Assay

KVH-14 and control drug were tested against J774.A1 macrophages using the CellTiter 96 AQueous one-solution cell proliferation assay (Promega, Madison, WI). In a 96 well drug plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, None-Treated, #3370) compounds were diluted in a series of 6 2-fold dilutions in media to produce a concentration range from 500µg/ml to 15.625µg/ml. Ten µL from each well was transferred to another 96 well plate (Costar, Assay Plate, 96 well with low Evaporation Lid, Flat Bottom, Tissue culture Treated, #3628) and 90µL of macrophages in media in a 50,000 cell per well concentration was added to produce a final concentration range from 50µg/ml to 1.562 µg/ml. The plates were then incubated at
37°C, 5% CO₂ for 72 hours. Twenty µL of MTS {3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium} (Promega, Madison, WI) solution was added to each well and incubated for an additional 4 hours. A Spectra Max M2e (molecular Devices, Sunnyvale, Ca) was used to measure optical density (OD) at 490nm. Non-linear regression via Trifox software was used to determine IC₅₀ values.

**Infected Macrophage Drug Sensitivity Assay**

In a 384 well assay plate (CellCarrier-384 Black, Optically Clear Bottom, Tissue Culture Treated, Sterile, #6007550), 2000 J774A.1 macrophages were seeded and incubated for at least an hour to allow adherence. The plate was then washed with pre-warmed media to remove the non-adherent cells. *L. donovani* axenic amastigotes were added to the plate in a ratio of 10:1 and incubated at 37 °C, 5% CO₂ for 24 hours. The excess extracellular amastigotes were then washed away using pre-warmed media. KVH-14 and Miltefosine (control drug) were prepared in a 384-drug plate (Thermo Scientific™ Nunc™ 384-Well Clear Polystyrene Plates with Non /treated Surfaces, #242757) with a starting concentration of 10 µM or 10 µg/ml and serially diluted in a 1:2 ratio. Drugs were then added to the assay plate and incubated at 37 °C, 5% CO₂ for 72 hours. After that, drugs were removed from the plate and the adhered cells were fixed with 2% paraformaldehyde (Alfa Aesar™ Paraformaldehyde, 16% w/v aq. soln., methanol free, #30525-89-4) and incubated for 15 minutes at room temperature. Fixative was then removed and cells were stained with 5 µM Draq5 (Thermo Scientific DRAQ5 fluorescent probe, DNA stain, #62251) and incubated for 5 minutes at room temperature. Stain was removed and fresh media was added to the plate. High content fluorescent imaging (Perkin Elmer Operetta) was used to capture images used
to perform cell counts. Harmony software calculated the number of amastigotes per 500 macrophages in each well and used these counts to generate IC$_{50}$ values.

In all three assays, Miltefosine was used as a negative growth control drug and culture media was used as a positive growth control.

**In-vivo Study**

A severe visceral leishmaniasis model was employed using 20 male Syrian hamsters ordered from Harlan laboratories (Livermore, CA). These hamsters were 4 weeks old and weighed around 40 grams at the time of arrival. They were randomly stored into four groups, Miltefosine 30 mg/kg, control, KVH-14 30 mg/kg and KVH-14 10 mg/kg. Cages with an average of 2 hamsters per cage were assigned for each group and maintained in a sterilized BSL2 facility. On day 0, each hamster was anesthetized by using Isoflurane (Isothesia, Butler Animal Health Supply, Dublin, OH) and 2x $10^8$ amastigotes suspended in amastigote media were inoculated into each hamster’s heart. Thirty days post infection each animal received treatment orally once daily according to its assigned group for 5 consecutive days. On day 36, the hamsters were sacrificed using CO$_2$ asphyxiation. Liver, spleen and bone marrow were collected and weighed following the protocol described in (Srivastava, Sweat, Azizan, Vesely, & Kyle, 2013). A piece of liver and spleen were cut and taped into slides. Bone marrow was collected in 100 µl media and smoothly spread on a slide as well. All slides were then methanol fixed and stained with 20% Gimsa stain. Microscopic examination of slides was used to determine parasite burden by calculating the number of amastigotes per 1000 macrophages multiplied by the organ weight in grams as described by Stauber (Huff et al., 1958), expressing results as Leishman Donovan Units (Moreira et al., 2012).
Hamsters were handled according to local and federal regulations, and the University of South Florida Institutional Animal Care and Use Committee approved research protocols.

**Results and Discussion**

In vitro assays in drug discovery projects are designed to screen hundreds of samples using small amounts of compounds with low cost in a timely manner. In these assays, compounds usually have a direct impact on the parasites. In some cases, the action of some compounds is affected by the host immune system. Therefore, the results may not be reflected when tested in vivo (Gupta & Nishi, 2011). However, the infected macrophage assay is more relevant since it reflects the physiological conditions, as in the clinically relevant form of the disease situated within the host macrophages (De Rycker et al., 2013). Moreover, the infected macrophage assay can evaluate the ability of the compound to pass through the infected macrophage cell membrane and test its effect on both the parasites and macrophages, which reflects a strong correlation to the in vivo results. Seifert et al, evaluated the in vitro activity of antileishmanial reference drugs on different types of macrophages (mouse peritoneal macrophages, mouse bon marrow derived macrophages, human peripheral blood monocyte derived macrophages and differentiated THP-1). The activity of these drugs was significantly different among macrophage populations, which revealed that the activity is host cell dependent (Seifert, Escobar, & Croft, 2010).

Compound KVH-14 showed no activity when tested in the axenic amastigotes assay. However, the same compound produced an IC$_{50}$ of 1.4 µM in the infected macrophage model (Figure 4.1). According to Vermeersch et al, some compounds
might need to be metabolized by the macrophages to gain activity or maybe the macrophage is the target, leading to the destruction of amastigotes (Vermeersch et al., 2009). An additional possibility is the compound accumulates to higher levels in the infected macrophage.

The finding of activity in the infected macrophage assay was confirmed in the severe hamster visceral leishmaniasis model in which KVH-14 inhibited parasitemia by 89.9%, 77.10% and 77.86% in the liver, spleen and bone marrow, respectively, when given at 30 mg/kg for 5 consecutive days. Compound KVH-14 (KVH-5-97) also was evaluated at 10 mg/kg given orally for 5 consecutive days. At this dose, KVH-14 reduced parasitemia by 60.51% in the liver, 47.36% in the spleen and 39.33% in bone marrow (Table 4.2), (Figure 4.2). Interestingly, in the acute murine visceral leishmaniasis model, KVH-14 reduced liver parasitemia by 37% when given at 15 mg/kg for 5 consecutive days. Therefore, KVH-14 activity was enhanced in the severe hamster model. The hamster visceral leishmaniasis model is considered to be more similar to human visceral leishmaniasis than to the murine model in terms of clinicopathological and immunopathological effects (Melby, Chandrasekar, Zhao, & Coe, 2001), which make KVH-14 an interesting antileishmanial candidate molecule. Seifert et al findings might explain the differences in the efficacy of KVH-14 between severe hamster model and acute murine model, as the activity of the drug is host cell dependent, which also could explain the efficacy differences on parasitemia between the 3 organs in the hamster model (Seifert et al., 2010). The outstanding activity of KVH-14 in the severe hamster model revealed that 2,4-quinazoline diamines is an important antileishmanial
candidate. More analogs of the same class need to be synthesized and evaluated in vivo.
Table 4.1: IC$_{50}$ Values in Drug Sensitivity Assays (µM)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Axenic amastigotes</th>
<th>J774A.1 Cytotoxicity</th>
<th>Infected macrophage</th>
<th>IM Selectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>KVH-14</td>
<td>&gt;20</td>
<td>45</td>
<td>1.4*</td>
<td>32</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>1.3*</td>
<td>&gt;50</td>
<td>1.79*</td>
<td>27</td>
</tr>
</tbody>
</table>

* Mean of triplicate runs, IM= Infected macrophage.
Figure 4.1: Operetta Pictures of Treated Infected Macrophages

Upper Right, 10 µM KVH-14 treated infected macrophages, upper left: 10 µM Miltefosine treated infected macrophage, lower left: Infected macrophage positive growth (no treatment- control), and lower right: macrophages only.
Table 4.2: Percentage of Parasite Inhibition in all Tissues in Severe Hamster Model

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Liver % inhibition</th>
<th>Liver St. dev</th>
<th>Spleen % inhibition</th>
<th>Spleen St. dev</th>
<th>Bone Marrow % inhibition</th>
<th>Bone Marrow St. dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miltefosine 30 mg/kg</td>
<td>95.59</td>
<td>1.28</td>
<td>92.22</td>
<td>2.67</td>
<td>96.70</td>
<td>2.28</td>
</tr>
<tr>
<td>KVH 30 mg/kg</td>
<td>89.92</td>
<td>0.98</td>
<td>77.10</td>
<td>4.03</td>
<td>77.86</td>
<td>4.52</td>
</tr>
<tr>
<td>KVH 10 mg/kg</td>
<td>60.51</td>
<td>7.18</td>
<td>47.36</td>
<td>10.2</td>
<td>39.33</td>
<td>2.28</td>
</tr>
</tbody>
</table>
Figure 4.2: Percentage of Parasite Inhibition in all Tissues in Severe Hamster Model

30mg/kg KVH-14, 10mg/kg KVH-14 and 30mg/kg miltefosine.

List of References


Chapter Five: **Summary**

**Introduction**

Leishmaniasis is one of the major neglected tropical diseases caused by *Leishmania* species parasites. There are more than 20 *Leishmania* species that cause the disease (Vijayakumar & Das, 2018). Transmission of leishmaniasis is caused by a bite of a female phlebotomine sand fly. There are more than 90 species are known to transmit leishmaniasis to human. Species of the genus Lutzomyia sand fly is found in the new world while species of Phlebotomus located in the old world (Bates et al., 2015). There are several clinical forms of the disease that depends on the infective species of the parasite. Visceral leishmaniasis (VL) is considered the severe form that infects internal organs and causes hepatomegaly, splenomegaly, weight loss and, fever and anemia. This form can be fatal if left untreated (Jain & Jain, 2015). Around 350,000 deaths from leishmaniasis reported annually worldwide (Kamhawi, 2017). An estimation of 0.2-0.4 million incidents of visceral leishmaniasis occurs annually. HIV- VL co-infection has been reported by 35 countries, which increased the burden of visceral leishmaniasis (Sundar & Singh, 2016). Pentavalent antimonials, amphotericin B, miltefosine, and paromomycin are the available treatments for visceral leishmaniasis (Figure 5.1). However, the widespread of resistance to some of these drugs, toxicity, decreased efficacy and limited availability encouraged the search for new drugs to treat this devastating disease (Sundar & Chakravarty, 2015).
In an effort to discover an antileishmanial agent, we have been participating in different drug discovery projects and collaborating with many universities and institutes. From these collaborations, we were able to evaluate a new series of novel compounds from synthetic and natural product sources that can lead to promising drug candidates for treating visceral leishmaniasis.

**Natural Products**

Natural products are a rich source for the identification of new active drugs, which prove its effectiveness in treating several diseases. They represent 9 of 13 antiparasitic drugs approved by the U.S. Food and Drug Administration in the past 30 years, emphasizing the vital role of this drug discovery resource for antileishmanial drug discovery research. Between the years 1981 to 2006, 1184 novel natural based drugs have been generated and 28% of them were from plants (Gutierrez-Rebolledo, Drier-Jonas, & Jimenez-Arellanes, 2017). *Catharanthus roseus* is one of the most studied medicinal plants. Its alkaloids led to the discovery of the powerful anticancer drugs vinblastine and vincristine (Zhu, Zeng et al. 2014). Furthermore, marine microorganisms and macroorganisms are another rich sources that are not well studied as natural product source for drug discovery (Tchokouaha Yamthe et al., 2017).

We tested *Catharanthus roseus* hairy root extracts against *Leishmania donovani* infected macrophage model. We also screened thousands of endophytic fungi extracts synthesized from mangrove plants in multiple *Leishmania donovani* assays. The initial screen demonstrated several active extracts that were then scaled up and tested for their activity. One fungal isolate was then scaled up and generated multiple pure compounds that led to the identification of Phomopsichromin C, which represented an
IC$_{50}$ of 0.67µM in the infected macrophage model with an excellent selectivity over J774A.1. In addition we evaluated the activity of Antarctic deep-sea coral; (Keikipukalides and Furanocembrane diterpenes) in the *Leishmania donovani* infected macrophage model. Several pure compounds exhibited antileishmanial activity with Pukalide aldehyde being the most potent one (Table 5.1).

Apparently, these sources have never been tested for their antileishmanial activity before. Thus, we were the first to identify novel antileishmanial hits from the Antarctic deep-sea coral, endophytic fungi from mangrove plants and *C.roseus* hairy root extracts.

**Synthetic Compounds**

By using Torry Pines scaffold ranking and positional scanning libraries, we were able to identify a series of guanidine-based compounds that possessed potent antileishmanial activity in multiple assays as well as selectivity over human macrophages, demonstrating that activity is unlikely to arise through simple promiscuous interactions. These compounds are structurally distinct from any of the currently used antileishmaniasis agents (Figure 5.1) and thus they represent a novel opportunity for lead optimization. We were subsequently able to use these libraries to focus in on compounds such as 64 that retain not only excellent activity (an IC$_{50}$ of 1.13 µM in the infected macrophage assay when run in quadruplicate with a standard deviation of 0.81 µM) and selectivity (an SI of 23 over J774 macrophages) but a reasonable molecular weight (245 g/mol) and physicochemical properties (e.g. cLogP = 3.26, tPSA = 39). Work toward the further optimization of these compounds with optimal pharmacokinetic and pharmacodynamics properties as well as attempts to ascertain
their target or mechanism of action is underway and will be reported in due course (Giulianotti et al., 2017).

We also have found a series of novel pyrazolo[3,4-c]pyrimidines based on the known Hsp90 agent SNX-2112 that show activity against *Leishmania donovani* in multiple assays. Significantly, several of these new compounds demonstrate more potent activity than the clinically used miltefosine in both of these assays, and excellent selectivity over any observed cytotoxicity against human J774 macrophage cells. Work toward a better understanding of this activity, including the biochemical target, is currently underway. Although the starting point for this project was a compound that demonstrated an excellent ADME/PK profile, this is an early drug discovery project and we will continue to assess these parameters as we seek a lead compound with optimal properties (Kanwar et al., 2017).

Furthermore, mono-amidine analogue DB2381 exhibited an exceptional antileishmanial activity in the infected macrophage model ($IC_{50} = 0.15 \mu M$, SI=181), substantially more potent than miltefosine.

Moreover, we discovered a new class of quinazoline that has antileishmanial activity. Around 60% of screened quinazoline analogues possessed activity in the *Leishmania donovani* infected macrophage model. Notably, QUI-1209 displayed an $IC_{50} = 0.27 \mu M$ and selectivity index =85.

Finally, we identified a new class of Thiazole with leishmanicidal activity against the infected macrophage model as well (KBA-16 $IC_{50} = 0.75 \mu M$, SI=237).
A summary of the most potent compound in each class of molecules is represented in (Table 5.2). The discovery of new validated hits could speed up drug discovery projects and hopefully generate new drugs against visceral leishmaniasis.

**Antileishmanial Lead Optimization of Quinazolines (KVH-14)**

So far, most of the reported drug discovery studies are mainly preliminary in vitro studies. Thus, in vivo studies are necessary in order to develop an effective leishmaniasis treatment (Singh, Mishra, Bajpai, Singh, & Tiwari, 2014).

In a previous in vivo study, KVH-14 (compound 23) reduced liver parasitemia level by 37% in an acute murine model of visceral leishmaniasis model when given orally at 15mg/kg for 5 consecutive days. The pharmacokinetics studies and the appropriate physiochemical properties of compound 23 (KVH-14) made it a suitable candidate for further investigation to develop a drug against *Leishmania* (Van Horn et al., 2014). Thus, we evaluated the efficacy of KVH-14 in a severe hamster visceral leishmaniasis model. This compound decreased parasitemia level in the liver by almost 90% when given orally at 30 mg/kg for 5 consecutive days. Apparently, the reduction of parasitemia level was enhanced in the severe hamster visceral leishmaniasis model compared to the acute murine visceral leishmaniasis model. It is important to note that the hamster visceral leishmaniasis model is considered more similar to human visceral leishmaniasis than to the murine model in terms of clinicopathologically and immunopathologically (Melby, Chandrasekar, Zhao, & Coe, 2001), which make KVH-14 a great antileishmanial candidate molecule. The outstanding activity of KVH-14 in the severe hamster model revealed that 2,4-quinazoline diamines is an important antileishmanial candidate.
Figure 5.1: Current Visceral Leishmaniasis Treatments

Liposomal amphotericin B (1), Sodium stibogluconate (2), Miltefosine (3), Pentamidine (4), and Paromomycin (5). (Giulianotti et al., 2017).
Table 5.1: Summary of Novel Natural Product-based Antileishmanial Hits

<table>
<thead>
<tr>
<th>Collaborator</th>
<th>Institute</th>
<th>Class</th>
<th>Identified Novel Hit</th>
<th>Infected Macrophage IC&lt;sub&gt;50&lt;/sub&gt; and Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Bill Baker</td>
<td>Department of Chemistry, USF</td>
<td>Mangrove Endophytic Fungal Extracts</td>
<td>Phomopsichromin C</td>
<td>0.67 µM, SI= 74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antarctic Deep Sea Coral (Plumarella delicatissima)</td>
<td>Pukalide aldehyde</td>
<td>1.9 µM, SI= 26</td>
</tr>
<tr>
<td>Dr. Carolyn W.T. Lee-Parsons</td>
<td>Northeastern University</td>
<td>C. roseus Hairy Root Extracts</td>
<td>Sample 5</td>
<td>2.42 µg/ml, SI= 20</td>
</tr>
</tbody>
</table>
### Table 5.2: Summary of Novel Synthetic Antileishmanial Hits

<table>
<thead>
<tr>
<th>Collaborator</th>
<th>Institute</th>
<th>Class</th>
<th>Identified Novel Hit</th>
<th>Infected Macrophage IC&lt;sub&gt;50&lt;/sub&gt; and Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Marc Giulianotti</td>
<td>Torry Pine Institutes</td>
<td>Guanidine</td>
<td>Monoguanidine 64</td>
<td>1.13 µM, SI= 23</td>
</tr>
<tr>
<td>Dr. Jim Leahy</td>
<td>Department of Chemistry, USF</td>
<td>SNX2112</td>
<td>Compound 18</td>
<td>1.06 µM, SI= 47</td>
</tr>
<tr>
<td>Dr. David Boykin</td>
<td>Georgia State University</td>
<td>Mono-amidine</td>
<td>DB2381</td>
<td>0.15 µM, SI= 181</td>
</tr>
<tr>
<td>Dr. Roman Manetsch</td>
<td>Northeastern University</td>
<td>Quinazoline</td>
<td>QUI-1209</td>
<td>0.27 µM, SI= 85</td>
</tr>
<tr>
<td>Dr. Kirpal Bisht</td>
<td>Department of Chemistry, USF</td>
<td>Thiazole</td>
<td>KBA-16</td>
<td>0.75 µM, SI= 237</td>
</tr>
</tbody>
</table>

### List of References


doi:10.1021/acsmedchemlett.7b00039


doi:10.1016/j.bmc.2013.11.048


Appendices:
Appendix A: Copyright Information

Title: Identification of a Hit Series of Antileishmanial Compounds through the Use of Mixture-Based Libraries
Author: Marc A. Giulianotti, Brian A. Vasely, Ala Azhar, et al
Publication: ACS Medicinal Chemistry Letters
Publisher: American Chemical Society
Date: Aug 1, 2017
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Author: Adonis McQueen, Lynn D. Blake, Ala Azhari, M. Trent Kemp, Tommy W. McGaha, Niranjan Namelikonde, Randy W. Larsen, Roman Manetsch, Dennis E. Kyle

Publication: Bioorganic & Medicinal Chemistry Letters

Publisher: Elsevier

Date: 15 October 2017

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MEMORANDUM

TO: Dennis Kyle, PhD

FROM: Farah Mouli, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 10/3/2013

PROJECT TITLE: Antileishmanial Lead Optimization of Quinazolines

FUNDING SOURCE: NIAID

IACUC PROTOCOL #: R IS00000344

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 10/3/2013:

Hamster: Mesocricetus auratus (4 wk., male and female, 40-80 g)

Please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

- All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons.

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128