Developing novel drug delivery methods for anti-leishmanial drugs.

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Developing novel drug delivery methods for anti-leishmanial drugs.

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Abstract

Background: Leishmaniasis is a DNDi listed disease caused by protozoan parasites of the kinetoplastida class. The disease currently spans over 80 countries, across the New World and Old World, potentially affecting 500 million people with 1 million new cases reported annually. Leishmaniasis is a vector-driven disease, utilizing two genus of sand fly, *Phlebotomus* sand fly is responsible for Old World transmission, whereas the *Lutzomyia* sand fly is responsible for New World transmission. The two main stages of leishmaniasis, are the diagnostic stage (promastigote in sand fly) and the infective stage (amastigote in mammalian cells). Dependent on strain, geography and location of infection, there are three main forms of leishmaniasis: cutaneous (subdivided into diffuse-cutaneous leishmaniasis (DCL) and disseminated-cutaneous leishmaniasis (DL)); mucocutaneous; and visceral (subdivided into post-kala azar dermal leishmaniasis (PKDL)).

The DNDi status of leishmaniasis indicates that the pharmaceutical interest into research and development is shockingly low, resulting in very little progress into new treatments, limited to current therapeutics that suffer from severe toxicities (cardio, nephro, hepato, oto). These issues can be circumvented by utilising liposomal drug-carriers, as part of an increased interest in nanoparticle research across all glycosciences, modifying these drugs to interact better with the target cell or liposomal carrier can be of great benefit.

Aims and Objectives: This project investigated the modification of current therapeutics in leishmanial treatment, paromomycin and compare the changes in antimicrobial efficacy. These modifications would revolve around enhanced binding affinity for macrophages and for liposomal carriers. This was achieved by modifying

paromomycin at its reactive primary alcohols, using previously explored chemistry to attach long-chain fatty acids (LCFAs) to these reactive groups to create potential prodrugs. These were then subject to comparative kill efficiency studies against *S. aureus*, *P. aeruginosa*, and *L. donovani* DD8 cells in MIC assays and a resazurin based assay. A further objective was to investigate novel drug targets in leishmania, using LCFA-ligase as a potential target, as it has been reported this protein is differentially expressed, showing prominence and a potential for inhibition. This compound was also tested against *L. donovani* DD8 in the resazurin based assay.

Methods: Paromomycin laurate and palmitate-based derivatives were synthesised by simple esterification, and the LCFA directive synthesised tert-butyl (4-(2-(decanesulfonyl)acetamido)butyl)carbamate) **(*N*-Boc DSA) w**as synthesised by known methods. These compounds were characterised and subjected to MIC assays on *S. aureus* and *P. aeruginosa*, and a resazurin-based high content imaging (HCI) assay on axenic amastigotes of *L. donovani* DD8. Parallel synthesis and testing of neomycin LCFA derivatives were made by Dylan Farr, and tested against the same pathogens, comparatively with paromomycin derivatives.

All experiments were conducted in triplicate and quadruplicate, with statistical differences being analysed by two-way analysis of variance (Two-way ANOVA). Values with $P<0.05$ were considered significant.

Results and Discussion: Paromomycin palmitate and dipalmitate were synthesised with preference on dipalmitate testing due to increased binding affinity for liposomal

carriers and macrophages. Laurate synthesis was much less effective under a multitude of conditions. Secondary compound Boc-DSA was synthesised for use in conjunction with the paromomycin derivatives. The paromomycin dipalmitate compound was tested against *S. aureus* and *P. aeruginosa*, with comparative aminoglycosides: neomycin palmitate, amikacin palmitate, and kanamycin palmitate. Against *S. aureus*, all compounds showed reduced activity at all concentrations, with paromomycin and amikacin being the least affected. Lower concentrations of antibiotic saw antagonistic effects with the lipid chain synergistically enhancing bacterial growth. *P. aeruginosa* testing was inconclusive due to increased pyocyanin expression, potentially increasing biofilm aggregation of the bacterial cells, reducing interactable surface-area for the aminoglycosides. Further testing with biofilm disruptors in conjunction may show improved results.

Candidates paromomycin dipalmitate, *N*-Boc DSA, and neomycin palmitate were tested by V.Avery group at GRIDD (Griffith Institute for Drug Discovery) against *L. donovani* DD8 axenic amastigotes. Results showed <50% activity among derivative candidates, with lower activity even for paromomycin, a known anti-leishmanial agent. Morphological and pathophysiological changes due to geographical variations in leishmanial strains have been reported to have different effects on therapeutic efficacy. Although the reduced activity of the candidates can be noted for the DD8 strain, further testing on a variety of geographically relevant strains may show different activities. Human monocyte cytotoxicity THP-1 assays were performed in conjunction, <50% activity was similarly found for the described compounds.

Conclusions and Future Remarks: Overall, the modification of ring-1 C6’ and ring-3 C5’ into a LCFA-derivative via esterification chemistry showed reduced activity at

all concentrations against *S. aureus, P. aeruginosa,* and *L. donovani* DD8 amastigotes. Similar for comparative aminoglycosides of neomycin, amikacin, and kanamycin, although results against *P. aeruginosa* indicate potential biofilm aggregation. The reference compounds, including DSA, tested against *L. donovani* DD8 showed <50% inhibition, this may be indicative of morphological and pathophysiological changes due to geographical differences in the test strain. Future avenues worth pursuing is a range of LCFA-derivatives such as C10,12,14,18 for synergistic studies. The use of biofilm disruptors in conjunction with the reference compounds may improve *P. aeruginosa* activity, in addition to biofilm disruptors, the addition of surfactants to improve solubility of the LCFAs, these additions may have antagonistic effects and are worth investigating. Further testing among various geographically relevant strains of *L. donovani* would prove the theory put forth by Stuart et al. and show the efficacy of the reference compound across multiple geographically-dependent strains. Incorporation of the test compounds into liposomes were not achieved within this project, however investigations against the aforementioned *L. donovani* DD8 amastigotes, and against RAW 264.7 cells using the encapsulated compounds as comparative data is warranted.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Acknowledgments

"Not all those who wander are lost." – J. R. R. Tolkien

"Nothing ventured, nothing gained." – John Heywood

This project has been a great learning experience and chapter in my long adventure through life. I explored areas people wouldn't think possible a decade ago, and I am still in disbelief of the work being conducted in the world today. A huge thank you to all the people that I met along the way and helped in my journey. I would like to extend a special thank you to my supervisors AProf. Todd Houston and Dr. Darren Grice, for their unwavering support and mentoring; I'd also like to thank members of the Houston group, Dylan, Tamim and Taylor for their helping hands in my endeavours; to Griffith University and the School of Medical Science for this once-in-a-lifetime opportunity; to my lifelong friends and family, both in Australia and back home in South Africa, for their support, unsolicited advice, and motivation throughout this project.

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Abbreviations

AAC	Acetyl CoA-dependent N-acetylation by acetyltransferases
ABLC	Amphotericin B lipid complex
ACR2	Antimoniate reductase
AG	Aminoglycosides
AIDS	Acquired immunodeficiency syndrome
AmB	Amphotericin B
AmB-L	Liposomal amphotericin B
AME	Aminoglycoside modifying enzyme
ANT	ATP-dependent O-adenylation by nucleotidyltransferases
APH	ATP-dependent O-phosphorylation by phosphotransferases
AQP1	Aquaglyceroporin 1
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
CFU	Colony forming unit
CL	Cutaneous leishmaniasis
Cys	Cysteine
Cys-Gly	Cysteinyl-glycine
d	Doublet
dd	Doublet of doublets
DCL	Diffuse-cutaneous leishmaniasis
DL	Disseminated-cutaneous leishmaniasis
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide

DNDi	Drugs for Neglected Diseases Initiative
DPBS	Dulbecco's phosphate buffered saline
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
ESI	Electro spray ionisation
EtOAc	Ethyl acetate
FBS	Fetal bovine serum
GSH	Glutathione
HCI	High content analysis
HIFBS	Heat inactivated fetal bovine serum
HIV	Human immunodeficiency virus
IC_{50}	50% inhibitory concentration
IFNγ	Interferon gamma
J	NMR coupling constant
L	*Leishmania*
LCFA	Long-chain fatty acid
LPG	Lipophosphoglycans
LPS	Lipopolysaccharides
m	Multiplet
m/z	Mass/charge ratio
MCL	Mucocutaneous leishmaniasis
MDR1	Multi-drug resistant protein 1
MeOH	Methanol
MIC	Minimum inhibitory concentration
mL	Millilitre
N-Boc DSA	tert-butyl (4-(2-(decanesulfonyl)acetamido)butyl)carbamate)
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
°C	Degree Celsius

OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEMT	Phosphatidylethanolamine N-methyl-transferase
PKDL	Post-kala azar dermal leishmaniasis
PPG	Proteophosphoglycan
PSG	Promastigote secretory gel
Sb(III)	Trivalent antimonials
Sb(V)	Pentavalent antimonials
SD	Standard deviation
T	Triplet
TDR1	Thiol-dependent reductase
THP-1	Human acute monocytic leukemia cell line
TLC	Thin-layer chromatography
SSG	Sodium stibogluconate
v/v	Volume per volume
V.	*Viannia*
VL	Visceral leishmaniasis
w/v	Weight per volume
WHO	World Health Organisation
µl	Microliter
µM	Micromolar

Chapter 1:

1.1 General Background

Announcements and concerns with the ever-growing issue of antibiotic resistance build-up, the media-dubbed ‘Superbug’ has necessitated an increase in development of novel potential antibiotics. These novel approaches aim to conserve the antibiotic’s mechanism of action as well as its intracellular targeting, thus creating a multitude of derivatives, with varying characteristics such as efficacy, selectivity, toxicity, and biodistribution. A general decline of global breakthroughs into novel drug development is a cause for concern, which is partly due the difficulty in making these breakthroughs, but also due to somewhat unappealing investment opportunities as few new classes of antibiotics are likely to be discovered. There are instances of approved antibiotic derivatives being commercially-restricted, put into reserves as a ‘last-resort-antibiotic’. As the second discovery of a new class of antibiotics was in the late 1940’s by Schatz and Waksman, streptomycin, introduced the new class of aminoglycosides to the world. This new class of antibiotic suffered from varying levels of toxicity, poor absorption, and quick pathogenic-resistance build-up. Other issues arise when considering that upwards of one billion people across 149 countries suffer from one of many neglected diseases. The term neglected disease means exactly that, a pathogen that receives very little public attention, funding, and research interest as the main endemic areas of these diseases are generally unappealing to profit-driven pharmaceutical companies. The WHO have listed a total of 17 neglected diseases including: Dengue virus; African sleeping sickness; Chaga’s disease; and Leishmaniasis. The Drugs for Neglected Diseases Initiative (DNDi) was formed to drive research and development in these impoverished areas containing neglected diseases.

1.2 Leishmaniasis

Leishmania donovani, a protozoan parasite of the kinetoplastida class, along with many other species, cause a complex of diseases known as leishmaniasis. These species can be organized into two broad categories known as New World or Old World. As the name would suggest, these categories relate to the geographic presence of the species, where New World relates to The Americas and Oceania, and Old World referring to Africa, Asia and Europe (Afro-Eurasia) (1). The disease spans over 80 countries, putting over 500 million people at risk of leishmaniasis. An estimated 650,000 – 1,300,000 new cases of leishmaniasis occur annually, with 70,000 of those cases being visceral leishmaniasis, also known as kala-azar, or 'black fever' (2,3). Kala-azar is responsible for over 30,000 deaths annually, with 90% of these cases being localized to seven countries: India, Kenya, Somalia, Brazil, Ethiopia, Sudan, and South Sudan. The majority of remaining cases occur in just six countries: Brazil, Iran, Syria, Afghanistan, Columbia, and Algeria (4). Although this is a widespread disease, it can be seen that most of the severe epidemics can be localized to very specific regions, which should make implementation and the logistics of treating leishmaniasis a simple endeavour; however, with the lack of accurate reporting and monitoring systems, it is difficult to ascertain the true incidence in these regions.

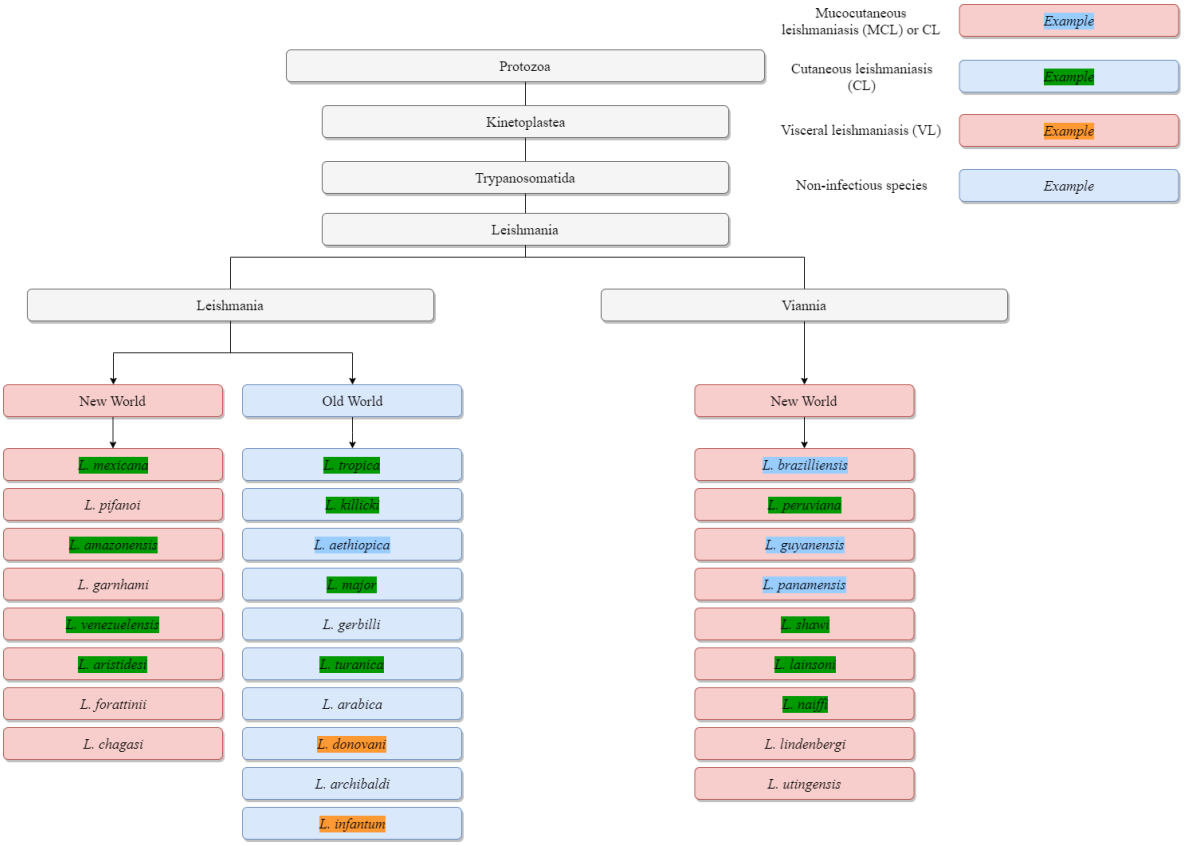


Figure 1.1: *Taxonomy and infectious-status of New World and Old World leishmania including vianna*

1.2.1 Leishmania parasite overview

As outlined in *Figure 1.1*, 18 of the 27 mentioned species are infectious, with all the infections being vector-borne. The vector in question is the sand-fly, with the genus being dependent on geographical classification. The *Phlebotomus* sand-fly is responsible for Old World transmission, whereas the *Lutzomyia* sand-fly is responsible for New World transmission (5). Zoonotic transmission is also possible and frequently seen in some species of *Leishmnia*. *Leishmania* alternates between two morphological forms, intracellular amastigotes and metacyclic promastigotes. These two morphological forms possess two distinct growth phases during the parasitic life cycle. Within the sand-fly vector, the parasite amastigote form needs to multiply to a critical density, and then transition into the

motile metacyclic promastigote form, which needs to be located in either the sand-fly's proboscis-area or midgut (6,7). Although the parasites activities within the sand-fly are largely ignored in regard to leishmania infection, important factors to note are the promastigotes defense against the sand-fly's immune response, and modifications to the sand-fly's proboscis to promote promastigote expulsion (8,9). This alternation of morphological forms is visualised in *Figure 1.2*, showcasing the life cycle of the parasite.

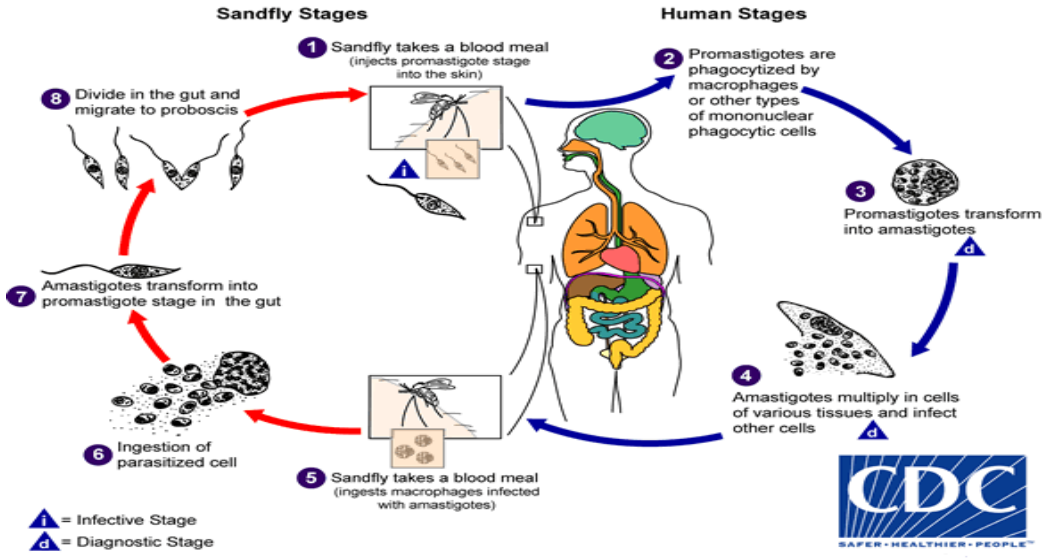


***Figure 1.2**:* Life cycle of leishmania through the diagnostic phase utilising the motile promastigote form, into the infective stage utilising the intracellular amastigote form. These stage transfers are mediated through a vector-carrier, the *Phlebotomus* sand fly. *Image available from CDC(10)*

For the parasites survival within the sand-fly's midgut, there has been investigations into some of the proteophosphoglycans (PPG) found in *Leishmania*. Although studies on every species of *Leishmania* is still required to have a comprehensive view of parasite-sand-fly interactions, current findings suggest that lipophosphoglycans (LPG), and more specifically the LPG2 and LPG2-related molecules are necessary to protect the parasite from proteolytic activity, as well as maintain infection of the vector upon secretion of the infective

promastigotes (11,12). The other major consideration in parasite-sand-fly interactions is the changes imparted onto the sand0fly's method of feeding, as this will directly affect the delivery of the mammalian infective form, the metacyclic promastigote. A noticeable change between uninfected sand-flys and heavily infected vectors were the presence of a gel-like substance containing an abundance of leishmanial PPGs, known as Promastigote Secretory Gel (PSG) (13). This gel builds up within the sand fly's thoracic midgut and abdominal midgut, in an area located right behind the salivary glands. The PSG serves two purposes, one as a signal for metacyclogenesis to occur, secondly as a blockage or plug to the sand fly's stomodeal valve which would promote regurgitation of any blood meal. The stomodeal valve acts as a regulator for the flow of food between the proboscis and thoracic midgut. The PSG plug also acts as a reservoir of mammalian infective promastigotes, resulting in mixture of blood meal and metacyclic promastigotes. The blockage would also result in partial uptake of blood meal, with the remainder being regurgitated back into the site of infection (6). *Figure 1.3* shows the cross-sectional view of this process, highlighting the position of the PSG plug, forcing the metacyclic promastigotes to expel during blood meals.

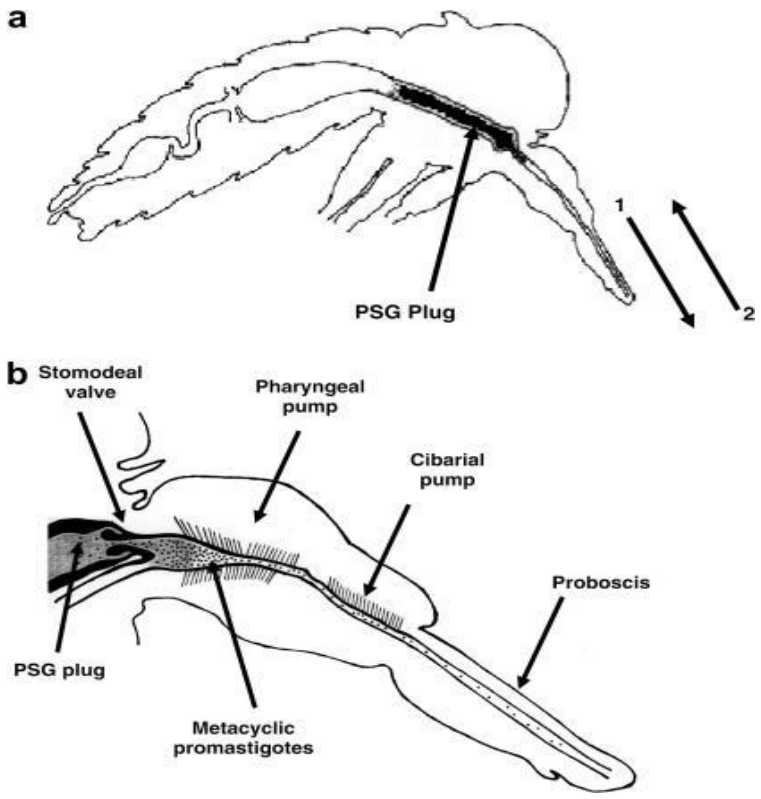


Figure 1.3: a) A leishmania-infected sand fly possessing a PSG plug behind the salivary glands, showing an exchange of metacyclic promastigotes during the egestion (1) and blood meal intake (2). b) Highlight of the PSG plug forcing the stomodeal valve open, allowing metacyclic promastigotes to flood an concentrate in the pharngeal pump, ready for egestion during a blood meal.

Image created by Paul Bates(5) and available via license: Creative Commons Attribution 3.0 Unported.

The method of alternating between vector and human host is described as a digenetic life cycle, where both vector and human can act as reservoirs for the parasite. The parasite's life cycle can be broken down into an infective stage, and diagnostic stage. The infective stage commences once the vector, sand-fly or otherwise, takes a blood meal and injecting the parasite's motile, promastigote stage, into the site of infection. At the site of infection, a strong type IV hypersensitivity (delayed type hypersensitivity) response can be observed, with enhanced IFNγ production, resulting in recruitment of neutrophils followed by macrophages (5,14–16). Although macrophages are the host cell for the promastigote, the

role that neutrophils play in phagocytic entry has been explored. At the site of infection, recruited macrophages are able to directly phagocytose majority of the promastigotes, however neutrophils may act as a temporary host-cell, or otherwise able to phagocytose promastigotes that would be inaccessible to other phagocytic cells(17–19).

After phagocytosis by the macrophage, the newly formed phagolysosome becomes the first point of metacyclogenesis within the human immune system. The infective promastigotes revert back to the proliferative amastigote stage (7,20). The parasite is able to reach critical densities and burst out of the infected macrophage, moving to neighbouring macrophages, neutrophils, dendritic cells and fibroblasts, exponentially increasing amastigote proliferation (21–23). To complete the life cycle, when an uninfected sand fly takes a blood meal from an infected human host, the amastigote containing macrophages are ingested by the sand fly, where the parasite will undergo metacyclogenesis again and repeat the cycle.

1.2.2 Clinically-relevant forms

Across all the species, the leishmaniases can be categorised into three clinically-relevant forms: cutaneous leishmania (CL); mucocutaneous leishmania (MCL); and visceral leishmania (VL). These three forms of leishmania are unique in terms of their infection site, cytokine responses, and mortality rates. Clinical manifestations can be visualised for the respective forms in figures *1.4, 1.6, 1.7,* and *1.9* with the epidemiology in figures *1.5*, and *1.8*

1.2.2.1 Cutaneous Leishmaniasis

CL is mainly caused by species *L. braziliensis*, and *L. mexicana* and is a generally acute version of the disease resulting in lesions and ulcers on the skin surface with reported cases of self-healing within 3-18 months. However, on the opposite spectrum, severe cases

included permanent disfigurement with high probability of relapse (24). In general, CL is the tamest form and is treated topically with no further incidence.

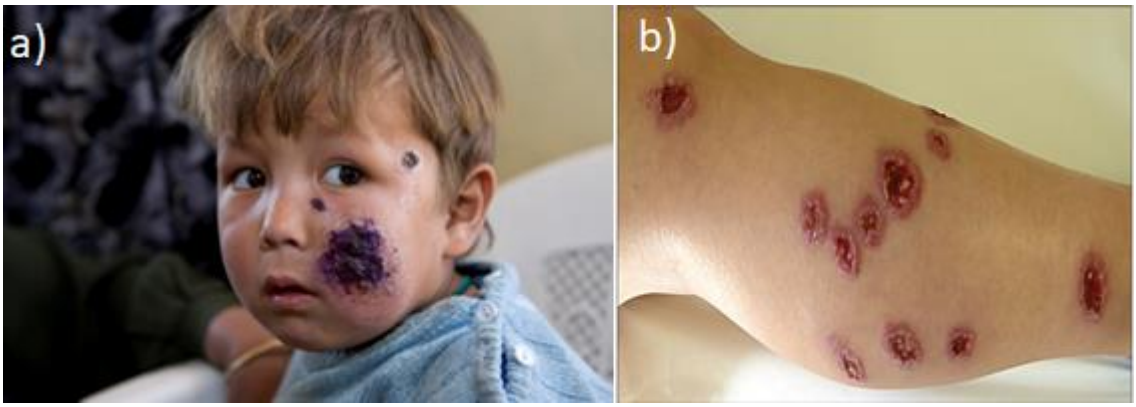


Figure 1.4: a) A child presented to an Afghanistan clinic with cutaneous leishmaniasis. b) A woman displaying multilesional cutaneous leishmaniasis three-weeks after initial contact with a *Leishmania major* endemic area in Israel. Images available from WHO*(25)*

1.2.2.1.1 Epidemiology and Pathology

CL, the more commonly reported form, affects between 600,000 – 1,200,000 people annually, with majority of the cases occurring in Afghanistan, Algeria, Brazil, Colombia, Iran, and Syria. CL results in the formation of lesions, most frequently around the exposed areas of the body. The most common areas are the face, scalp, extremities, and in some cases, back, abdomen and gluteal region. The size of the lesion is variable ranging from 0.5cm to 10cm depending on type of lesion, with the number of lesions being anywhere from one, to as high as five in some patients (24,26,27). The most common form of lesion is noduloulcerative, followed by papulonodular, vegetative, verrucous, and plaque. The duration of the lesion varied between 1 month – 16 months. Although the vast majority of CL cases are localized (>96%), within the last decade or so, two new forms have emerged. Diffuse-cutaneous leishmaniasis (DCL) and disseminated-cutaneous leishmaniasis (DL)

(28). DL is characterized by multiple papulonodular manifestations around exposed areas, namely the face and extremities. The number of papulonodules can reach numbers in the hundreds. These lesions are generally low in parasitic density, caused exclusively by *L. braziliensis*, and induce greater inflammatory responses than CL cases. DCL, is characterized by numerous non-ulcerative nodular lesions, abundant parasitic load within the nodules, negative results on conventional leishmanial skin tests, and failure to conventional treatments that otherwise would have worked in a CL diagnosis (29–31).

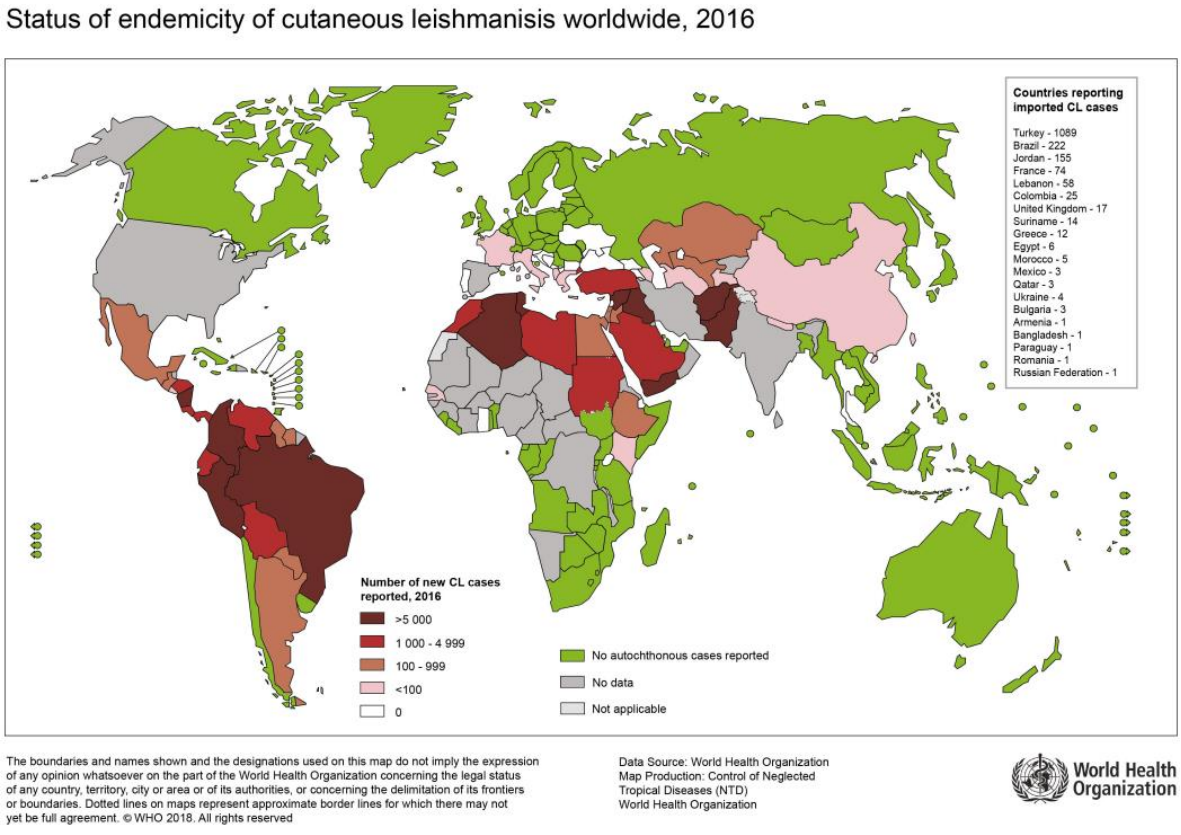


Figure 1.5: Global epidemiology of cutaneous leishmaniasis, and by extension mucocutaneous leishmaniasis, adapted from WHO*(32)*.

1.2.2.2 Mucocutaneous Leishmaniasis

MCL, which can be described as a derivative of CL, is caused by species *L. guyanensis* with overlapping from species *L. braziliensis* and *L. mexicana* depending on if the site of infection of CL is within proximity of a mucosal membrane. MCL, as disfiguring as CL, can also be life-threatening due to the destruction of the mucosal regions: such as the nasal mucosa; skin of the lips and nose; and oropharyngeal mucosa. This disfigurement can cause death due to sepsis, pneumonia or starvation in regions with insufficient medical intervention (24,33).

1.2.2.2.1 Epidemiology and Pathology

Of the reported CL cases, 35,000 or <5% of them result in mucocutaneous leishmaniasis, with majority of cases being localized in and around the South American continent. MCL usually occurs years after manifestation of CL, however can also occur simultaneously. MCL affects the mucosal regions, namely the nasal, oral and pharyngeal (34,35). The most important factor in MCL diagnosis is prior history with CL. Ninety-percent of patients with MCL have been treated for CL previously (36). Common symptoms include nasal inflammation, which progresses into ulceration and perforation around the nasal-oral mucosal membrane, such as the hard and soft palate, and the nasal septum. The effects are quite debilitating, with chance of permanent disfigurement around crucial organs. Similar to DL, the parasitic abundancy present in the lesions are comparatively lower, making it harder to diagnose by visualization of the amastigotes (37). The alternative method of diagnosis is by identification of leishmania DNA via polymerase chain reaction (PCR).

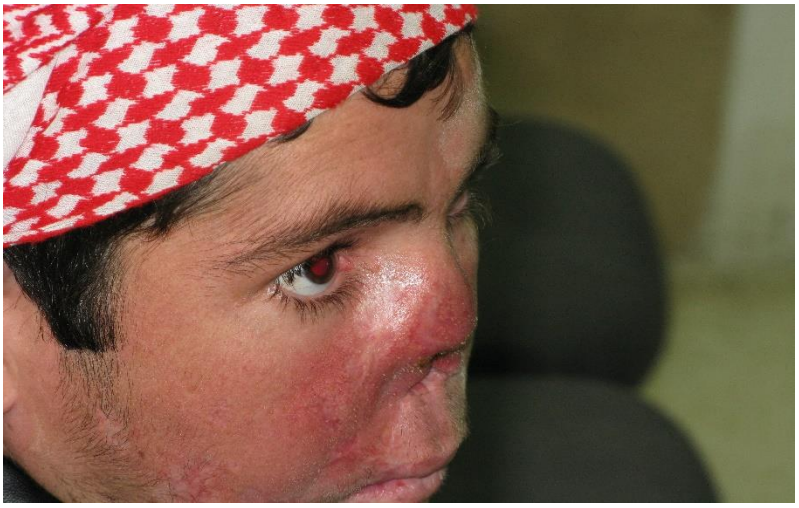


Figure 1.6: Partial destruction of the mucous membrane surrounding this male's nasal area. Image available from WHO*(36)*.

1.2.2.3 Visceral Leishmaniasis

Finally, VL is the most severe form of leishmania, caused mainly by species *L. donovani* and *L. infantum*, consisting of the parasite infecting macrophages throughout the viscera, consisting of the spleen, liver, and surrounding bone marrow. If left untreated, VL almost certainly results in 100% mortality, with other economic and social factors influencing the prognosis. Even with treatment, VL mortality rate can range between 10% and 90%, dependent on the treatment and the aforementioned factors (38).

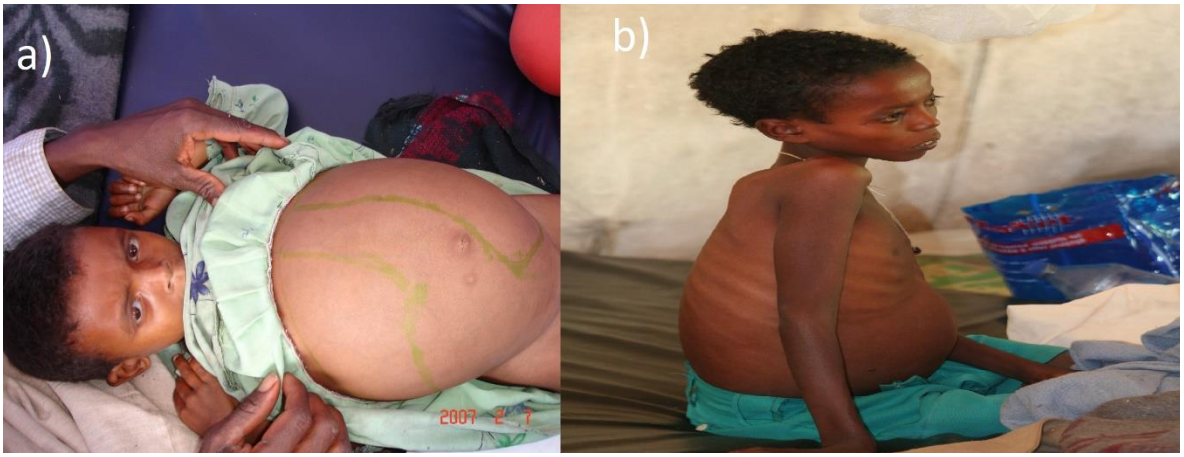


Figure 1.7: a) An Ethiopian girl suffering from visceral leishmaniasis, presenting with typical markets of liver and spleen enlargement. b) A boy presenting with similar spleen and liver swelling, with additional weight loss and potentially more markers such as anaemia. Images are available from WHO*(25,39)*.

1.2.2.3.1 Epidemiology and Pathology

Although VL is the deadliest, it's endemicity can be localized to just 5 countries where just over ninety percent of reported cases occur: Brazil, India, Sudan, Nepal, Bangladesh. In India and the surrounding areas, high infection rates can be due to humans being the main reservoir for the parasite. As for Brazil and its surrounding areas, zoonotic transmission is the main concern, with canines being the major reservoir of infection (39).

Upon infection by anthroponotic or zoonotic delivery, symptoms can develop as soon as 2 weeks or as late as 18 months. With early symptoms being emergence of a skin lesion at the site of infection, anaemia, fever, hepatomegaly, cachexia, splenomegaly, and lymphadenopathy (40,41). Hepatomegaly and splenomegaly are the main physiological signs of viscera infection. Biochemical and serological tests can further conclude the visceral infection, with isoenzyme analysis and rK39 rapid immunochromatographic test (ICT) respectively (40). One of the main pathological concerns with VL is the chance of

co-infections, as the parasite directly effects the phagocytic mononuclear system, making the patient susceptible to further infection.

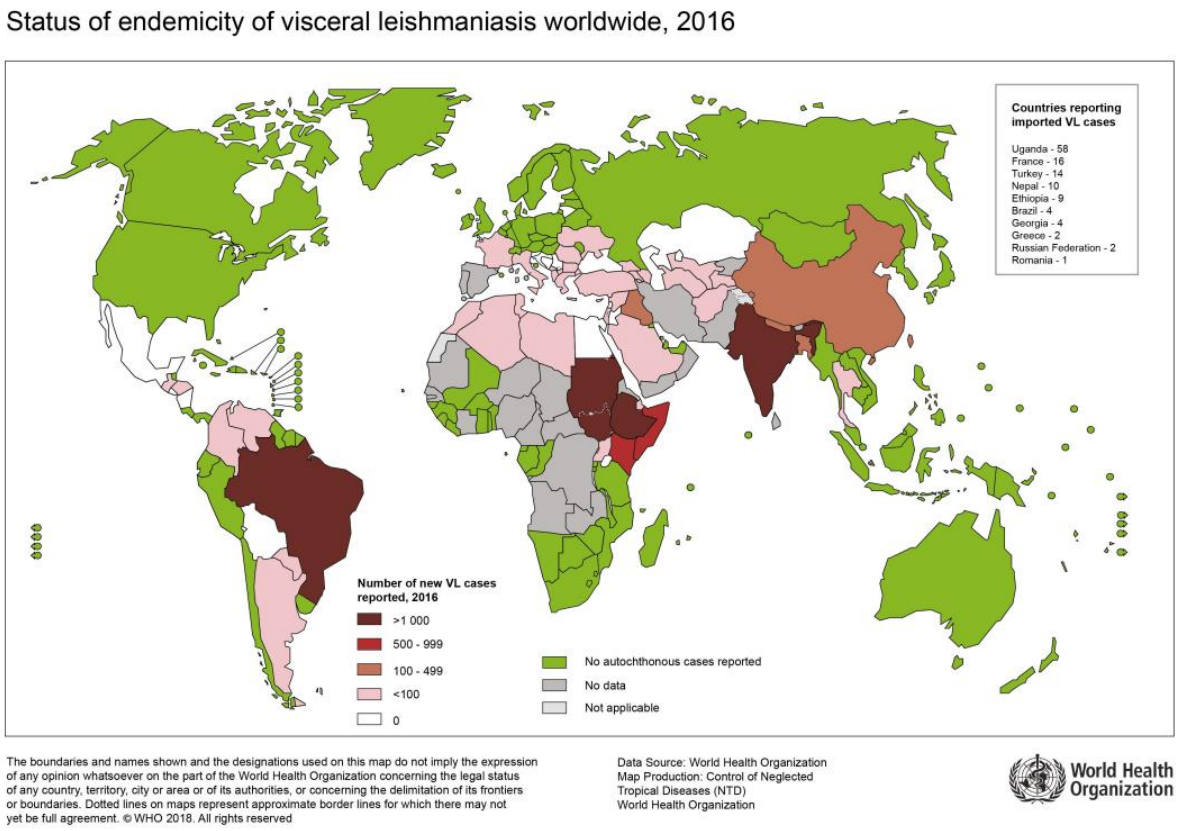


Figure 1.8: Global epidemiology of visceral leishmaniasis, adapted from WHO*(32)*.

1.2.2.4 Post-Kala azar dermal Leishmaniasis (PKDL)

PKDL can be regarded similarly to CL and MCL, where PKDL is a lesion that can occur in VL patient's post-treatment. However, PKDL is currently not well understood, with it being heralded as a 'phenomenon'. PKDL is caused mainly by *L. donovani* with rare reports of *L. infantum* infection (42).

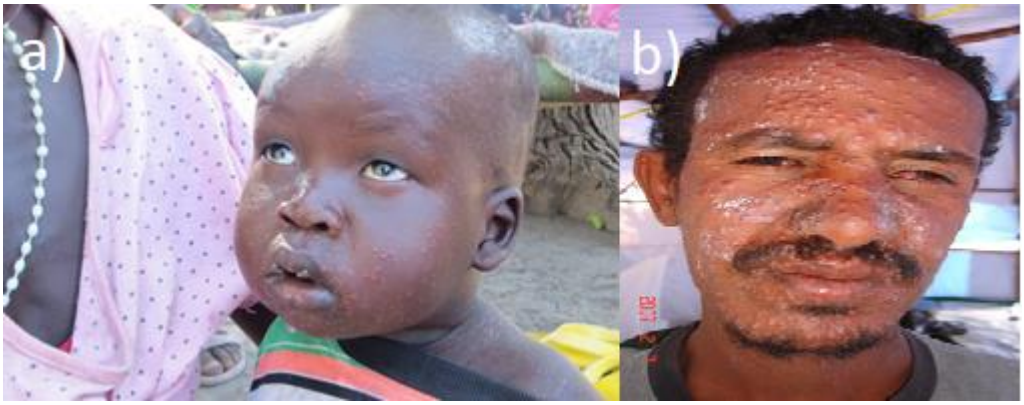


Figure 1.9: a) South Sudanese child presenting with PKDL, the area suffered from severe VL outbreaks 4 years prior. b) Ethiopian man formerly treated for VL and cured, presented later with PKDL. Images available from WHO*(25)*.

1.2.2.4.1 Epidemiology and Pathology

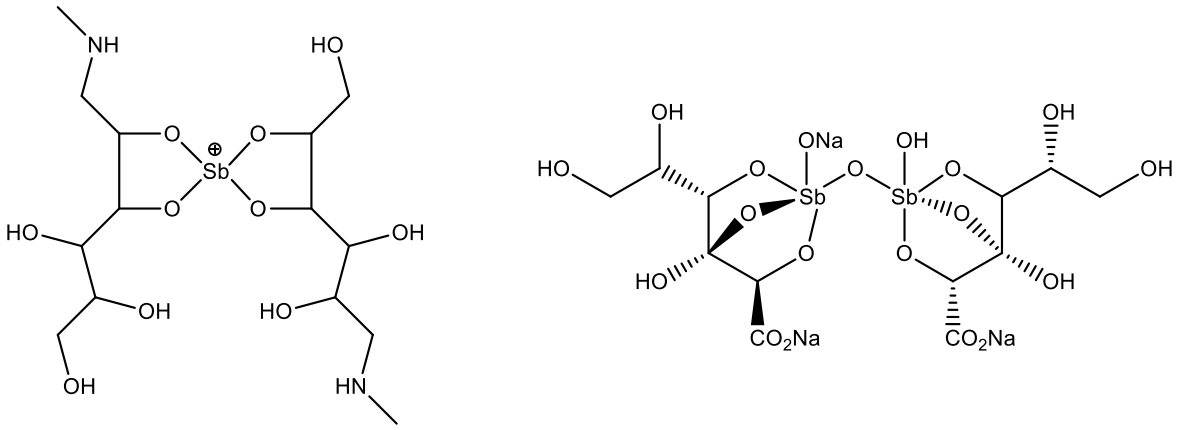
PKDL occurs mainly in the Sudan, Indian and Chinese localities. The reason PKDL is regarded as 'phenomenal', is the incidence and reproducibility of symptoms and treatment. In India, 5-10% of VL patients develop PKDL, with a follow-up period of 6 months to several years. In Sudan, PKDL occurs in 50% of VL patients, with a follow-up period of 0-6 months (42,43). There has currently been no accurate reporting of VKDL cases in China. In most patients across both regions, the lesion heals spontaneously without medical intervention, but for the minority of cases, the lesion can be debilitating in similar fashion to severe cases of CL (44). Of all reported PKDL cases, none have resulted in VL relapse, but rather remained at a cosmetic level. Investigations into treatment association found that, although majority of PKDL cases occurred following pentavalent antimonial treatment, one case of miltefosine treatment PKDL was reported. Further investigations into the mechanism and etiopathogenesis is of great importance, as PKDL can act as a recurring reservoir for further infection.

1.2.3 Current Therapeutics

The range of treatments for the leishmaniases have been getting refined over the decades due to less toxic, more efficacious treatments being developed.

1.2.3.1 Pentavalent Antimonials (Sb(V))

Originally, treatment of CL, MCL, and VL were limited to the antimonials, meglumine antimoniate and stibogluconate. These heavy-metal based drugs are expectantly highly toxic, with adverse side effects. During the drugs decades of use, its mechanism of action, and even structure, remained unclear. This was due to the amorphous properties of the antimonial complex. Advances over the years in structure characterisation techniques, mass-spectrometry and nuclear-magnetic resonance spectroscopy, has allowed researchers to determine that the complex sits in a pentavalent state Sb(V) (45). *Figure 1.10* illustrates the proposed structures of meglumine antimoniate and sodium stibogluconate.



Meglumine Antimoniate

Sodium Stiboglucanate

Figure 1.10: Chemical structures for meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) in aqueous solution.

1.2.3.1.1 Mode of Action and Drug Resistance

Although the antimonials are packaged in the pentavalent complex, there have been studies which show that Sb(V) acts as a prodrug, being reduced to the trivalent form, Sb(III), a more biologically active state (46). There have been multiple proposed methods for how this conversion takes place. A range of thiols across different cell types: parasitic cell, mammalian lysosome, and in the cytosol of mammalian cells have been regarded as essential in the prodrug reduction(45–47). The thiols in question are: Trypanothione ($T(SH)_2$), for reduction within parasitic cells; cysteine (Cys) and cysteinyl-glycine (Cys-Gly) for reduction within the lysosome; and finally, glutathione (GSH) for reduction within the cytosol of mammalian cells. Other studies have suggested the usage of parasite-specific enzymes, thiol-dependent reductase (TDR1) and antimoniate reductase (ACR2) (48,49). Although the Sb(V) prodrug model is the most supported, alternative studies have shown Sb(V) to have intrinsic antileishmanial activity without the need for reduction to the trivalent form. *Figure 1.11* shows the aforementioned models for Sb(V).

Although the antimonials were regarded as a first-line defense against the leishmaniases, the drug has a multitude of limitations. The method of administration, parenterally; side effects such as, hepatotoxicity, abdominal colic, myalgia and most concerningly cardiotoxicity; and even resistance build-up, *L. major* and *L. donovani* exhibited ten-fold resistance to both the prodrug form Sb(V), and the more toxic, active form, Sb(III). This resistance was found to occur in isolates with a deactivated aquaglyceroporin. For both Sb(V) and Sb(III), the major form of cell-entry is through the aquaglyceroporin, *AQP1* (50).

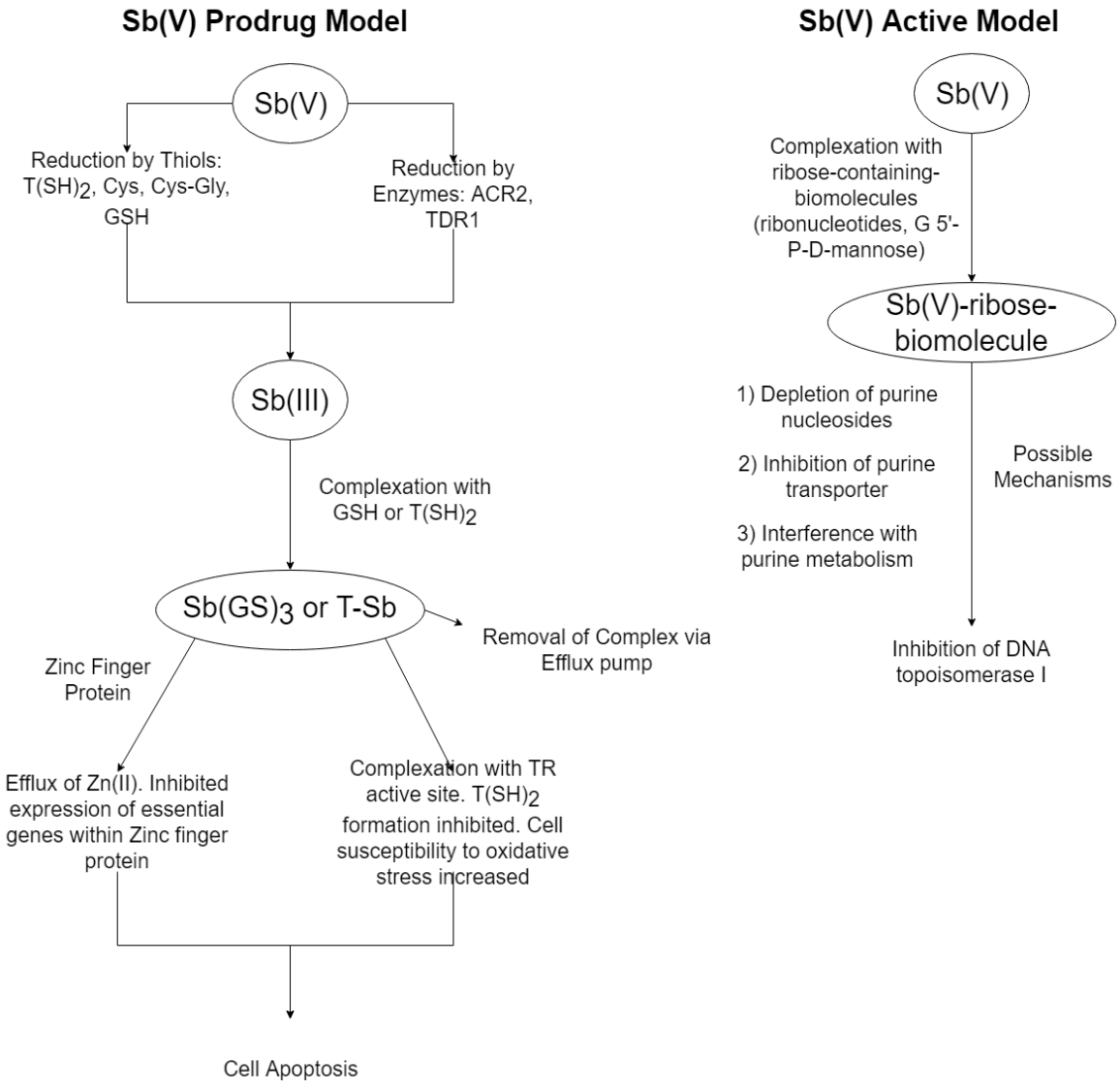
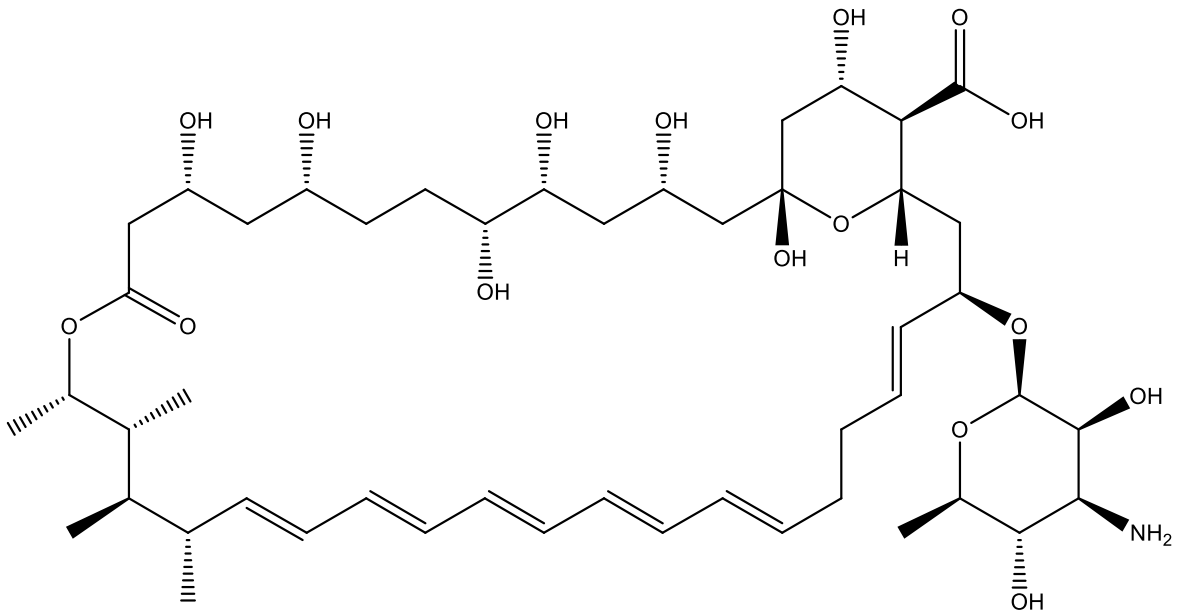


Figure 1.11: The two proposed mode of actions for the pentavalent antimonials, with the prodrug model indicating that Sb(V) is reduced in the cell to the more clinically active trivalent-form Sb(III) versus the active model where Sb(V) does not undergo reduction and complexes with ribose-containing-biomolecules. Image adapted from Frezard et al*(51)*.

1.2.3.2 Amphotericin B

A less-toxic, highly efficacious alternative to the pentavalent/trivalent antimonials, is the polyene macrolide antibiotic, Amphotericin B (AmB) as shown in *Figure 1.12*. The drug exists in a multitude of forms: raw form; AmB lipid complex (ABLC), and liposomal AmB (AmB-L) (52,53). Although AmB can be described as a superior choice to the antimonials, the biggest limitation is that AmB is extremely expensive relative to the antimonials. As previously mentioned, ninety percent of VL cases can be localized to 5 countries, these countries all fall into a category that contains the following attributes: inadequate healthcare systems; endemic areas inaccessible to commercial transportation; sand-fly vector population; prevalence of HIV/AIDS co-infection; poor nutrition; prevalent poverty; lack of NGO presence; poor hygiene and highly communal lifestyles. These attributes create a situation where, AmB is too expensive to purchase, too difficult to distribute and monitored administration is unfeasible, leading to incomplete dose regimes which promote drug-resistant strains (54). Recent solutions to some of the issues are WHO-negotiated pricing for VL patients, with a 10-fold decrease in pricing; development of liposomal formulations which decrease effective dosage, and potentially shortens regime duration. However, other issues such as the economic burden VL itself imposes, distribution and administration of the drug are still prevalent, as AmB and all forms are administered parenterally. Similarly, to the antimonials, modification to the cell membrane composition conferred AmB resistance.



Amphotericin B (Fungizone)

Figure 1.12: Chemical structure of the lipid-based polyene antibiotic Amphotericin B (Fungizone)

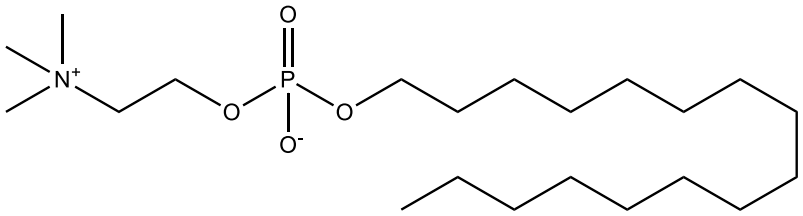
1.2.3.2.1 Mode of Action and Drug Resistance

AmB's mechanism of action is a well-studied area, with a consensus that AmB is a membrane-active drug. AmB binds to membrane sterols and forms two types of ion channels, non-aqueous and aqueous (55). These ion channels increase cell membrane permeability for ions and small solutes, leading to cell death. AmB, with higher binding affinity for ergosterol, and comparable binding affinity for cholesterol, allows for it to also compete for binding with macrophage cholesterols, effectively sequestering them from interacting with membrane receptors, some responsible for leishmanial entry (56). Although AmB has higher binding affinity for ergosterol, the sterol found on fungal and protozoan cell membranes, its comparable binding affinity for cholesterol, the mammalian sterol, confers cytotoxicity problems for the drug (57). In vitro studies on AmB cytotoxicity

found abnormal cell morphology and decreased proliferation at locally delivered concentrations of 5-10µg/ml(57). As for AmB resistance, amphotericin-B-resistant clinical isolates of *L. donovani* showed a reduction in parasitic membrane ergosterol, replaced by cholesta-5,7,24-trien-3β-ol, reducing AmB binding affinity, evidenced by reduced AmB uptake. Other observed features in AmB-resistant strains, were increased expression of Multi-Drug Resistance Gene (MDR1), increasing rate of efflux for AmB (58).

1.2.3.3 Miltefosine

The only orally-active anti-leishmanial drug, miltefosine as shown in *Figure 1.13*, is a recent addition to the arsenal of leishmaniasis treatment. Originally an anticancer candidate, miltefosine was later repurposed after it was found to have in vivo antileishmanial activity. The drug has been subject to extensive academic and clinical research, especially in and around the Indian subcontinent. In terms of efficacy, miltefosine showed comparable six-month cure rates to current therapeutics (miltefosine-94% vs AmB-97% vs Sb(V)-83%) (59,60). Acute toxicity for miltefosine was comparably higher than AmB (miltefosine-38% vs AmB-20% vs Sb(V)-60%) (59,60). Worth noting, however, is that in cases involving VL/HIV co-infection, studies have found miltefosine to lose effectiveness (initial treatment failure: Sb(V)-10% vs miltefosine-18%) (61). A major hallmark with miltefosine treatment is the possibility of outpatient care. With oral administration and low toxicity, it can be considered for outpatient care, although the long half-life of the active compound, one week, opens risk of resistant strains developing. However, the interaction of these drugs in HIV/VL co-infected patients is an even more under explored area. As with AmB, miltefosine suffered from issues pertaining to availability, distribution and cost. For pricing, WHO-negotiated pricing has relieved the burden in poverty-stricken countries.



Miltefosine

Figure 1.13: Chemical structures of the alkylphosphocholine drug, Miltefosine (Impavido)

1.2.3.3.1 Mode of Action and Drug Resistance

The mode of action of miltefosine has been largely underexplored until recently, leading to more information on trypanosomatid parasite biochemical pathways being explored. Some of the currently reported mechanisms for miltefosine are shared among other lysophospholipid analogues (LPAs) of which miltefosine is a part of(62). With regards to the activity of miltefosine, a major mechanism of action is the compounds effect on the phospholipid and sterol composition of trypanosomatid cell membranes(63). It was found that miltefosine inhibited phosphatidylethanolamine *N*-methyl-transferase (PEMT), which inhibited the synthesis of phosphatidylcholine(64,65). Although this transmethylation is present in mammalian cells, it was found that the IC_{50} required to induce a similar effect was 10-20-fold higher. This selective effect seems to be extended to both the promastigote and amastigote forms of the parasite (62,63,66).

Another important mechanism is one that is shared across a variety of drugs targeted for leishmanial treatment, where at least one of the targets is the mitochondrion(67,68). Studies carried out with cytochrome-c oxidase as a potential target found miltefosine inhibits oxygen consumption rate and reduces the mitochondrial membrane potential ($\Delta\Psi_m$)(69–72)

. A multitude of studies into other mechanisms is constantly developing for leishmanial treatments, with more recent studies finding additional avenues including more recently, disruption of Ca^{2+} homeostasis via leishmanial plasma membrane Ca^{2+} channels, ultimately leading to rapid alkalinisation of parasitic organelles(73).

In similar fashion to the Sb(V) resistant strains of leishmania, a drastic reduction in drug-uptake is the major characteristic of miltefosine resistant strains. Within *L. donovani* promastigote and amastigotes, all short-chain glycerophospholipids are internalised via the same protein, P-glycoprotein-like transporters(74,75). Reduction and/or inactivation of the P-glycoprotein-like transporters will reduce uptake of the antibiotics, stemming from mutations involving P-glycoprotein-like transporters which are related to MDR1. Regarding long-chain glycerophospholipids, more research into the field is needed.

1.2.3.4 Paromomycin

Finally, the latest addition to the anti-leishmanial arsenal, paromomycin, part of the previously discussed class of broad-spectrum antibiotics, aminoglycosides. Paromomycin as seen in *Figure 1.14*, like other aminoglycosides, exhibits a wide range of activity against most gram-negative bacteria, and many gram-positive bacteria. Paromomycin is unique among common aminoglycosides in the fact that it exhibits antileishmanial activity. The most important side-effects associated with aminoglycosides are nephrotoxicity and ototoxicity. The risk factors associated with these drug-induced conditions often brings into question clinical regimes and dosage justification when applied to treatments. The first reported case of aminoglycoside-induced ototoxicity was in a clinical trial conducted in 1945 utilizing streptomycin in the treatment of tuberculosis (76). Tinnitus was frequently reported as an early-onset symptom; however, the parameters for characterizing hearing

damage were not well defined or distinguished. These clinical trials would often associate hearing loss with ototoxicity although more recently, clinicians have further defined the parameters for ototoxicity to include vestibulotoxicity and cochleotoxicity. Vestibulotoxicity refers to damage induced to the cochleovestibular mechanisms in the inner-ear, which are responsible for balance and detecting spatial movements of the head, damage to this system will still only result in hearing loss and tinnitus, with cochleotoxicity being defined as hearing loss within the <8 kHz frequency range, also resulting in hearing loss and tinnitus.

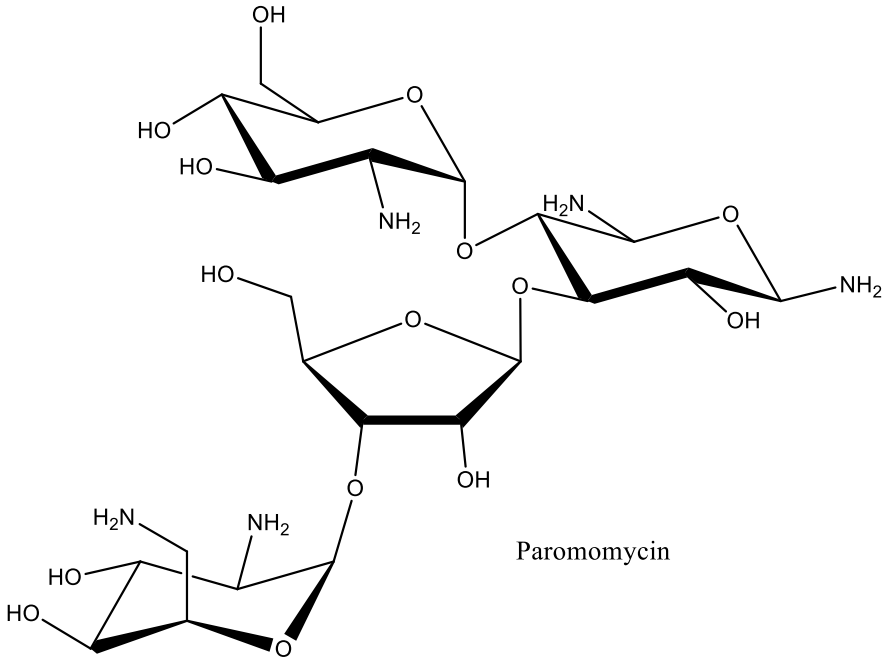


Figure 1.14: Chemical structure of paromomycin

1.2.3.4.1 Mode of Action and Drug Resistance

The mode of action for paromomycin has been extensively studied in *E.coli* models. The earliest known observations on the aminoglycosides showed that they were bactericidal but

by a different mechanism than initially thought(77). Using penicillin as the comparison, the bactericidal properties weren't due to cell lysis but rather irreversible membrane damage, attributed to inhibition of protein synthesis. This inferred action was later localised to the S12 protein in the 30S subunit of the ribosome in addition to 70S subunit (78–80). This would cause the incorporation of misread proteins into the cell membrane, increasing small-molecule permeability to the now leaky membrane(81,82).

Interactions with *Leishmania* are not as well studied, with current studies on paromomycin uptake suggesting the role of lipopolysaccharides (LPSs) and lipophosphoglycans (LPGs), which are highly abundant anionic components of the leishmanial promastigote cell surface. It is indicated that paromomycin, which is cationic, interacts with these LPSs and LPGs on the promastigote glycocalyx, internalising within the cell via endocytosis, with incorporation of endocytosis inhibitors such as vinblastine having a notable effect on paromomycin uptake, backing up this hypothesis (81,83–85). It is worth noting that similar observations are made in both promastigote and amastigote forms, with the amastigote form having drastically lower levels of LPG, leading to the involvement of other surface components(83).

Paromomycin's effect on protein synthesis via ribosomal subunits is similar between *E. coli* and *L.donovani*, showing paromomycin's binding to the 91S subunits for cytoplasmic ribosomes and to 75S for mitochondrial ribosomes. These subunits are unique to *L. donovani*, with paromomycin binding to 88S and 75S respectively, in *L. tropica* (86–89). Paromomycin promotes the association of both cytoplasmic and mitochondrial ribosomal subunits thus hindering protein synthesis(86,90). It was found that neomycin, a close analogue of paromomycin, does interact with the aforementioned ribosomal subunits, but

shows no activity against leishmanial promastigotes, indicating a need for paromomycin's uptake mechanism in addition to protein synthesis inhibition(86). Additional bactericidal properties occur from paromomycin's effect on mitochondrial membrane potential, showing a disruption of reductants needed in the respiratory chain, particularly upfield of the respiratory chain after the Krebs cycle (91). This shortage of reductants and substrates would result in an overall lower ATP supply, slowing down electron transfer. This cascade failure of metabolic events would decrease the mitochondrion membrane potential, eventually leading to cell death (83,91–93).

Paromomycin-resistant strains share similar mechanisms to previous described antibiotic-resistant strains, including the following: a drastic reduction in drug-uptake either by reduced binding affinity or increased efflux; mutations to the 30S, 70S, 75S, 88S, 91S ribosomal binding sites; or with the use of aminoglycoside modifying enzymes (AMEs) (83). The use of AMEs can go through three different modifications: ATP-dependent *O*-phosphorylation by phosphotransferases (APH); ATP-dependent *O*-adenylation by nucleotidyltransferases (ANT); and acetyl CoA-dependent *N*-acetylation by acetyltransferases (AAC), with enzymes possessing hybrid functions such as the case in staphylococci and enterococci species containing APH/ANT AMEs (94–96).

1.2.3.5 Liposomal drug carrier systems

The issues plaguing antileishmanial treatments revolve around the treatment plans of each therapeutic, with cases of resistant-strains forming due to poor influx of the drug or long half-lives. Although the straight-forward solution to poor influx is to increase the dosage, this leads to greater toxicity to cells and greater risk of resistance forming. Liposomal drug

delivery is a field that has seen extensive research and development in recent years, completely revolutionising treatment plans of current therapeutics. Liposomes act as vesicles for drug delivery, imparting benefits such as improved efficacy, lower cell cytotoxicity, adjustable drug release, intracellular targeting and protection from biological processes (97–99). Liposomes are rapidly cleared from the blood, making the bioavailability of the encapsulated drugs poor prior to intracellular delivery (100). The improved efficacy and lower cytotoxicity are due to the biodistribution and bioavailability of the encapsulated drug. With specific targeting of the vesicle, the bioavailability of the drug to specific target organs can be improved and reduce the necessary dose required, lowering overall toxicity and improving issues of influx (101). The targeting can be either passive or active, with passive targeting being due to the vesicles physico-chemical properties such as vector size, and active targeting utilizing specific targeting ligands dependant on the target organ (102,103).

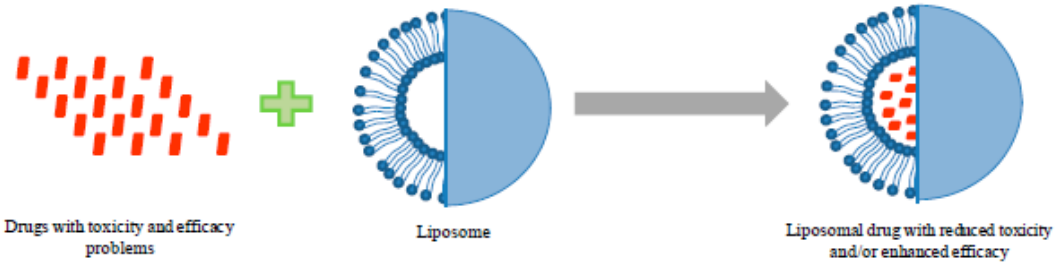


Figure 1.15: Encapsulation of a drug, with varying levels of cytotoxicity and efficacy, inside a phospholipid bilayer – liposome. Improved therapeutic indexes for the drug are the result from this drug-carrier system.

Image created by Upendra Bulbake (99).

1.2.3.6 Recent advances within the decade ~2010-2020

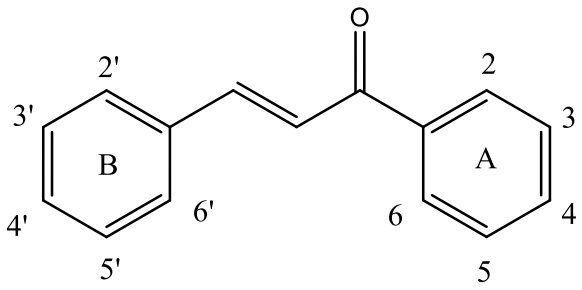
As illustrated in *Table 1*, there are many drawbacks associated with the currently established list of therapeutics for the various leishmaniases, demanding research into novel therapeutics outside the realm of heavy-metal antimonials, lipid-based polyenes, alkylphosphocholines, and aminoglycosides.

1.2.3.6.1 Chalcone

Chalcone and chalcone derivatives, also known as αβ-unsaturated ketones, are a pharmacologically diverse group of drugs hailing as prodrugs of flavonoids and isoflavonoids in naturally occurring sources such as Chinese Glycyrrhiza spp. “liquorice root”(104). These chalcone-containing roots have been used in the treatment of cancer, and the subject of antileishmanial studies with the pharmacological activity of chalcones being attributed to the structures double bond in addition to the carbonyl functionality, which has been visualised in *Figure 1.16* (105,106).

The mechanism of action and drug target of chalcone isn’t definitive, with findings such as Zhai et al. reporting that chalcones morph the ultrastructure of L. *major* and L. *donovani* promastigote mitochondria thus inhibiting mitochondrial dehydrogenase activity (107). Additional studies by Chen et al. found Fumerate Reductase (FR) to be the more specific target enzyme within the L. *major* promastigote mitochondria. Inhibition of this enzyme leads to a decrease in succinate levels as fumarate is no longer converted during glucose metabolism. A promising feature of this is that FR does not exist in mammalian cells, allowing for nontoxic targeting for these prospective leishmanicidal compounds (108). As of recently, Ogungbe et al. virtually screened 352 compounds against 24 enzyme targets of

L. *major*, L. *donovani* and L. *infantum*. Conclusively, the screening showed promising enzyme targets for a variety of chalcone derivatives such as: dihydroorotate dehydrogenase of the pyrimidine synthesis pathway; methionyl-tRNA synthetase for protein biosynthesis; oligopeptidase B for hydrolysis of arginine and lysine bonds in oligopeptides; and nucleoside hydrolase for the hydrolysis of specific RNA nucleotides such as uridine and inosine(109–111).



Chalcone (alpha beta-unsaturated ketones)

***Figure 1.16**:* Chemical structure of the open chain flavonoid, chalcone.

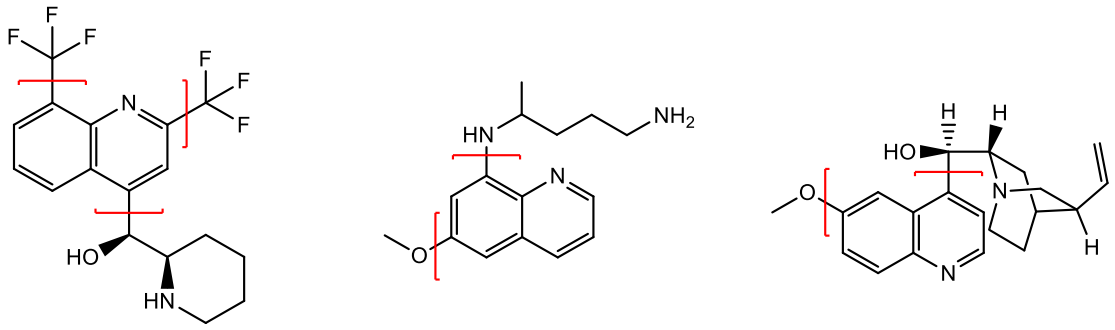
1.2.3.6.2 Quinoline

Quinoline and quinoline derivatives have long been the basis for chemotherapeutic agents against malaria, while also possessing a vast array of antimicrobial activity including anti-bacterial, anti-fungal and anti-leishmanial(112). The front-line therapeutics against malaria as outlined in *Figure 1.17*, these compounds have generated added interest into the leishmanicidal properties of quinoline-based compounds. Preliminary results by Eswaran et al. and Vanderkerckhove et al. showed adequate activity against *E.coli*, *S.aureus*, *P.aeruginosa, C.albicans, M.tuberculosis* (113,114). It is well established the biochemical and phylogenetically commonalities between *Plasmodium, M.tuberculosis*, and *Leishmania*

spp., which has vested interest into these compounds as potential anti-leishmanial compounds.

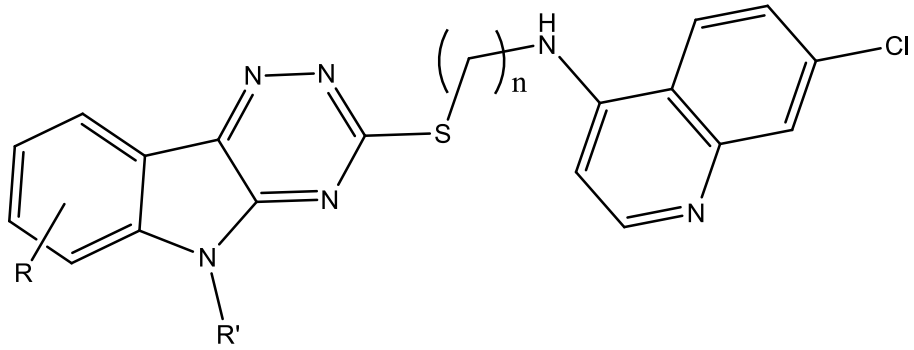
Chloroquine and amino-based quinolines have been tested against *Leishmania* spp. and other protozoans like *Trypanosoma* spp. Studies done by Miguel et al. onto the virulence factor *LHR1* within *L. amazonensis*, mediating the uptake of heme, found direct correlation to replication inhibition when knocking out *LHR1* (115). Further studies by Thomè et al. on *Plasmodium* found chloroquine to interact with heme, preventing subsequent polymerization to hemozoin (116). As *Leishmania*, *Trypanosoma* and *Plasmodium* are unicellular organisms and thus lacking the complete set of enzymes required in the heme-biosynthesis pathway, which could allow for a promising drug target. In the case of *Leishmania* spp, it is lacking all but the last three of the eight steps in the de-novo heme biosynthesis pathway. The seventh step, utilizing protoporphyrinogen oxidase, has been conjectured as a potential drug target due to *Leishmania* spp, and some other trypanosomatids being the only bacterial eukaryotes to possess this enzyme (117–119).

Additional studies on quinoline hybrids have been reported. Sharma et al. examined the effectiveness of triazino indole-quinoline hybrids on *L. donovani*. Using previously reported data by Sunduru et al. on the antileishmanial activity of the triazine moiety (120), and data done by Gupta et al. and Kgokong et al. on the efficacy of a triazino[5,6-b]indole scaffold against *Plasmodium* spp. and *L. donovani* (121,122), Sharma et al. synthesized a series of hybrid compounds as showcased in *Figure 1.18*, this scaffold was found to be more potent than conventional therapeutics against the amastigote form of *L. donovani* (123).



Mefloquine Primaquine Quinine

***Figure 1.17**:* Chemical structure of quinoline derivatives: Mefloquine, Primaquine, Quinine with the quinoline moiety highlighted.



7-chloro-N-(2-(5-alkyl-5H-[1,2,4]triazino[5,6-b]indol-3-ylthio)alkyl)quinolin-4-amine

***Figure 1.18**:* Chemical structure of the triazino indole-quinoline hybrid synthesised by Sharma et al. *(123)*

1.2.3.6.3 Azoles

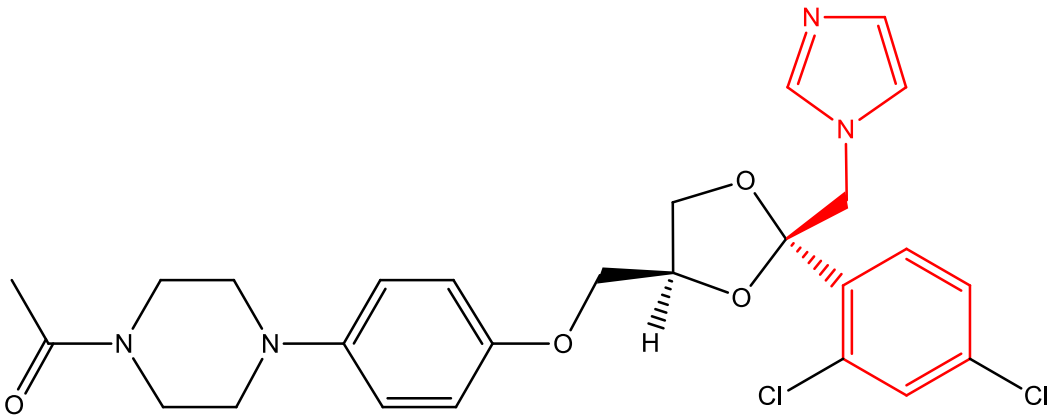
Azoles have a long-standing use as anti-fungal medication, with reported success as a topical therapeutic against CL in a clinical trial against *L. braziliensis panamensis*. Saenz et al. reported that ketoconazole had a 76% cure rate, comparable to pentostam, with 20% lower incidence of mild side effects (124). The mode of action for azoles is on the sterol biochemical pathway, inhibiting the cytochrome P450-dependent lanosterol 14α-demethylase enzyme(125). Similar to AmpB, targeting of ergosterol is a major convenience

due to the crucial role in membrane viability and parasitic cell growth that it has, as well as ergosterol being absent from mammalian cells, instead utilizing cholesterol as the predominant component of the cell membrane. Inhibition of this fungi-trypanosomatida-exclusive enzyme can disrupt the synthesis of the parasitic cell membrane, reducing viability (126).

The imidazole-based scaffolds of already commercialised antifungal therapeutics such as ketoconazole, shows a recurring pharmacophore, *N*-phenethyl-imidazole, as illustrated in *Figure 1.19*, thus a promising scaffold to utilize as it is apparent this pharmacophore is essential in binding with the enzyme(127). Shokri et al. synthesised a series of novel 3-imidazolylflavanones (IFs) containing the aforementioned pharmacophore. Shokri found 8-fold increased effectiveness for the synthesised IFs, including oxime derivatives (IFOs) against both promastigote and amastigote forms of *L. major* (128).

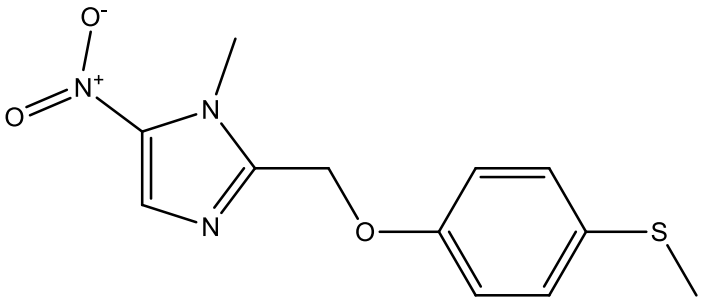
Baiocco et al. reported *L. donovani* and *L. infantum* cell growth inhibition utilizing an array of imidazolyl derivatives containing the same aforementioned pharmacophore. Baiocco reported a different mechanism of action, with a focus on the parasite's antioxidant protection mechanisms. Disruption of the *L. infantum* trypanothione reductase enzyme interrupts a key component of trypanothione metabolism, required to neutralize hydrogen peroxide made by the host macrophages (129).

A clinical trial by DNDi (NCT01980199) in Sudan for the treatment of visceral leishmaniasis patients using a new azole-based therapeutic, Fexinidazole, was approved through to Phase 2 trials before it was terminated in late October 2015. Cited reasons were for lack of efficacy and high cytotoxicity at efficacious concentrations. Fexinidazole is outlined in *Figure 1.20*.



Ketoconazole

Figure 1.19: Chemical structure of the antifungal ketoconazole with the pharmacophore *N*-phenethyl-imidazole highlighted



Fexinidazole

Figure 1.20*:* Chemical structure of the azole-based drug that underwent Phase II clinical trials in Sudan for VL treatment

Table 1: Summary of the listed therapeutics for leishmanial treatment, highlighting main features for each.

Drugs	Route of administration	Efficacy	Resistance	Toxicity	Strengths	Limitations	Cost	Reference
Pentavalent Antimonials Sb(V)	IV, IM, IL	30-90% based on geographic location	Resistance in Bihar, India	Severe cardiotoxicity, nephrotoxicity, hepatotoxicity	Cheap, globally available	• Rampant resistance in Bihar, India • Dosage regime issues due to sever side-effects, administration, and length of treatment	US$165 per treatment	(130–132)
Amphotericin B deoxycholate	IV	> 90%	Resistance in vitro	Severe nephrotoxicity	High efficacy	• Storage due to heat instability • IV cannot be self-administered, hospitalisation required	US$360 per treatment	(54,133,134)
Liposomal Amphotericin	IV	> 96%	Undocumented	Mild nephrotoxicity	High efficacy, relatively low toxicity	• Storage due to heat instability • Expensive	US$1700 per treatment	(54,133,134)
Amphotericin B lipid complex	IV	> 86%	Undocumented	IV related side-effects	High efficacy, virtually no toxicity	• Storage due to heat instability • Less efficacious than liposomal amphotericin	N/A	(54,133,134)
Miltefosine	Oral	> 93 % in India > 60% in Eastern Africa	Resistance in Bihar, India and Ethiopia	Hepatoxicity, nephrotoxicity	Oral administration	• Resistance emerging in Indian endemic regions • Resistance possibility due to long drug half-life • Dosage regime issues due to side-effects	US$84.8 per treatment	(135,136)
Paromomycin	IM, Topical	> 94% in Asia and 46-85% in Africa	Resistance in vitro	Nephrotoxicity, ototoxicity, hepatotoxicity	High efficacy, cheap	• Variable efficacy among different strains • Potential for resistance	US$7.5 per treatment	(137,138)

Table 2: Summary of the listed recent discoveries for novel therapeutics against leishmania, highlighting main features for each.

Drugs	Recency of publications	Proposed Efficacy	Clinical Trial Status	Toxicity	Strengths	Limitations	Target species	Reference
Chalcone	2018	More effective than standard	N/A	Excellent activity at non-cytotoxic concentrations	Pharmacologically diverse group, with a unsaturated αβ-ketone scaffold	• Large multi-step reactions without suitability for "one-pot synthesis" • High catalyst loading	*L. donovani* *L. major*	(105,108,109,111)
Quinoline	2019	More effective than standard (intracellular amastigotes only)	N/A	Severe cytotoxicity, narrow therapeutic index	Already established efficacy against biochemically similar *Plasmodium*	• Unappealing synthesis scheme: poor yields and selectivity, hazardous organic solvents and harsh reaction conditions	*L. amazonensis*	(115,116,119)
Quinoline Hybrids	2014	Several folds more effective than standard (amastigotes only)	N/A	Mild cytotoxicity	Better therapeutic index compared to lone-quinoline	• Unappealing synthesis scheme: poor yields and selectivity, hazardous organic solvents and harsh reaction conditions	*L. donovani*	(120,121,123)
Azoles	2017	Several folds more effective against both amastigotes and promastigotes	Terminated in Sudan 2015 (Lack of efficacy)	Cytotoxic at efficacious dosage	Simple *N*-phenethyl-imidazole scaffold	• Severe toxicity reported during phase II clinical trials	*L. braziliensis panamensis* *L.* infantum *L. major*	(125,128,129)

Chapter 2:

Synthesis and characterization of lipid-conjugated aminoglycosides for liposomal drug delivery and LCFA-ligase as potential drug target

2.1 Introduction

Aminoglycosides continue to serve a pivotal role in the treatment of life-threatening infections such as leishmanial, mycobacterial, enterococcal, and a variety of gram-negative bacteria. Although it has been well-documented the irreversible toxicity caused by the aminoglycosides, the high potency, broad-spectrum characteristics makes this class of antibiotic invaluable to the treatment of these serious infections. The high associated toxicities with aminoglycosides have made justification for their usage difficult; however, recent breakthroughs and discoveries in the glycosciences and carbohydrate-based modification have brought renewed interest in the synthesis of novel carbohydrate-based candidates. Some of these breakthroughs include substitution of certain functional groups that have increased efficacy and reduced cytotoxicity. In regards to the reference compound of paromomycin, modification of the ring-1 4'-hydroxyl, to a ethylthio or propyl group yielded a variety of beneficial changes: increased efficacy; enhanced ribosomal subunit selectivity; better activity against resistant strains; and lower ototoxicity (139,140). Modification of other functional groups can be of interest, when considering the impact of liposomal drug-carriers. Covalent bonding to these liposomes via long chain fatty acids could yield increased stability, efficacy and binding affinity. Other avenues of research include the synthesis of drug targets based around long-chain fatty acid-CoA ligase (LCFA-ligase). In *L. donovani* amastigotes, LCFA-ligase is differentially expressed in resistant strains, showcasing itself as a crucial protein and a potential target for inhibition (141).

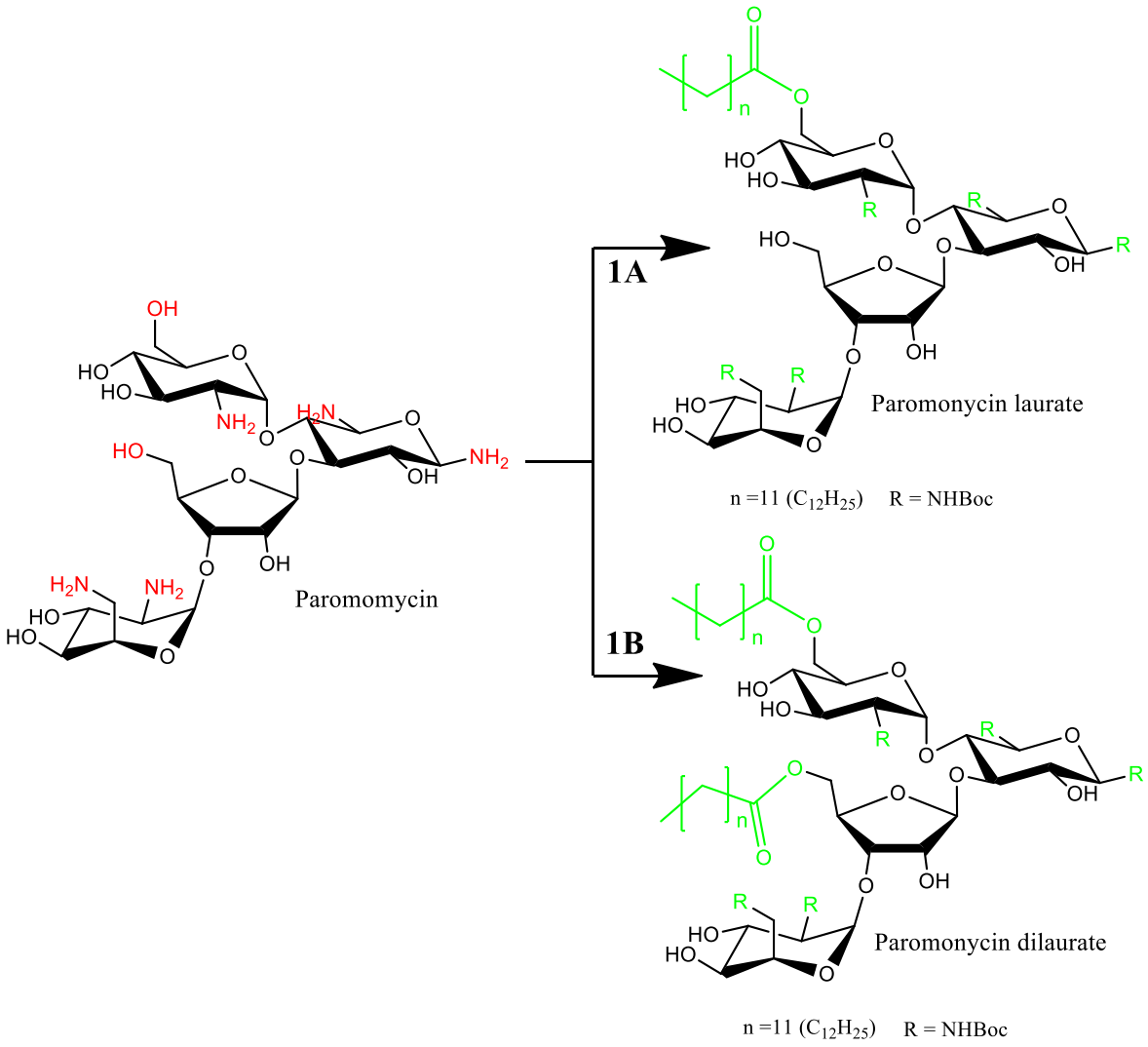


Figure 2.21: Synthesis schema of compounds **1A** and **1B**.

Paromomycin, as previously cited, has the unique characteristic of inhibiting leishmanial ribosomal subunits, with other aminoglycosides having similar ribosomal subunit inhibition, but lacking in penetrating leishmanial cell surfaces. In vitro resistant strains had significantly reduced uptake of paromomycin, via reduction of membrane transporters and increase in drug efflux. Synthesis **1A** and **1B** adds LCFAs to the primary hydroxyls of paromomycin via esterification chemistry. These additions can then be used to explore novel drug delivery methods to circumvent current resistance.

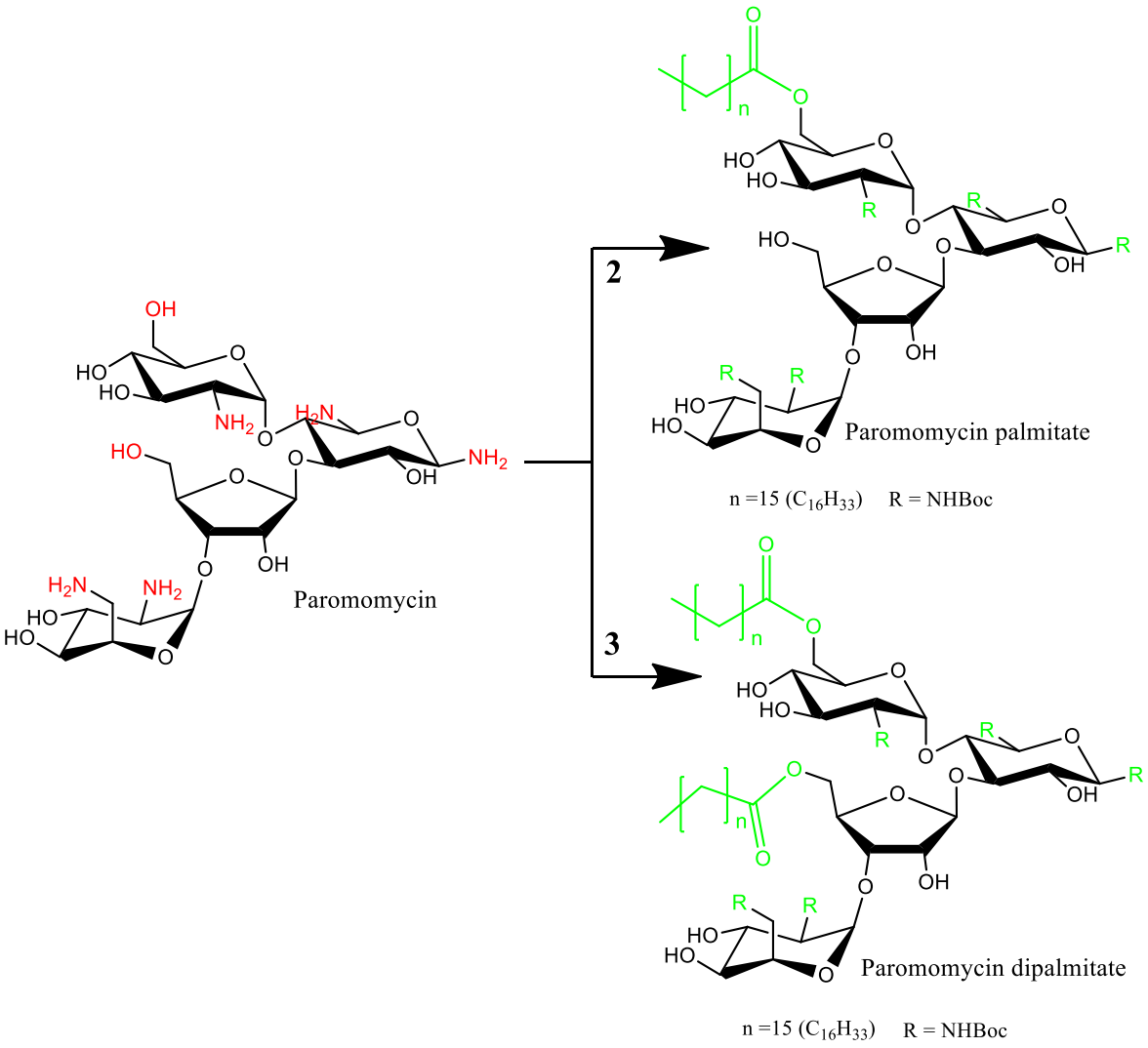


Figure 2.22: Synthesis schema of compounds **2** and **3.**

In contrast to *Figure 2.16*, a different LCFA is added to the primary hydroxyls of paromomycin via esterification chemistry. Usage of different LCFAs can be used to optimise the paromomycin prodrug, by investigation interactions of the LCFA against antimicrobials and for incorporation into liposomes. Synthesis **2** and **3** will also see the addition of a singular LCFA to a primary hydroxyl, and a LCFA to both primary hydroxyls, respectively.

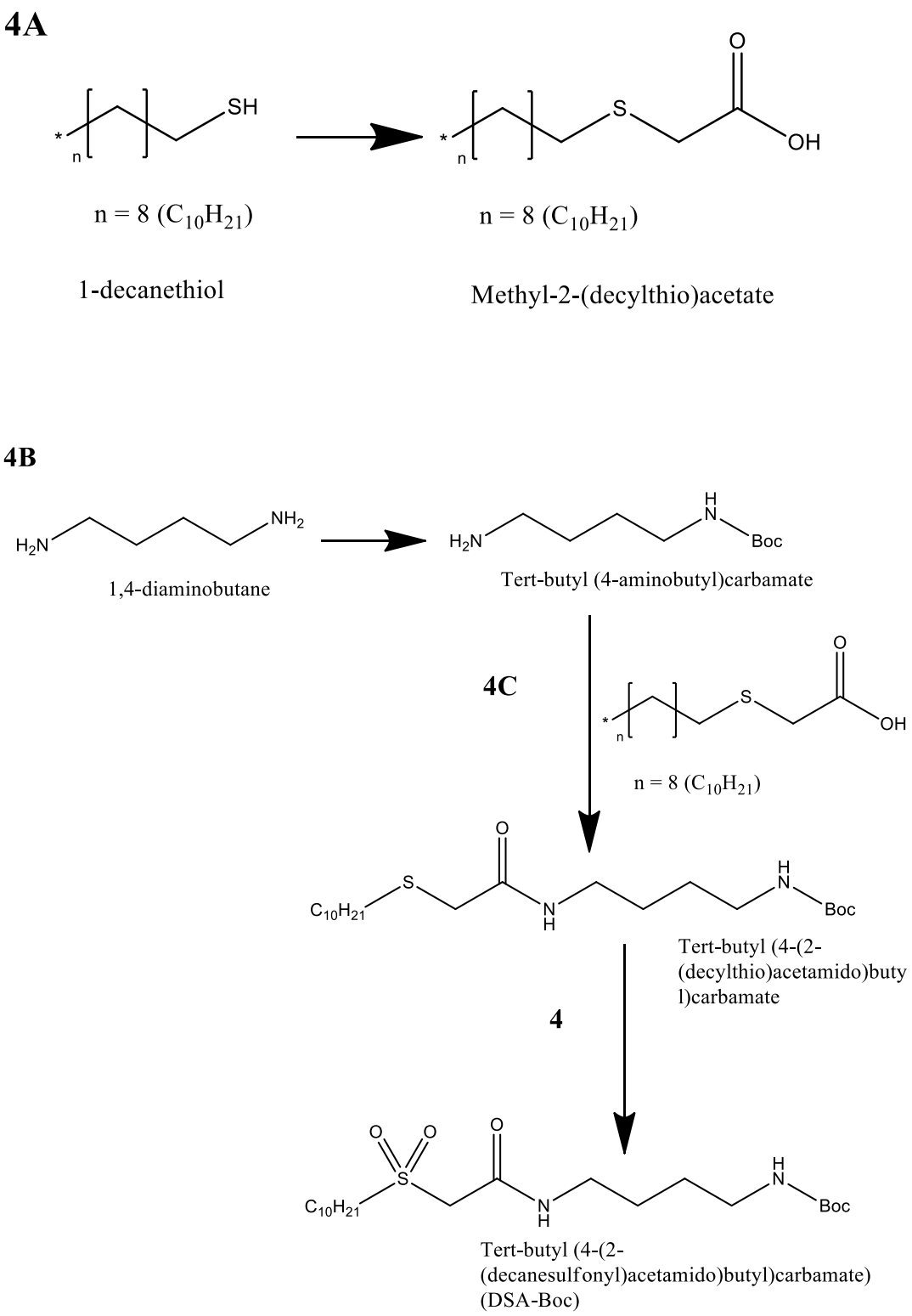


Figure 2.23: Synthesis of compound **4** (*N*-Boc DSA) visualised, including synthesis steps **4A, 4B,** and **4C**.

Synthesis of *N*-Boc DSA as a antileishmanial drug is based off research on fatty acid synthesis inhibition in *M. tuberculosis*. The compounds that showed the most activity, comparable to current antituberculosis therapeutics, were alkylsulfonyl-containing acetamides (142). As seen in *Figure 2.19*, the target in fatty acid synthesis, fatty acyl CoA contains the pantothenic acid group, with *N*-Boc DSA having structural similarities to fatty acyl CoA, containing a sulfonyl acetamide, and a structurally similar pantothenic acid group.

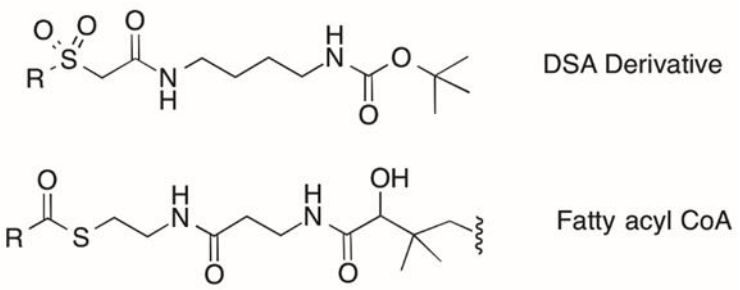


Figure 2.24: Comparison between the fatty acid synthesis target fatty acyl CoA and the *N*-Boc DSA derivative, based on novel antitubercular compounds synthesised by Jones et al. *(142)*

2.2 Experimental Section

2.2.1 General

Electrospray ionization low resolution mass spectrometry (ESI-LRMS *m/z*) was recorded on a Bruker Daltronics Esquire 3000 Ion-Trap LC MS, using Bruker Daltronics Esquire Control 5.0 software. All spectra were recorded in positive-ion mode at a concentration of 0.5-1.2mg/mL with sample introduction at 300µL/hr.

^{1}H and ^{13}C NMR spectra were recorded in the relevant solvents and temperatures on a Bruker Ascend 400 MHz and 600MHz respectively. Chemical shifts (δ) are expressed to two decimal places in parts per million (ppm) relative to tetramethylsilane (0 ppm) and coupling constants (*J*) are in hertz (Hz). Abbreviations for multiplicities are indicated using standard notation: single (s), doublet (d), doublet of doublets (dd), doublet of doublet of doublets (ddd), triplet (t), quartet (q), and multiplet (m). Standard prefixes are used including broad (br.) and apparent (app.).

All reactions were monitored under thin layer chromatography (TLC) using Merck silica gel plates (GF_{254}, cat No. 1.055545 on aluminium) and spots were visualized by UV light at 254nm and then developed by exposure to a solution of 5%Z H_2SO_4 in ethanol, then heating the plate at ~200°C. Flash column chromatography was performed using Merck 230-400 mesh silica gel for purification. Reaction products were purified by flash chromatography with silica gel 60 (0.040-0.063 micron, cat No 1.09385).

All solvents used in reactions were of analytical grade, distilled, or dried prior to use. All solvents used for chromatography were distilled prior to use. The reactions were conducted under dry N_2 wherever anhydrous conditions were required.

2.2.2 Materials

Paromomycin sulfate, di-tert-butyl dicarbonate (BOC), 1,4-diaminobutane, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 1-decanethiol, N-hydroxysuccinimide (NHS), 1,2-dichloroethane (DCE), lauroyl chloride, palmitoyl chloride were purchased from Sigma-Aldrich.

2.2.2.1 Synthesis of BOC-protected paromomycin

Paromomycin sulfate (1 g, 1.4 mmol) was readily dissolved in water (5 mL). A solution containing triethylamine (4.5 mL, 25.1 mmol) and methanol (5 mL) was added to the dissolved paromomycin sulfate solution. BOC (2.5g, 11 mmol) was added to the reaction mixture and stirred at 55°C for 16 hours. The reaction mixture was evapourated for methanol removal and the residue partitioned between ethyl acetate (100 mL) and water (50 mL) and washed with ethyl acetate (2 x 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under vacuo to produce a white-ish powder (1.28g, 91%). Further purification by silica column chromatography using dichloromethane: methanol (DCM: MeOH) = 9:1 as eluent.

TLC (DCM/MeOH 9:1) $\mathbf{R_f}$ = 0.0, **ESI-MS**: *m/z* = 1179.3 $[M + Na]^+$.

*2.2.2.2 Synthesis of paromomycin-laurate (**1A**) & paromomycin-dilaurate (**1B**)*

Boc-protected paromomycin (0.05g, 0.09 mmol) was dissolved in dry 2,6-Lutidine (5 mL) and lauroyl chloride (0.1g, 0.46 mmol) was added dropwise at 0°C and stirred for 18 hours. After reaction completion, the reaction was quenched with methanol, and solvent evapourated under vacuo. Crude compound was dissolved in CH_2Cl_2 (10 mL) and washed with 1M HCl (2 x 25 mL), $NaHCO_3$ (10 mL), brine (10 mL), and dried over Na_2SO_4 and concentrated under vacuo.

TLC (DCM/MeOH 9:1) $\mathbf{R_f}$ = 0.49, **ESI-MS**: *m/z* = 1331.1 $[M]^+$ 0.5x10^5 Intensity.

Condition changes on **1** synthesis: acid chloride ratio 5:1 → 10:1; temperature and time 0°C 18 hour → RT 6 hour; 2,6-Lutidine → Pyridine / 2,3,5-Collidine. Reaction still remained as starting material.

*2.2.2.3 Synthesis of paromomycin-palmitate (**2**) & paromomycin-dipalmitate (**3**)*

Boc-protected paromomycin (0.05g, 0.09 mmol) was dissolved in dry 2,6-Lutidine (5 mL) and palmitoyl chloride (0.1g, 0.36 mmol) was added dropwise at 0°C and stirred for 18 hours. After reaction completion, the reaction was quenched with methanol, and solvent evapourated under vacuo. Crude compound was dissolved in CH_2Cl_2 (10 mL) and washed with 1M HCl (2 x 25 mL), $NaHCO_3$ (10 mL), brine (10 mL), and dried over Na_2SO_4 and concentrated under vacuo. Yielding an orange oily residue with further purification by silica column chromatography using dichloromethane: methanol (DCM: MeOH) = 9:0 → 9:1 as eluent.

TLC (DCM/MeOH 9:1) $\mathbf{R_f}$ = 0.38, 0.45, **ESI-MS**: *m/z* = 1377.5 $[M]^+$ & 1615.8 $[M]^+$

The aforementioned silica column chromatography yielded a mono-lipid (**2**) (0.01g, 0.008 mmol, 15%) and di-lipid (**3**) (0.05g, 0.03 mmol, 33%) pairing.

Compounds **3** (0.02g, 0.015 mmol) and **4** (0.05g, 0.03 mmol) were subjected to boc-deprotection, the compounds were dissolved in DCM (1 mL) and treated with TFA (0.5 mL) for 45 minutes stirred. The solvent was evapourated and the crude compound was concentrated under vacuo.

ESI-MS: *m/z* = 854.7 $[M]^+$ & 1093.2 $[M]^+$,

^{1}H NMR (600 MHz, CD_3OD). δ 0.80 (m, 6H), 1.20 (d, *J* = 6.0 Hz, 36H), 1.29 (s, 10H), 1.30 (m, 6H), 1.37 (m, 31H), 1.50 (m, 2H), 2.19 (dt, *J* = 2.1, 7.4 Hz, 2H), 2.9 (m, 2H), 3.01 (m, 2H), 3.50 (m, 3H), 3.64 (m, 4H), 3.83 (m, 2H), 4.16 (m, 2H), 4.27 (m, 3H), 4.45 (s, 2H), 4.49 (s, 2H)

2.2.2.4 Synthesis of tert-butyl (4-(2-(decanesulfonyl)acetamido)butyl)carbamate ***(4) (N-Boc DSA)***

Methyl-2-(decylthio)acetate (**4A**) was synthesized using K_2CO_3 (2096mg, 15 mmol) and adding to MeCN (10 mL) under argon gas. 1-decanethiol (1.03 mL, 5 mmol) was added to the solution followed by methylbromoacetate (0.68 mL, 6 mmol) and stirred at 50°C over 24 hours. Solvent was evapourated under vacuo and partitioned between ethyl acetate (50 mL) and 1M HCl (100 mL) and washed with ethyl acetate (2 x 50 mL). The combined organic layers were washed with HCl (3 x 30 mL), water (30 mL), brine (30 mL) and dried over Na_2SO_4, and concentrated to yield a yellowish liquid (1.07g, 86%).

^{1}H NMR (400 MHz, $CDCL_3$) δ: 0.88 (t, 3H, *J* = 6.59 Hz), 1.27 (m, 12H), 1.37 (m, 2H), 1.59 (m, 2H), 2.62 (t, 2H, *J* = 7.43 Hz), 3.22 (s, 2H), 3.74 (s, 3H)

Tert-butyl (4-aminobutyl)carbamate (**4B**) was synthesized using 1,4-diaminobutane (2.0g, 22.69 mmol) dissolved in DCM (50 mL), then added dropwise to a solution of di-tert-butyl-dicarbonate (0.587g, 5.38 mmol) dissolved in DCM (5 mL) over 1 hour. After complete addition, the reaction was carried at RT over 24 hours. Reaction was partitioned between water (100 mL) and DCM (100 mL) and washed with DCM (3 x 50 mL), dried over

Na_2SO_4. The solvent was evapourated under vacuo yielding a colourless oily residue (189.3 mg, 1.003 mmol, 4.4%)

^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.38 (m, 13H), 2.69 (t, 1H, *J* = 6.6 Hz), 3.11 (q, 2H, *J* = 6.5 Hz), 4.65 (s, 1H)

Tert-butyl (4-(2-(decylthio)acetamido)butyl)carbamate (**4C**) was synthesized by activating **4A** (0.6g, 2.58 mmol) in EDC (0.744g, 3.88 mmol), NHS (0.297g, 2.58 mmol) and DCE (10 mL) over 1 hour. After activation, **4B** (0.49g, 2.58 mmol) was dissolved in DCE (10 mL) and added to the reaction mixture, this combined mixture was stirred at 50°C over 72 hours. Solvent was evapourated under vacuo and partitioned between water (50 mL) and ethyl acetate (50 mL) and washed with ethyl acetate (2 x 25 mL), dried over Na_2SO_4 and concentrated under vacuo. Yielding a white powder (0.66g, 1.65mmol, 64%) with further purification by silica column chromatography using (DCM: MeOH) 98:2 as eluent.

TLC (DCM/MeOH 98:2) $\mathbf{R_f}$ = 0.67. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J* = 6.8 Hz), 1.26 (s, 12H), 1.35 (m, 2H), 1.44 (s, 13H), 1.52 (m, 2H), 2.51 (t ,2H, *J* = 7.46 Hz), 3.14 (m, 2H), 3.21 (s, 2H), 3.31 (m, 2H)

Compound **4** was synthesized using **4C** (0.5g, 1.25 mmol), oxone (3.14g, 5 mmol), aluminium chloride (0.33g, 2.55 mmol) ground together for 0.5 hour, then dissolved in DCM (30 mL). Reaction mixture was washed with $NaHCO_3$ (10 mL), water (10 mL), dried over Na_2SO_4 and evapourated under vacuo. Yielding a white solid (0.11g, 0.325 mmol, 74%) with further purification by silica column chromatography using (DCM: MeOH) 100:2 as eluent.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J* = 6.8 Hz), 1.26 (s, 12H), 1.44 (s, 13H), 1.52 (m, 2H), 1.86 (m ,2H), 3.15 (m, 4H), 3.32 (m, 2H), 3.83 (s, 2H)

2.2.3 Additional comments

For the synthesis of the laurate/palmitate and the dilaurate/dipalmitate derivatives of paromomycin, adjustment of reaction conditions shifted the ratios into the appropriate direction, with longer reaction time forcing excess acid chloride to bind to the alternate primary alcohol. Although the N-Boc protection groups are very stable, there were instances of the excess acid chloride knocking off protection groups and attaching. These tri-laurate/tri-palmitate instances were not investigated further. It was required that humidity control of the experiments was maintained as the aminoglycoside esterification chemistry is extremely sensitive to moisture along with quarter-hourly control of reaction conditions.

Although ^{1}H NMR showed no indication of lipid-chain conjugation, it was a possibility discussed during synthetic steps as the lipid-chains could potentially conjoin onto an existing lipid chain instead of an alternate primary hydroxyl. The turbulence induced by MeOH solvent however should negate the possibility in most cases.

Selection of the laurate and palmitate brings to mind the other major lipid, stearate. Previous work conducted by T.Mosaiab and D.Farr within the Houston group influenced the decision to disregard the C_{18} lipid chain. Alternative lipid chains such as decanoate, undecanoate and myristate were currently under investigation in a parallel project, deemed unnecessary and outside the scope of works. With the results outlined in *Table 3*, additional studies into gram-negative and gram-positive bacteria with differing paromomycin

conjugates can add valuable insight into the research area, however results outlined in *Table 4*, with the discussion, major modifications are needed in order to effectively test against *Leishmania* spp. with geographical strains being a main focus.

As mentioned previously, liposomal drug targeting was investigated in order to adjust the therapeutic index of aminoglycosides, which have been mentioned as extremely nephrotoxic, ototoxic and hepatotoxic. Addition of these synthesised final targets into liposomal systems to widen therapeutic indexes are of great interest and future work into this field will undoubtedly investigate it.

Chapter 3:

Antimicrobial studies against *S. aureus, P. aeruginosa, L. donovani* and pro-inflammatory studies against murine macrophage cells

3.1 Introduction

Intracellular pathogens pose a major global health issue due to their ability to infect host cells, proliferate and disperse without causing damage to these host cells. By virtue of how host antibodies are incapable of passing through cellular or subcellular membranes, the ability for pathogens to spend a portion or all of their life cycle within host cells offers a very safe haven for these pathogens to multiply and spread. Some pathogens that have developed mechanisms to penetrate and survive in the host cell include: *Staphylococcus aureus, Pseudomonas aeruginosa, Leishmania* spp., and *Mycobacterium* spp. Additional issues that have arisen from intracellular infections relate to general antibiotic effectiveness. Once a pathogen is within the host cell, not only is it protected from the host immune response, it is also difficult to treat with conventional antibiotics due to the inability for antibiotic compounds to influx into the cell at regular concentrations, inability to remain in the cell due to efflux channels, and inability to disperse effectively to subcellular structures. These factors result in increased dosage of the aforementioned therapeutics so as to avoid the potential of drug-induced resistance, and this in turn poses greater risk as the treatments associated with intracellular infections carry many side effects. Aminoglycosides for example are associated with nephrotoxicity and ototoxicity when used at relatively high dosages. The conditions of the host cell may also affect stability and antimicrobial activity of the antibiotics, with changes in cell temperature, enzymatic presence, and the variable range of pH's across different cells. Targeted drug delivery can overcome many of these issues.

3.2 Experimental Section

3.2.1 Materials

- Luria-Bertani (LB) medium (Sigma-Aldrich, Australia)
- DMEM (1X High Glucose) Dulbecco's Modified Eagle Medium (Sigma-Aldrich, Australia)
- Heat-inactivated fetal bovine serum (HI-FBS) (ThermoFisher, Australia)
- Penicillin-Streptomycin solution (10,000 U/mL) (Sigma-Aldrich, Australia)
- DPBS (1X, (-)$CaCl_2$, (-)$MgCl_2$) Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, Australia)
- DMSO, Dimethyl Sulfoxide (CAS 67-68-5) (Purity ≥99.7%) (Sigma-Aldrich, Australia)
- Paromomycin sulfate-salt (CAS 1263-89-4) (Sigma-Aldrich, Australia) stored at RT as a powder. Bacterial stock solutions of 1 mg/mL were made up in sterilised MilliQ® water, supplemented with ≤ 5% DMSO where indicated.
- Neomycin trisulfate-salt (CAS 1405-10-3) (Sigma-Aldrich, Australia) stored at RT as a powder. Bacterial stock solutions of 1 mg/mL were made up in sterilised MilliQ® water, supplemented with ≤ 5% DMSO where indicated.
- Amikacin hydrate (CAS 1257517-67-1) (Sigma-Aldrich, Australia) stored at RT as a powder. Bacterial stock solutions of 1 mg/mL were made up in sterilised MilliQ® water, supplemented with ≤ 5% DMSO where indicated.
- Kanamycin sulfate (CAS 70560-51-9) (Sigma-Aldrich, Australia) stored at RT as a powder. Bacterial stock solutions of 1 mg/mL were made up in sterilised MilliQ® water, supplemented with ≤ 5% DMSO where indicated.

3.2.2 Bacterial strains and mammalian cell lines

S. aureus strain (ATCC 25923) were cultivated in Luria-Bertani Broth medium at 37°C shaking at 160 rpm. Colony forming units per mL (CFU/mL) was determined by plating the *S. aureus* cells on LB agar plates after serial dilution.

*P. aeruginosa (*ATCC 27853) were cultivated in Luria-Bertani Broth medium at 37°C shaking at 160 rpm. Colony forming units per mL (CFU/mL) was determined by plating the *S. aureus* cells on LB agar plates after serial dilution.

L. donovani MHOM/IN/DD8 (ATCC 50212) were utilised by the V.Avery group at GRIDD as per protocol by Zulfiqar et al. (143)

RAW 264.7 (ATCC TIB-71) murine macrophage cell line were cultured in complete medium containing DMEM (Sigma-Aldrich), 10% heat-inactivated fetal bovine serum (FBS), 100µg/mL penicillin, 100µg/mL streptomycin, stored at 37°C, 5% CO_2. Cultures were discarded when they reached passage 20.

3.2.3 Maintenance of bacterial and mammalian cells

Cryopreservation stocks were created of *S. aureus* and *P. aeruginosa* utilising 15% v/v sterile DMSO and liquid culture in 2 mL cryopreservation tubes, stored long-term at -80°C. Samples were revived from frozen stocks and added to fresh media, incubated over 18 hours, subcultured and incubated for a further 18 hours. Absorbance was measured, and stocks were adjusted to 0.5 McFarland (~0.1 Absorbance @ 600nm) using single strength LB broth.

RAW 264.7 (Murine macrophage cells) were maintained in DMEM Complete medium (Supplemented with 10% v/v HI-FBS, 1% Strep-Pen solution), incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were passaged every 48-72 hours (Confluency never exceeding 90%). Cultures were discarded after reaching passage 20.

3.2.4 Statistical Analysis

Data from the experiments were grouped and combined with means ± standard deviation (SD). All data was averaged from experiments containing at least 4 replications. Statistical significance was determined by two-way analysis of variance (two-way ANOVA) and the effect among the groups were compared by sidak's multiple comparison's test using GraphPad Prism (version 8.2.1, La Jolla, CA). Values with $P<0.05$ were considered significant.

3.3 Results and Discussion

Antibacterial susceptibility testing was performed on *S. aureus* and *P. aeruginosa*, gram-positive and gram-negative bacteria, respectively. The chosen candidates included a range of aminoglycosides: with neomycin; amikacin; and kanamycin derivatives being synthesised and provided by Dylan Farr. The palmitate lipid derivatives were chosen for comparison against their respective control in killing efficiency against the aforementioned bacteria.

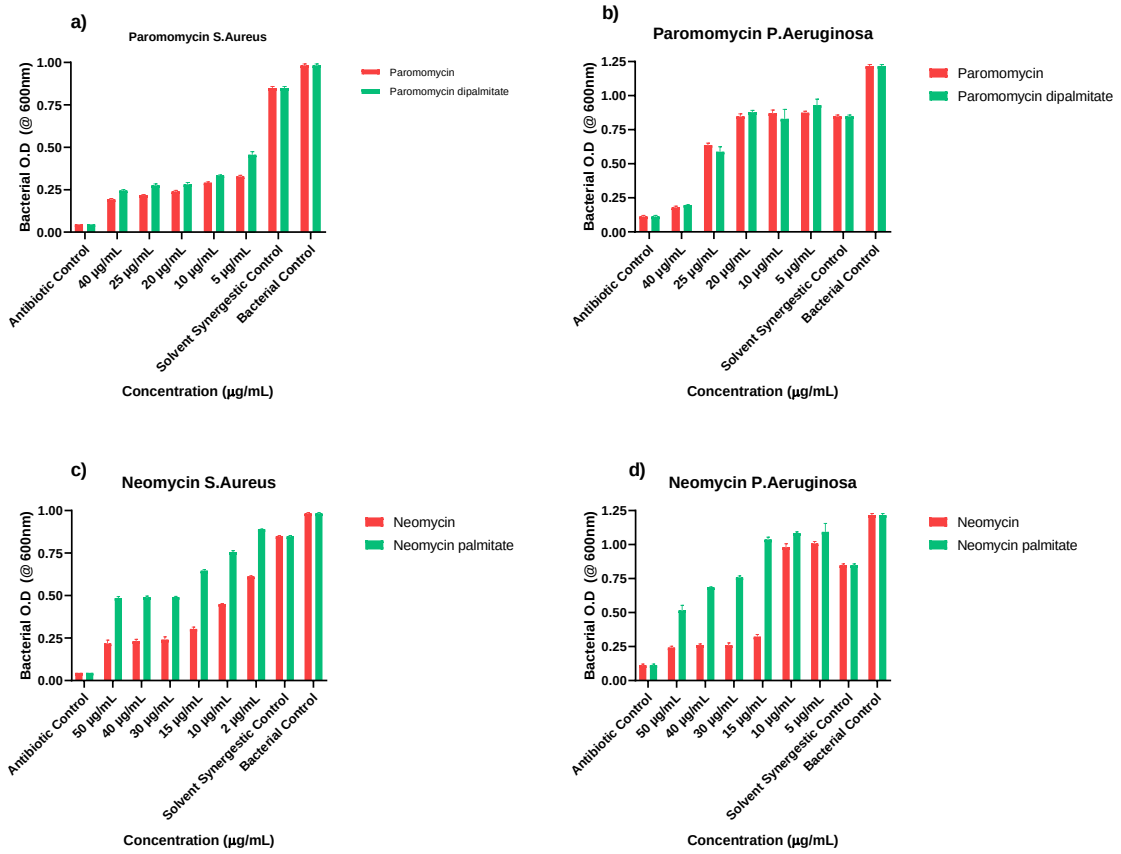


Figure 3.25: Antimicrobial susceptibility of two aminoglycoside controls and a lipid-derivative versus two strains of bacterium a) Paromomycin sulfate and paromomycin dipalmitate versus *S. aureus*, b) Paromomycin sulfate and paromomycin dipalmitate versus *P. aeruginosa*, c) Neomycin trisulfate and neomycin palmitate versus *S. aureus*, d) Neomycin trisulfate and neomycin palmitate versus *P. aeruginosa*. A range of concentrations were compared versus a streptomycin positive control and a bacterial negative control

For paromomycin against *S. aureus*, the lipid derivative showed lower activity at all concentrations, with a noticeable trend of lower concentrations having a greater loss in activity between the paromomycin and the dipalmitate. The concentration range beginning at 100 µg/mL with 12 intervals to 1 µg/mL showed a scaling decrease in lipid activity, with the lowest deviation in the top 25% of concentrations (5.2% decrease), followed by a 9.73% decrease in activity in the second quartile, 12.74% in the third quartile, and 19.39%

in the bottom 25% of concentrations. The IC_{50} values for paromomycin is 5 µg/mL, which matches up well with established literature for paromomycin's IC_{50} against *S. aureus* and *E. coli* being 3 µg/mL(144). The IC_{50} for the dipalmitate is 15 µg/mL, which immediately brings to mind, the issue of potential cytotoxicity in mammalian cells. As mentioned extensively, the toxicity issues plaguing aminoglycosides are exasperated when there is a five-fold increase in minimum inhibitory concentration. Studies investigating the ribosomal inhibition between protozoan ribosomes and mammalian ribosomes have shown a ten-fold increase in misreading frequency in *L. mexicana* ribosomes when compared against rat liver ribosomes(90). The scaling decrease in antimicrobial activity with the lipid derivative is of interest when analysing the efficacy between the two drugs, as this is another variable acting upon the data. One likely hypothesis is that, with the decrease in concentration of antibiotic, the synergistic effect of the lipid chain starts having an inverse effect on bacterial growth. The idea is that, as the potency decreases below IC_{50} concentrations, the lipid chains will act as a secondary nutrition source for the bacteria. Proving this hypothesis would require a range of lipid-derivatives to see the interactions of bacterial growth when subjected to shorter fatty acid chains (FAC) (C_{10}, C_{12}, C_{14}) and longer fatty acid chains (C_{18}). Overall, the reduced activity induced by the lipid-derivative shows a basis for modification of the primary alcohols on paromomycin without major breakdown of antimicrobial activity.

For paromomycin against *P. aeruginosa,* a similar trend can be observed between the base antibiotic and the lipid-derivative; however, inconsistencies can be seen between the concentrations, especially at the lower concentrations. Phenotypic discrepancies observed during testing showed increased pyocyanin production, a blue pigment secreted *P.*

aeruginosa, pyoverdine a green pigment is also secreted by *P. aeruginosa;* however, it was observed that levels of pyocyanin were higher after introduction of antibiotic. As there is very little literature on the interactions between aminoglycosides and *P. aeruginosa,* one theory is the interactions of pyocyanin and cell surface aggregation. Increased pyocyanin production influences the binding of extracellular DNA binding to cells, affecting hydrophobicity and physico-chemical interactions thus promoting increased cell-aggregation(145,146). Increased aggregation of bacterial cells in a bacterial susceptibility test can pose issues with antibiotic's accessing the target cell. Additional assays focussed around biofilm disruption may be of great benefit to see the synergistic effect of pyocyanin and aminoglycosides.

Neomycin, which differs from paromomycin by having an amino group at the C6 position of ring 1 (glucosamine), whereas paromomycin has a hydroxyl group. As mentioned in previous literature, this single functional group change rendered neomycin unable to penetrate leishmanial cells but still retained activity against leishmanial ribosomes(86). Neomycin showed overall lower activity with a similar scaling decrease in antimicrobial activity, although the quarterly decrease wasn't as exasperated as seen in paromomycin, with the first through fourth quarters being: 26.72%; 28.31%, 27.83%, 32.74% respectively. The IC_{50}'s for neomycin and neomycin palmitate were 4 µg/mL and 40 µg/mL. Following on the lower scaling decrease, 8.02% difference between bottom and top, versus paromomycin's 14.19%. The same hypothesis of the synergistic effect of the lipid chain applies to neomycin but with a potentially lower proportionality as neomycin has one primary alcohol with a bound FAC on C5 of ring 3, whereas paromomycin contains two, one on C6 of ring 1, and C5 of ring 3. Similar observations on neomycin

against *P. aeruginosa* were made, following on the biofilm aggregation, making drug uptake difficult.

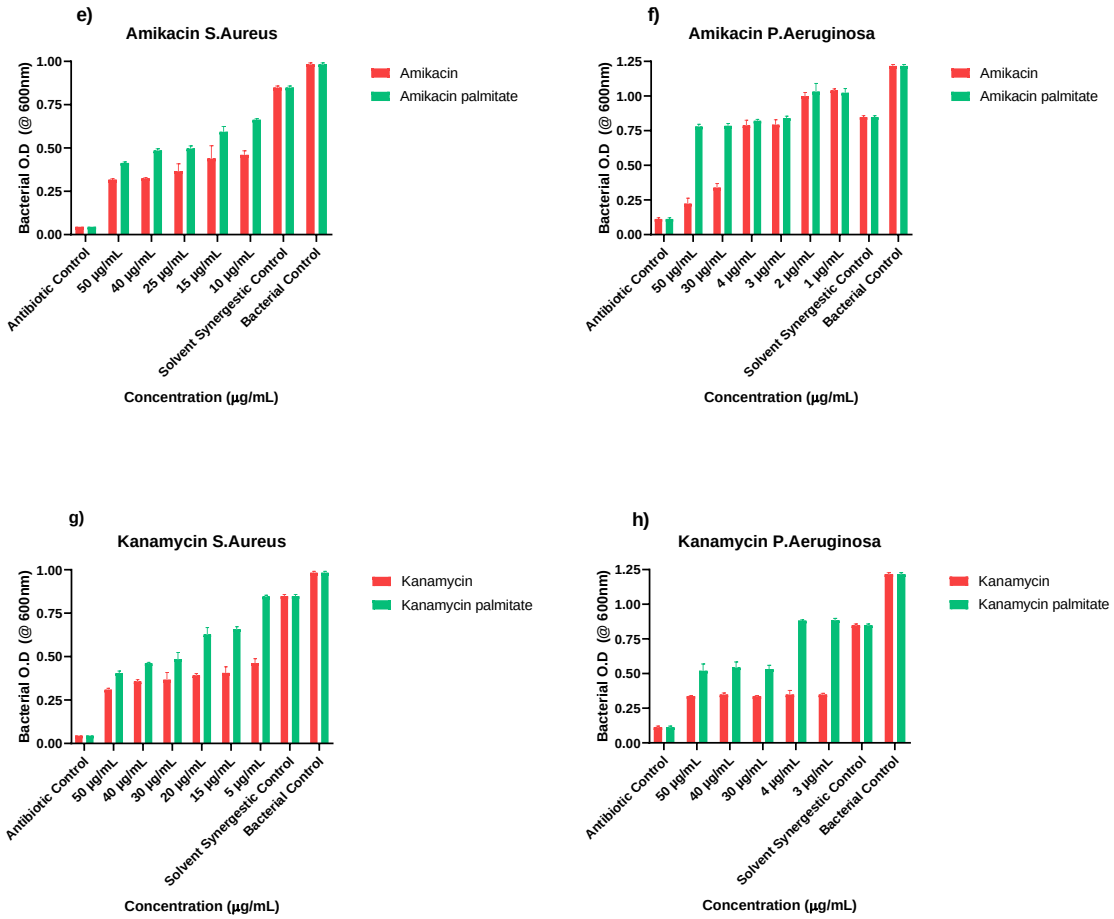


Figure 3.26: Antimicrobial susceptibility of two aminoglycoside controls and a lipid-derivative versus two strains of bacterium d) Amikacin hydrate and amikacin palmitate versus *S. aureus*, e) Amikacin hydrate and amikacin palmitate versus *P. aeruginosa*, g) Kanamycin sulfate and kanamycin palmitate versus *S. aureus*, h) Kanamycin sulfate and kanamycin palmitate versus *P. aeruginosa*. A range of concentrations were compared versus a streptomycin positive control and a bacterial negative control

Amikacin and kanamycin against *S. aureus* showed consistent activity over the range of concentrations with amikacin showing an average drop in activity of 17.50% over the quartiles, while kanamycin showed the greatest loss in activity averaging at 31.75%

between the top and bottom quartiles. The IC_{50} values for amikacin, amikacin palmitate, kanamycin, and kanamycin palmitate were: 11 µg/mL, 26 µg/mL; 30 µg/mL, and >50 µg/mL respectively. Established literature on the two aminoglycosides report 8 µg/mL for amikacin and >15 µg/mL for kanamycin against *S. aureus*(147,148). Paromomycin and amikacin show the most stability when comparing base antibiotic to palmitic-derivative, with kanamycin showing the largest drop-off in antimicrobial activity. As for the drugs against *P. aeruginosa*, the previously outlined discrepancies were observed in this MIC assay, weighing toward the biofilm aggregation mentioned previously.

Table 3: MIC Breakdown of the four aminoglycosides: paromomycin, neomycin, amikacin, kanamycin; and their palmitate derivatives. Indicating IC_{50}'s and the average activity decrease between the base antibiotic and its respective derivative.

Antibiotic/Test compound	***S. aureus*** **ATCC 25923**	***S. aureus*** **Activity Decrease**	***P. aeruginosa*** **ATCC 27853**	***P. aeruginosa*** **Activity Decrease**
Paromomycin	5 µg/mL	14.19%	>50 µg/mL	ns
Paromomycin dipalmitate	15 µg/mL		>50 µg/mL	
Neomycin	4 µg/mL	28.25%	>50 µg/mL	ns
Neomycin palmitate	40 µg/mL		>50 µg/mL	
Amikacin	11 µg/mL	17.50%	>50 µg/mL	ns
Amikacin palmitate	26 µg/mL		>50 µg/mL	
Kanamycin	30 µg/mL	>30.00%	>50 µg/mL	ns
Kanamycin palmitate	>50 µg/mL		>50 µg/mL	

Further testing was done by collaborators in the V.Avery group at GRIDD (Griffith Institute for Drug Discovery, Nathan Australia). The testing was done using a high content imaging (HCI) assay on axenic amastigotes of *L. donovani*. This axenic amastigote model provides the best mimicry of intracellular models showing a 43.2% hit rate using a database containing known compounds that are cytotoxic to intracellular amastigotes (149,150). Previous assays were based around promastigote models, showing only 4% hit confirmations using the same compound database(151,152). This assay is complemented by a THP-1 (human monocyte cell line) cytotoxicity assay. The main compounds tested were paromomycin, paromomycin dipalmitate, *N*-Boc DSA, neomycin palmitate, and established anti-leishmanial compounds as controls. The reported inhibition of paromomycin and paromomycin dipalmitate were 55% at top dose of 160µM, and <50% at 80µM. The literature IC_{50} of paromomycin against *L. donovani* DD8 intracellular amastigotes is 161.9 µM (153). The dipalmitate showed lower inhibition than the base aminoglycoside, with paromomycin showing lower inhibition than reported values. Studies on the variations in clinical responses of different drugs in diverse geographical settings has been well documented for a multitude of leishmanial treatments. It was found that there were significant variations in drug susceptibility among strains of the same species, and geographical variations among the same strains(60,154,155). Further testing on more geographically relevant strains may yield different susceptibility to *L. donovani* DD8 amastigotes.

Table 4: Resazurin based assay on *L. donovani* DD8 using 4 reference compounds, and 3 established control compounds. The intracellular amastigote assay is completed by a THP-1 cytotoxicity assay for human monocytes.

Antibiotic/Test compound	***L. donovani* R1 (IC_{50})**	***L. donovani* R2 (IC_{50})**	***THP-1* (1) (IC_{50})**	***THP-1* (2) (IC_{50})**
Paromomycin	55% at 160µM	56% at 160µM	<50% at 160µM	<50% at 160µM
Paromomycin dipalmitate	<50% at 80µM	<50% at 80µM	<50% at 80µM	<50% at 80µM
Decanesulfonylacetamide	<50% at 160µM	<50% at 160µM	<50% at 160µM	<50% at 160µM
Neomycin palmitate	<50% at 17.7µM	<50% at 17.7µM	<50% at 17.7µM	<50% at 17.7µM
Amphotericin-B	0.073	0.072	0.902	0.994
VL-2098	0.701	0.836	<50% at 40µM	<50% at 40µM
DNDI-1044	0.340	0.284	<50% at 40µM	<50% at 40µM

3.4 Conclusions and Future Remarks

Synthesis work on the aminoglycoside paromomycin, using esterification chemistry to make LCFA-based derivatives, in addition to DSA derivatives based off antitubercular work done by Jones et al. (142). These compounds have been tested for their antimicrobial activity; using *S. aureus*, *P. aeruginosa*, and *L. donovani* DD8 axenic amastigotes. Results found reduction in activity for the derivatives across all concentrations, with the dipalmitate paromomycin derivative retaining significant activity against *S. aureus*. For the testing against *L .donovani*, significant reduction in activity across all compounds, with pathophysiological differences due to geographical variance in strains potentially having a role in the activity. *P. aeruginosa* showed inconclusive results, with massive reduction in activity across all compounds and concentrations. Phenotypic anomalies noticed during testing showed significant increase in pyocyanin expression after incubation, increasing potential biofilm aggregation of the cells as a result.

Exploration into different geographical strains to test drug efficacy is important as outlined in *Table* 1, even current therapeutics such as Sb(V), miltefosine, and paromomycin have huge variations in efficacy across continents. Further exploration incorporating these aminoglycosides and DSA derivatives into liposomes may yield a range of benefits to these compounds; such as, increased efficacy via better biodistribution and uptake from intracellular targeting, protection from biological processes such as extracellular lipase interactions to the LCFAs, and lower doses for drug administration to lower overall cell cytotoxicity. This research is currently underway for a range of aminoglycosides and for the DSA derivative, with a variant of LCFAs also being tested for the aminoglycosides.

Optimising the reference aminoglycoside with LCFAs and liposomal conditions can prove beneficial in improving efficacy, cytotoxicity and effective biodistribution.

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