Synthesis and antileishmanial activity of novel benzothiadiazine-1,1-dioxide derivatives

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PREFACE

This dissertation is submitted in article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University.

An article in the form of a manuscript is included in this dissertation:

Chapter 3: Submitted article

Synthesis and *in vitro* antileishmanial efficacy of novel benzothiadiazine-1,1-dioxide derivatives

The article was submitted to the *Archiv der Pharmazie* - Chemistry Life Sciences journal. The manuscript was prepared using the author's instructions obtained and available in the Author Information pack on the journal's homepage (Annexure B): https://onlinelibrary.wiley.com/pb-assets/assets/15214184/ArchPharm_Guidelines_2020-1583143819340.pdf

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ABSTRACT

Leishmaniasis claims thousands of lives of the human populace every year. Globally, it infects approximately 12-15 million people annually and estimations have indicated that approximately more than 350 million people are at high risk of being infected (Dubie & Mohammed, 2020:12). Most cases occur in poverty-stricken tropical regions where poor water, adequate sanitation and hygiene (WASH) remain a challenge. Furthermore, it leads to an estimated 2.4 million disability adjusted life years (DALYs) (Charlton *et al*., 2018:219-236) which influence the increases in depression, anxiety and decreases in the overall quality of life (Tamiru *et al*., 2019:855).

Currently, pentavalent antimonials, amphotericin B, miltefosine, pentamidine and paromomycin are the only drugs available for antileishmanial chemotherapy, since no vaccine has been developed yet. However, the emergence of resistance of several *Leishmania* species, high costs associated with parenteral administration, and adverse side effects have deemed these medicines less effective, which has led to poor patient compliance. Therefore, this highlights the need for more research studies into the development of novel oral antileishmanial agents.

Leishmania parasites successfully colonise human cells through the manipulation of carbonic anhydrase enzymes. These enzymes monitor blood pH through a reversible reaction that increases proton and carbon dioxide concentrations. Carbonic anhydrase enzyme is vital in the *Leishmania* life cycle because it prevents the creation of an acidic environment, thus inhibiting hydrolytic degradation processes initiated by macrophages. Conversely, hydrochlorothiazide is an orally administered anti-diuretic drug that is classified as a benzothiadiazine-1,1-dioxide derivative. It confers potent antileishmanial activity through the inhibition of carbonic anhydrase, suggesting that benzothiadiazine-1,1-dioxide derivatives are not easily degraded by an acidic environment such as the one in the gastrointestinal tract.

As a contribution to leishmaniasis research, this study reports a simple two-step method for the synthesis of benzothiadiazine-1,1-dioxide derivatives via a nucleophilic substitution reaction. All synthesized compounds followed Lipinski's rule of 5, i.e. molecular weight, lipophilicity, hydrogen donating and accepting potential and aqueous solubility, as predicted on SwissADME, http://www.swissadme.ch. Their druglikeness is increased due to enhanced aqueous solubility, which is indicative of potential increased of bioavailability and gastrointestinal tract absorption. They were screened *in vitro* against promastigotes of *Leishmania donovani* strains (1S and 9515) and an *L. major* strain (IR-173) for antileishmanial activity determination. The synthesized compounds did not outperform the standard drug Amphotericin B (AmB). However, in general, they were nontoxic on Vero cells and possessed antileishmanial activities with inhibitory

concentration IC_{50} values ranging from 7 to 74 µM against 1S; 12 to >100 µM against 9515, and 0.20 to 3.5 µM against IR-173 promastigotes.

Compounds **1**, **2b**, **2c** and **2d** were the most potent against *L. donovani* 1S, with activity IC_{50} < 10 µM and selectivity index, SI > 10. Thus, they were identified as visceral leishmaniasis (VL) antipromastigote hits, which encourages their further intracellular amastigote screening. On the other hand, compounds **2**, **2c** and **2d** possessed nanomolar activities *L. major* parasite with associated selectivity indices SI > 100. Therefore, they could be identified as anti-cutaneous leishmaniasis (CL) leads that need to be confirmed in amastigote screening of *L. major* parasite. Compounds **1** and **2** with IC_{50}= 0.2 and 6.5 µM, respectively, stand as anti-promastigote hits which encourages more studies focusing on their development as new antileishmanial agents.

Keywords: *Leishmaniasis, carbonic anhydrase, benzothiadiazine-1,1-dioxides, oral antileishmanial agents.*

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LIST OF ABBREVIATIONS

µM	Micromolar
ADME	Absorption, Distribution, Metabolism and Excretion
AmB	Amphotericin B
ATP	Adenosine Triphosphate
ATQ	Atovaquone
AZA	Acetazolamide
Br	Bromine
CA	Carbonic Anhydrases
CO_2	Carbon dioxide
CDC	Centre for Disease Control
Cl	Chlorine
CL	Cutaneous leishmaniasis
CRT	Cluster Randomised Trial
DAT	Direct Agglutination Tests
D-AMB	Deoxylate-Amphotericin B
DCL	Diffused cutaneous leishmaniasis
DDT	Dichlorophenyl trichloroethane
DNA	Deoxyribonucleic Acid
DHFR	Dihydrofolate Reductase
DHFS	Dihydrofolate Synthetase
ELISA	Enzyme-Linked Immunosorbent Assay
EWG	Electron-Withdrawing Group
EZA	Ethoxzolamide
FA	Folic Acid
F	Fluorine
Gp63	Glycoprotein 63
GDP	Glucose diphosphate-pyrophosphorylase
H^+	Hydrogen ion
H	Hydrogen
IC_{50}	Inhibitory concentration
hCA	Human Carbonic Anhydrase
HCT	Hydrochlorothiazide

HCO^{-}_{3}	Bicarbonate ion
HIV	Human immune Virus
$HO^{\bullet}$	Hydroxyl radical
$HOCl^{-}$	Hypochlorite ion
H_2O_2	Hydrogen peroxide
IFAT	Immunofluorescence Antibody Tests
IM	Intramuscular
iNOS2	Inducible Nitric Oxide Synthetase 2
IR	Indoor residual
ITNs	Insecticide-Treated Nets
IV	Intravenous
K^{+}	Potassium ion
K_i	Inhibition constant
kDNA	kinetoplast DNA
L-AMB	Lipid-encapsulated Amphotericin B
LCL	Localised cutaneous leishmaniasis
mm	Millimetre
MCL	Mucocutaneous leishmaniasis
N	Nitrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NOS	Nitrogen Oxygen species
NTD	Neglected Tropical Disease
NW	New World
O_2^{-}	Superoxide anions
OW	Old World
$ONOO^{-}$	Peroxynitrite
PCR	Polymerase Chain Reaction
POP	Persistent Organic Pollutant
PQ	Primaquine
PV	Parasitophorous vacuoles
ROS	Reactive Oxygen Species
S	Sulphur

SAR	Structural Activity Relationship
Sb^{5+}	Pentavalent antimony
SO_2	Sulfonyl
spp.	Species
$t_{1/2}$	Half-life
TB	Tuberculosis
T_h	T helper
TR	Trypanothione Reductase
var.	*Variant*
V-ATPase	Vacuolar-type Adenosine Triphosphatase
VL	Visceral leishmaniasis
WASH	Water, Adequate Sanitation and Hygiene
WHO	World Health Organisation
Y/N	Yes/No

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Background

Leishmania parasite is the causative agent for leishmaniasis, classified in the family *Trypanosomatidae*, order *Kinetoplastida* (Van der Auwera & Dujardin, 2015:266). It is transmitted through the bite of female sand-flies of the *Psychodidae* family (Patino *et al.,*2017:2). Sand-flies of the genera *Phlebotomus* and *Lutzomyia* spread Old World (OW) and New World (NW) leishmaniasis to countries in the Eastern Hemisphere and Western Hemisphere, respectively (Kevric *et al.*, 2015:579).

Leishmaniasis is a poverty-related neglected tropical disease (NTD) (Esteves *et al.*, 2018:195) affecting more than 90 countries (WHO, 2012). It is endemic to Middle East, Mediterranean, Central-South American, African, Asian, and Southern European regions (Figure 1-1) (Thakur *et al.,* 2018:2). OW leishmaniasis is highly prevalent and endemic in the three latter regions (Kevric et al., 2015:579). Annually, leishmaniasis is responsible for 0.7–1 million new infections (Burza *et al.,* 2018:951), 50, 000 deaths and 3.3 million disability-adjusted life years (DALYs) (Gillespie *et al.,* 2016:2992). In addition, it has been estimated that more than 350 million of people in endemic regions are at risk of more than one form of infection (Ortalli *et al.,* 2019:126). This is attributed to the presence of more than 20 *Leishmania* (*L.*) species (spp.) that can infect humans (Thakur *et al*., 2018:2).

Leishmania donovani (OW)*, L. major* (OW) and *L. vianna* subgenus (NW) are among spp*.* responsible for causing the three main clinical manifestations, namely visceral (VL), cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, respectively (Piscopo and Azzopardi, 2006:650). CL and MCL parasites replicate within cutaneous and mucocutaneous tissues, respectively, while VL parasites spread through the bloodstream to the internal organs (Esteves *et al.,* 2018:197). Leishmaniasis infects persons of all ages and genders (Gupta *et al.,* 2017:215). However, its spread is limited by factors relating to the geographical distribution of species and their reservoirs (Van der Auwera & Dujardin, 2015:266) and host immunity (Kafetzis, 2003).

CL is the most dominant while VL is the most virulent (PAHO, 2017), and they are the most prevalent forms of leishmanial infection (orange in Figure 1-1) (Esteves *et al.,* 2018:196). North America, South America and European countries have low incidences of both infections, in comparison to West African and Asian regions. Brazil is a home to all three clinical forms of infections (maroon in Figure 1-1) (Esteves *et al.,* 2018:196). In endemic regions, 50% of VL cases

may occur in children (Prestes-Carneiro *et al.,* 2019:2), whereas only one-sixth of CL infections (48 915 cases) have been reported for those in the Americas (Uribe-Restrepo *et al*., 2018:2). The virulence and pathogenicity of *Leishmania spp.* is attributed to the coinfection of leishmaniasis with immune system-targeting infections such as Human Immune Virus (HIV), which deplete host innate defences against opportunistic pathogens (Galgamuwa *et al.,* 2018:2) such as the tuberculosis (TB)-causing *Mycobacterium tuberculosis* (Van Griensven *et al.,* 2018:1).

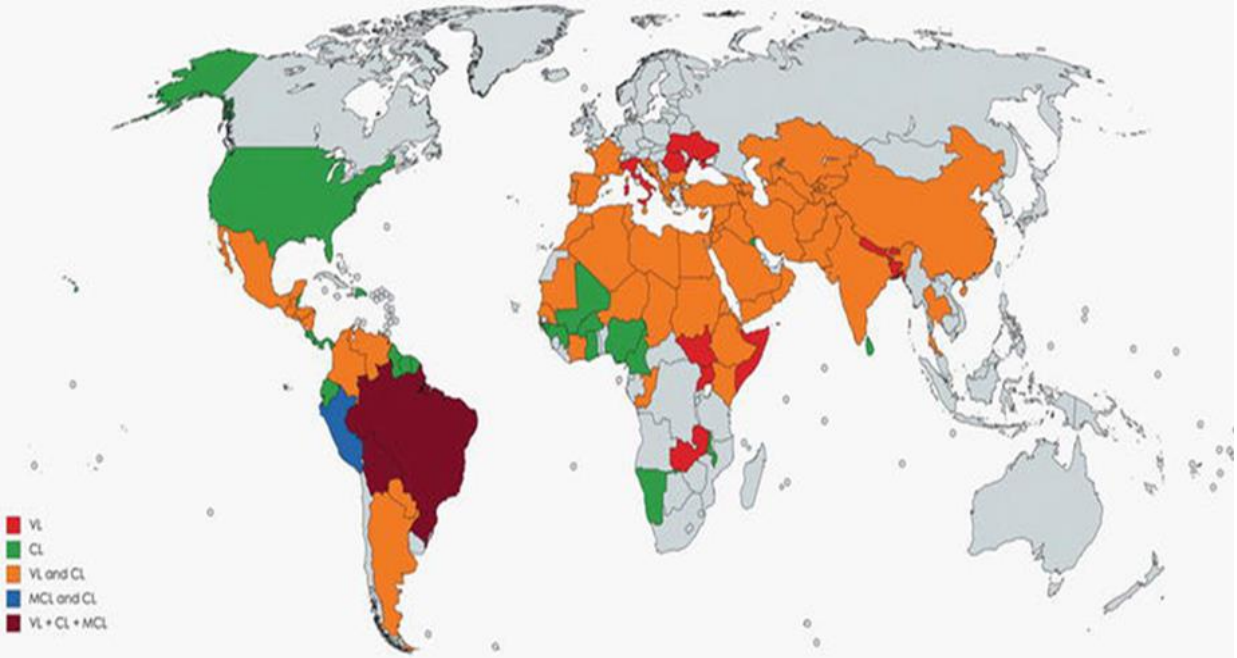


Figure 1-1: The geographic distribution of human VL,CL and MCL (Esteves *et al.,* 2018:196).

There are various efforts already in place aimed at eliminating leishmaniasis, in both the sand-fly vector and the mammalian host. Vector control is the usage of dichlorophenyl trichloroethane (DDT), indoor residual (IR) and insecticidal sprays, or insecticide-treated nets, to prevent human-sand-fly interactions (Gillespie *et al.,* 2016:2293). On the other hand, antileishmanial chemotherapeutic treatment inhibits the replication of parasites in host immune cells (Haldar *et al*., 2011:2). Currently, clinical drugs for leishmanial treatment include pentavalent antimonials, amphotericin B, miltefosine, paromomycin and pentamidine. However, factors such as toxicity, severe side effects, development of drug resistance by *Leishmania* parasites (Kapil *et al.,* 2018:343), prolonged hospitalisation and high costs, limit the effectiveness of these drugs (Akbari *et al.,* 2017:87).

Therefore, the search for new antileishmanial drugs continues including intense studies on the quinazolinones (Figure 1-2). Quinazolinones are *N*-containing heterocyclic compound

pharmacologically active against cancer (Wani *et al.,* 2016:2), inflammation (Rakesh *et al.,* 2016:1072), diabetes (Wei *et al.,* 2017:1303), microbial infections (Dinari *et al.,* 2018:43) and leishmaniasis (Tiwary *et al.,* 2015:3). *In vitro* studies on *L. donovani* promastigotes by Birhan *et al*., (2014) identified 2,3-disubstituted-4(*3H*)-quinazolinone derivative, compound **1 (**Figure 1-2) as a potent anti-leishmanial agent, with an IC_{50} = 12.80 ng/ml, which is 250 times higher than that of miltefosine (Birhan *et.al.,* 2014:3). An interesting property about the heterocyclic quinazolinone moiety is its ability to facilitate salt formation and increase compound solubility that increases drug absorption within cells and bioavailability upon oral administration (Khan *et al.,* 2014:193).

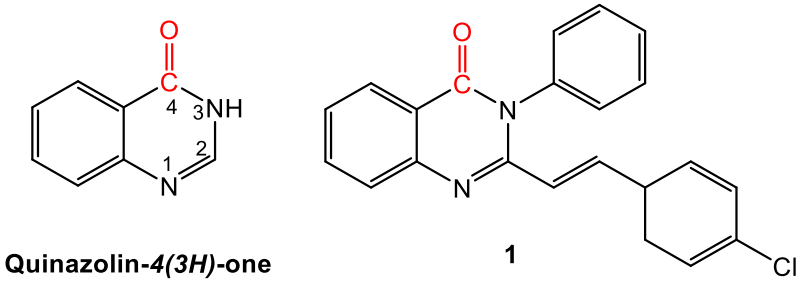
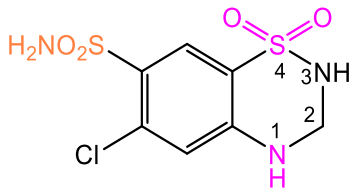


Figure 1-2: Molecular structures of quinazolin-4*(3H*)-one and its 2,3-disubstituted derivative.

Benzothiadiazine-1,1-dioxides (Figure 1-3) are quinazolinone analogues obtained when the cyclic carbonyl group (-C=O) group at position 4 of the quinazolinone moiety (Figure 1-2) is replaced with a sulfonyl group (-SO_2) (Cohen *et al.,* 1960:2731). Benzothiadiazine-1,1- dioxides are classified as sulphonamides due to the presence of the A-SO_2NHR functional group, where R can be an aromatic, heterocyclic or aliphatic group (Supuran, 2017:1). They have anti-hypertensive (Gupta, 2018:1099), antifungal (Morrow, 2008:353), antibacterial (Hunsen, 2008:297) and diuretic (Cody, 2010:482-483) activity. Although low toxicity is a property associated with sulphonamides, over-consumption can cause long-term side effects such as constant urination and allergic reactions (He & Yan, 2018:1).



Hydrochlorothiazide, HCT

Figure 1-3: Molecular structure of HCT, a benzothiadiazine-sulfonamide.

Hydrochlorothiazide (HCT, Figure 1-3) is a diuretic sulfonamide drug (Herman and Bashir, 2019) with potent *in vitro* activity against *L. donovani chagasi* promastigotes (Syrjänen *et al*., 2013:7372). HCT is a strong inhibitor for the *Leishmania* carbonic anhydrase (CA) enzyme with an inhibition constant (K_i) of 50.3 nM. *Leishmania* parasites rely on CA for resistance against an acid produced by host immune cells as part of their anti-parasitic tactics. Furthermore, the inhibition of human CA (hCA) by HCT was studied using recombinant isozymes, hCA I and II, and results indicated HCT as a poor hCA I and II inhibitor with inhibition constant (K_i) values, K_i = 328 nM and K_i=290 nM, respectively Therefore, given characteristics of quinazolinones and the mechanism of action by sulphonamides against *Leishmania,* drug development based on these two scaffolds is worth exploring.

1.2 Aims and objectives of the study

The aim of this study is to investigate novel benzothiadiazine-1,1-dioxide derivatives as anti-leishmanial agents.

The objectives of this study are:

- To synthesise novel 2,3-disubstituted-benzothiadiazine-1,1-dioxide derivatives.
- To assess *in vitro* anti-leishmanial activity of the synthesised compounds against *L. donovani* and *L. major* strains.
- To assess cytotoxicity profiles of synthesised compounds in Vero cells.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Leishmaniasis is an intracellular, protozoal, vector-borne infectious disease that has caused approximately 20 000 to 30 000 annual deaths (PAHO, 2017) in 90 regions of tropical and subtropical countries (Akbari *et al*., 2017: 86; Molyneux *et al*., 2017:312). It causes three main clinical forms, namely visceral (VL), cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, all of which have different global distributions (Piscopo and Azzopardi, 2006:650). More than 90% of new VL cases occur every year in India, Nepal, Sudan, Bangladesh and Brazil (Oliveira, *et al.,* 2018:1). About 95% of annual CL cases are from the Americas, the Mediterranean basin, Middle East and Central Asia. MCL causes over 90% of infections in Bolivia, Brazil, Ethiopia and Peru (Esteves *et al.,* 2018:196-197).

Leishmaniasis is a neglected tropical disease (NTD) (Deribew *et al*., 2017:3) that has been recognized as an emerging and uncontrollable category-1 disease by the World Health Organisation (WHO) (Jain & Jain, 2015:1). It is mostly prevalent in poverty-stricken regions where limitations to water, basic sanitation and hygiene (WASH) (Boisson *et al*., 2016: 19) have been shown to exacerbate the transmission of vector-borne infections (Akbari *et al*., 2017:86; Molyneux *et al*., 2017:312). It does not come as a surprise that leishmaniasis continues to flourish and restrict itself to the poorest human populations, because it has not been prioritised or highlighted as a political- and research-worthy infectious disease on the agendas of many countries (Hailu *et al*., 2016: 142). However, globalisation has resulted in the spread of leishmaniasis to well-developed continents, such as Europe, forming new endemic locations. This has played a significant role in leishmanial research (Dujardin *et al*., 2008:1014) as endemism to more than one continent (Africa, Europe, Asia) serves as a reason to shed more light upon this disease (WHO, 2019b). Across the globe, poor or the lack of leishmanial surveillance and control have resulted in slow antileishmanial pharmaceutical developments (Dujardin *et al*., 2008: 1016). However, this is bound to change because leishmaniasis is deemed a global public health threat as it is ranked next to malaria–a leading parasitic infectious disease in the world (Esteves *et al*., 2018:196).

The WHO has set a goal to reach mortality and morbidity rates of 0% for VL in 2030 (WHO, 2019a). A more realistic milestone towards achieving this goal could include the implementation of a holistic control approach to promote (i) patient compliance and (ii) the use of clinically- and cost-effective medicines (Sundar *et al*., 2014 :1-3). Current anti-leishmaniasis medicines are

becoming less effective because of the emergence of resistance by several *Leishmania* species (*spp.)* (Bouyahya, *et al*., 2018:51). These medications are also expensive, associated with side effects (Hendrickx *et al*., 2019:2743-2744), and only 1 (miltefosine) out of 5 clinically approved medicines is orally administered (Sunyoto *et al*., 2018:2). Other medications require parenteral administration which ultimately increase hospital and patient expenses. Therefore, this highlights the need for more research studies with aims to develop novel oral antileishmanial medicines.

To follow this introductory section are detailed discussions of the leishmanial life cycle, pathogenesis and pathophysiology. A variety of diagnostic, control and treatment methods are outlined and the development of antileishmanial chemotherapeutic agents using Schiff-bases is discussed.

2.2 Life cycle

A female phlebotomine sand-fly bites a human host on exposed areas of the skin (e.g. arms, legs, neck and face), creating an entry portal for *Leishmania* parasites (Figure 2-1, step 1) into the human body if the sand-fly is infected (Kevric *et al*., 2015: 580). *Leishmania* parasites have two morphologically distinct forms namely, flagellated, motile promastigotes (infective form) and non-flagellated amastigotes (clinical form). Infected sand flies inject promastigotes through their proboscis as they feed on the host's blood (Figure 2-2) (Maspi *et al*., 2016: 248).

An immediate response of skin-based macrophages is to accumulate at the injured site where they recognise and engulf promastigotes (Sunter & Gull, 2017:3; Lavoie & Levy, 2017:1208). Promastigotes also use their receptor-enriched flagella to quickly initiate signalling events leading to their engulfment (Figure 2-1) (Sunter & Gull, 2017:3). They are contained within parasitophorous vacuoles (PV), which fuse with lysosome membranes to form phagolysosomes and this triggers the transformation to amastigotes (McGwire & Satoskar, 2014:8; Sunter & Gull, 2017:3). This morphological change marks the beginning of the parasite's replication by binary fission (Kevric *et al*., 2015: 580). Amastigotes continue to use macrophage cellular contents for replication, then later burst out of cells for extracellular deliverance (Figure 2-1) (Sunter & Gull, 2017:3). Continuous engulfment of amastigotes by uninfected macrophages and macrophage-macrophage interactions spread the infection to other tissues in the body (Maspi *et al*., 2016:248; Mcgwire & Satoskar, 2014:8).

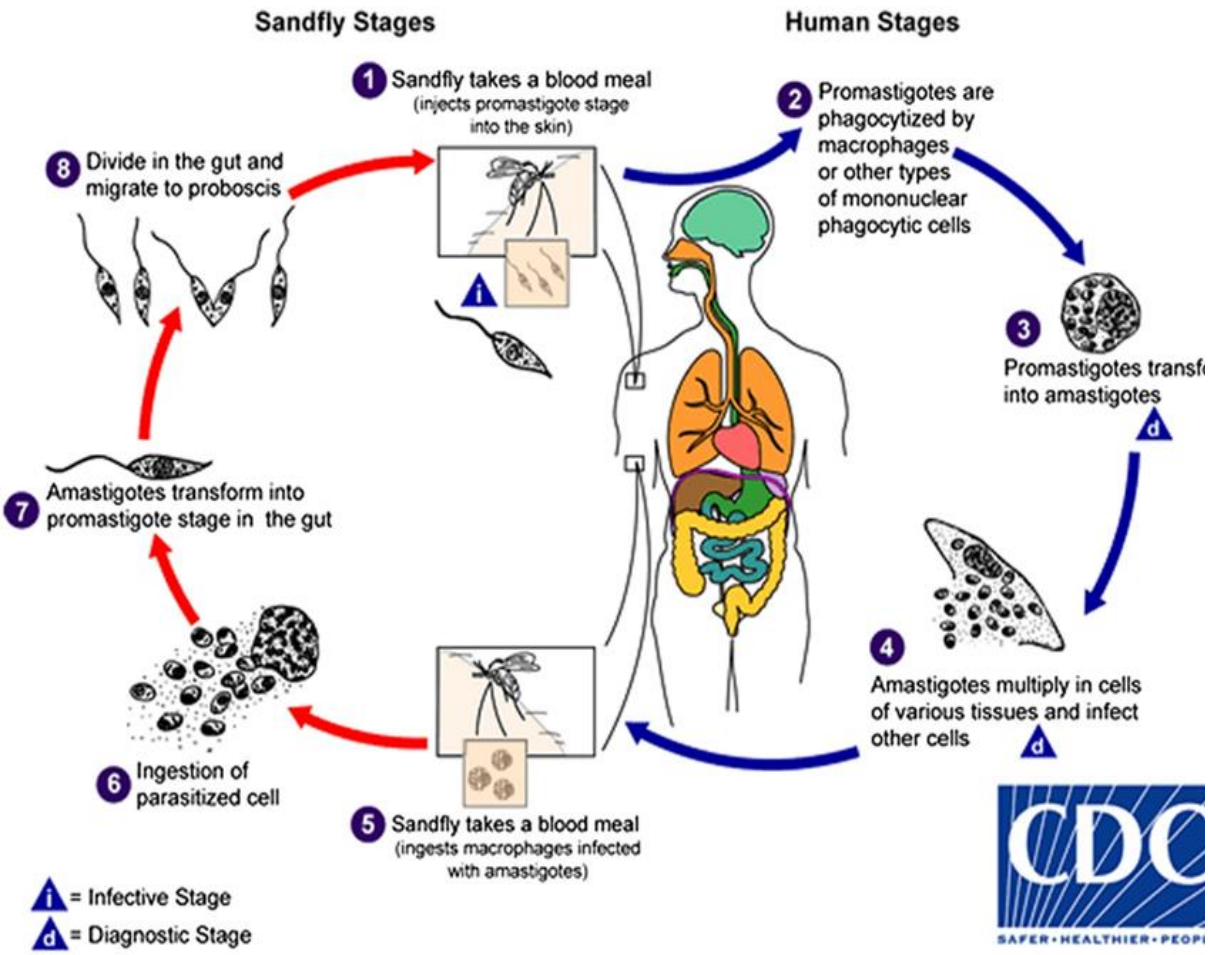


Figure 2-1: A diagram showing *Leishmania* life cycles (CDC, 2002). Step 1 represents an interaction between human and vector stages.

2.3 Pathogenesis

The human body has a set of immune cells, such as monocytes, macrophages and dendritic cells, that it uses for generating responses against invasion by foreign organisms (Lavoie & Levy, 2017:1208). Macrophages are of the utmost importance in the case of a *Leishmania* infection, because they (i) present *Leishmania* antigens to T helper cells (T_h) for immunologic memory by the adaptive immune system and (ii) engulf promastigotes into phagolysosomes for later degradation using hydrolytic enzymes.

The phagolysosome membrane has high levels of an enzymatic protein known as Vacuolar-type Adenosine Triphosphatase (V-ATPase) whose function is to translocate protons (H^+ ions) into the internal environment of the phagolysosome (Uribe-Querol & Rosales, 2017:4). This decreases

pH and activates the production of hydrolytic enzymes within the PV. Additionally, V-ATPase induces the production of superoxide anions (O_2^-) that react with H^+ ions to produce reactive oxygen species (ROS). Furthermore, macrophages produce nitrogen species (NOS), such as nitric oxide (NO), that, along with ROS molecules, are highly toxic (Almeida *et al*., 2012:492; Carneiro *et al*., 2016:2). The O_2^- molecules are used by macrophages to form hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^•$) and hypochlorite ($HOCl^–$) (Almeida *et al*., 2012:492). These products later serve as intermediates for a reaction with NO to produce peroxynitrite ($ONOO^–$), a metabolically active molecule that is highly toxic and acidic against *Leishmania* parasites. However, a toxic environment is not an effective mechanism against *Leishmania* parasites, because they have developed mechanisms to detoxify oxidants and suppress superoxide production by infected macrophages (Lodge & Descoteaux, 2006:2736). Consequently, parasites can thrive within the host, resulting in an active leishmaniasis infection. An active leishmanial infection can be any of the three main clinical manifestations of leishmanial infections, i.e. CL, MCL and VL, which are discussed in detail below.

2.3.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis occurs due to intensive amastigote replication that is restricted to skin-based macrophages. During a blood meal, sand flies inject promastigotes through blood vessels such that macrophages and other immunity cells, including phagocytic dendritic cells, become infected. This helps spread parasites to epithelial cells coating the skin, hence, ulcers and lesions appear on the skin surface (Figure 2-2) (Ayi, 2007:4; Marieb & Hoehn, 2013:151-153).

A CL infected area reddens and swells to form a papule that enlarges into a nodule (Linton, 2011:102). Papules are raised lesions of less than 10 mm in diameter, and they only form within upper skin layers. As parasite replication continues, the papule diameter increases to more than 10 mm, thus spreading the infection to subcutaneous tissues and transforming papules into nodules. There are two CL clinical representations, namely localised CL (LCL) and diffused CL (DCL) (Reithinger *et al*., 2007:581). LCL causative agents include *L. minor* and *L. major* and it occurs when replication is restricted to areas near the infected site (Figure 2-2) (Linton, 2011:102). DCL is commonly caused by *L. aethiopica*, *L. mexicana* and *L. amazonensis* (Cobo, 2014:237) and occurs when the infection disseminates to other parts of the body (Figure 2-2) (Bogitsh *et al*., 2013:99). The absence of secondary bacterial contamination could lead to CL ulcers and lesions healing within a year (Bogitsh *et al.,* 2013:99); however, disfiguring scars often remain (Cobo, 2014:237). Healing with or without medication does not completely clear parasites from patients as the deoxyribonucleic acid (DNA) remains detectable on scars (Scorza *et al*., 2017:1299).

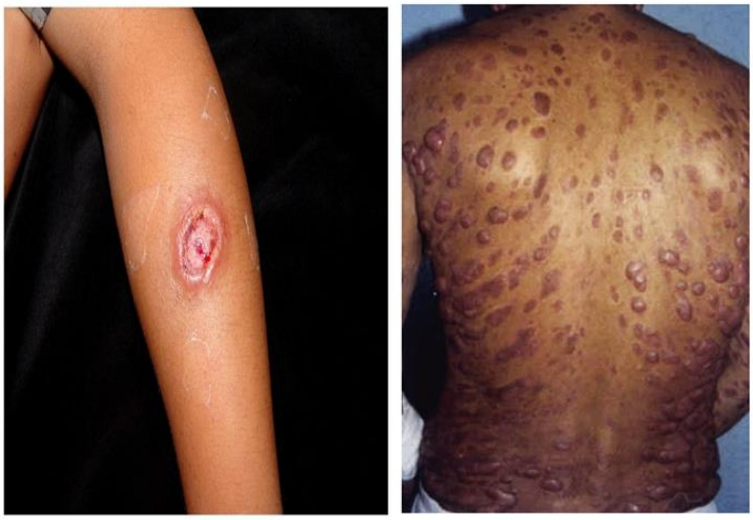


Figure 2-2: Images of patients with localised CL ulcer (left) and DCL (right)(Torres-Guerrero *et al*., 2017:8-9).

2.3.2 Mucocutaneous leishmaniasis

MCL, also known as espundia, occurs in 5-20% patients residing in CL endemic regions. It is often a complication of the first episode of untreated CL infection by *L. viannia* (also known as the *L. braziliensis complex*) (Piscopo & Azzopardi, 2006:651). A primary MCL infection occurs when amastigotes travel from the site of infection via lymphatic and blood vessels to mucous membranes in the respiratory tract (Ahluwalia *et al*., 2004:842), and the mouth (Piscopo & Azzopardi, 2006:651). They later extend to the larynx and pharynx where they continue with intra-macrophage replication. Non-self-healing ulcerative lesions in respiratory cutaneous tissues are observed months or a year later (Chappuis *et al*., 2007:7). Consequently, patients eat with difficulty (Piscopo & Azzopardi, 2006:651) and are prone to secondary bacterial and viral infections (Ayi, 2007:2-4).

2.3.3 Visceral leishmaniasis

VL occurs when *L. donovani, L. infantum* (Old World), and *L. chagasi* (New World), amastigotes disseminate to internal organs (Ayi, 2007:2-4). These parasites use macrophage-macrophage interactions to migrate from cutaneous and mucocutaneous tissues to reach the circulatory system. This spreads the infection to internal organs, such as the liver, spleen, bone marrow, lymph nodes of intestines, heart, lungs and the stomach. VL infections give parasites an opportunity to remain active without their clinical presence ever being observed through physical symptoms (Bi *et al*., 2018:1). However, a very small and unnoticeable papule may occur at the feeding site. First clinical symptoms are flu-like and include low-grade recurrent fever coughing (Davidson, 2005:44). They are followed by progressive weight loss and tissue enlargement, due to the accumulation of infected macrophages in the spleen, liver, intestine, bone marrow and lymph nodes. Other symptoms include severe anaemia, haemorrhagic manifestations, cardiac abnormalities, jaundice, and diarrhoea (Peixoto *et al*., 2015:334). Lesions in the lymphatic system, oral and nasopharyngeal tissues also occur (Buckner & Schwartz, 2017:510). Untreated VL infections increase the likelihood for chronic complications in patients and predispose them to secondary bacterial infections (Peixoto *et al*., 2015:334).

2.4 Diagnosis

Leishmaniasis and infections, such as malaria, schistosomiasis, leprosy and tuberculosis, usually present with similar clinical signs and symptoms (Reithinger & Dujardin, 2007:587). Therefore, more than one diagnostic tool (Table 2-1) is always used to allow clear a distinction between these infections. This section outlines a variety of diagnostic tools applicable for leishmanial diagnosis.

Table 2-1: Diagnosis of leishmaniasis.

Diagnostic technique	Parasite specific (Y/N)	Method	Target	Advantages	Disadvantage
Non-Leishmanin tests	No [1,2]	Hemogram test (assesses organ functionality) [2]	Haemoglobin, leucocyte and platelet counts, as well as erythrocyte sedimentation rates [3].	Vital for VL diagnosis [2]	Requires additional testing to confirm infection [1,2].
Leishmanin skin test	Yes [4]	Skin hypersensitivity test [4]	*Leishmania* antigens [5]	Faster diagnosis (48 hours) [5]	Negative result not always indicative absence of *Leishmania* antigens [2].
Parasitology	Yes [6,7]	Histopathology [6,7]	*Leishmania* cells in biological samples, e.g. skin scrapings and biopsies [6,7].	Vital for CL and MCL [6,7], affordable and accessible [10].	Sensitivity (53 to 99%) [8] dependent on affected organ [2], haemorrhage and death due to needle aspirates[2, 9].
		Cell culture	*Leishmania* cells from biopsy samples [11].	More informative [2]	Results affected by parasite dispersion and parasite load [10]. Slow and requires expertise.
Serology	Yes [12,2]	Direct agglutination tests (DAT), immunofluorescence antibody tests (IFAT), enzyme linked immunosorbent assay (ELISA) [11].	*Leishmania* antigens (ELISA) and anti-leishmanial antibodies (DAT, IFAT) in body fluids [11].	Sensitive DAT:91-100%, IFAT :55-70% [12] and ELISA:95% [2].	Expensive [13]
Molecular	Yes [11]	Polymerase chain reaction (PCR)	Non-species- and species-specific target leishmanial genes [14].	Sensitivity of 98% [1], and faster diagnosis [9].	Labour intensive [13]

[1] Buckner & Schwartz, 2017:510; [2] Cobo, 2014:233; [3] Mathur *et al.*, 2008:1170; [4] Krolewiecki *et al.*, 2017:2; [5] Antonio *et al.*, 2014:375; [6] Markle & Makhoul, 2004 :1457-1458; [7] Sharifi *et al.*, 2015:301; [8] Marlais *et al*., 2018:2; [9] Sundar, 2015; [10] Rashid, 2014:231; [11] Torres-Guerrero *et al*., 2017:10; [12] Sundar & Rai, 2002:954 & 956; [13] Tsokana *et al.*, 2014:164; [14] Galluzzi *et al*., 2018:1.

2.5 Prevention and control

Leishmaniasis prevention and control is currently achieved through vector control methods, since no human vaccine is available yet. This section outlines differences between the two strategies and gives an update on anti-leishmanial vaccine research.

2.5.1 Vector control

Vector control prevents the transmission of *Leishmania* spp. between sand-flies, animals (wild and domestic mammals) and human hosts (Van der Auwera, 2015:266). Sand flies choose their habitats based on social and environmental conditions that include under-developed housing (e.g. mud houses), closeness to water bodies, cattle farming, pollution, sewage leakages, deforestation and poor irrigation habits (Oryan & Akbari, 2016:925). Therefore, most widely used vector control measures include indoor residual (IR) and insecticidal sprays with dichlorodiphenyltrichloroethane (DDT), and insecticide-treated nets (ITNs) (Gillespie *et al*., 2016:2993).

DDT is usually sprayed on the walls of houses and animal shelters to control indoor and outdoor sand-flies (Coleman *et al*., 2015: 8573-8574). In a cluster randomised trial (CRT) performed in India, Bangladesh, and Nepal, DDT has effectively reduced the clustering of indoor sand flies by 72.4%. However, DDT, which is made from a combination of chemicals including malathion, deltamethrin, cyfluthrin, alpha-cypermethrin, and lambda-cyhalothrin, has been categorised as a persistent organic pollutant (POP). POPs can accumulate in human and wildlife to cause toxic side effects, hence the phasing out of DDT usage is being considered in India. In addition, a multi-country intervention study has shown that consistent usage of nets treated with an insecticide tablet, K-O TAB 1-2-3, reduced sand-fly densities by 60-80% (Chowdhury, *et al*., 2019:4. However, a CRT conducted in India and Nepal indicated that sand-fly density was not influenced by the usage of ITNs, which also suggested that sand flies were adapting to the outside environment.

2.5.2 Vaccines

Antileishmanial vaccine research is still an on-going process (Wittner & Tanowitz, 2000:196-197). Vaccines are important for preventing accidental transmissions by contact with samples in laboratories, and mother to child transmissions. Human vaccine clinical trials were recently conducted on healthy adult individuals in Washington, and second-generation vaccines namely, LEISH-F1 and LEISH-F3 passed the initial phase (Moafi *et al*., 2019:98). LEISH-F1 was effective

for treating and inducing protective immunity against CL or MCL, while LEISH-F3 induced immunity against VL parasites. However, newly developed vaccines are expensive, which limits their access to low- and middle-income endemic regions.

2.6 Antileishmanial chemotherapeutic drugs

Chemotherapeutic treatment is the usage of drugs to inhibit parasite replication in host cells (Haldar *et al*., 2011:2). Leishmaniasis could be fatal if left untreated, hence the reliance on chemotherapeutic drugs (Figure 2-3, Table 2-2). However, the emergence of drug resistance has led to few drugs being available to treat *Leishmania* infections (Ouellette *et al.*, 2004:262). Therefore, a variety of studies are aimed at developing novel antileishmanial agents. Currently, pentavalent antimonials are the first line of treatment (Haldar *et al*., 2011:2), while second-line drugs include Amphotericin B and pentamidine (Ouellette *et al.*, 2004:257). Alternative drugs include miltefosine (Branco *et al*., 2016:4) and paromomycin (Bhargava & Singh, 2012:5). In this section, details about their pharmacokinetics and pharmacodynamics are given (Harvey & Champe,2009:1-2). Pharmacokinetics discusses drug absorption, distribution, metabolism and excretion (ADME), while pharmacodynamics traces the drug's biologic effect through its interaction with cellular structures, such as enzymes and receptors. Attention is drawn to factors influencing treatment effectiveness, such as drug chemistry, dosage, formulation, affordability and duration.

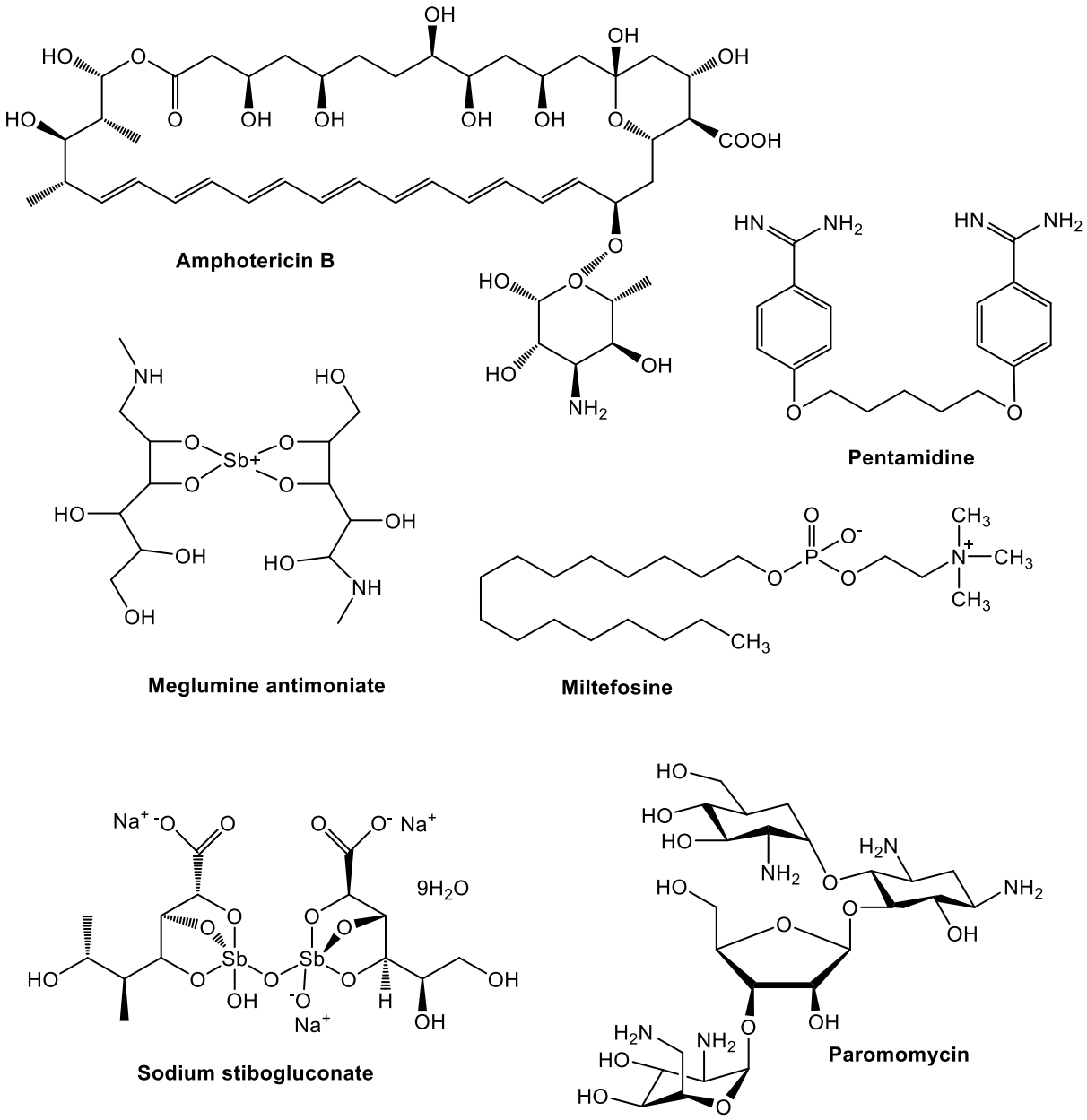


Figure 2-3: Molecular structures of antileishmanial drugs.

2.6.1 Pentavalent antimonials

Pentavalent antimonial drugs include meglumine antimonate and sodium stibogluconate which have been identified as gold standard antileishmanial drugs (Haldar *et al*., 2011:2-3). They are complexed drugs where antimony (Sb^{5+}) serves as the central atom. Sb^{5+} binds to sodium gluconate and *N*-methyl-D-glucantime to form sodium stibogluconate (Pentostam®, $C_{12}H_{38}O_{26}Sb$) and meglumine antimoniate (Glucantime®, $C_{14}H_{29}O_{10}N_2Sb$), respectively.

2.6.2 Amphotericin B

Amphotericin B (AMB) has two main formulations namely, deoxylate-AMB (D-AMB) and lipid-encapsulated AMB (L-AMB). AMB is used as a second-line treatment for VL, CL and MCL in regions with high levels of pentavalent antimonial resistance (Ouellette *et al.*, 2004:257&261), such as India (Croft & Olliaro, 2011:1479).

2.6.3 Miltefosine

Miltefosine is a highly stable, alkyl phospholipid-derived compound also called hexadecyl phosphocholine. Miltefosine has good activity against CL and VL, even though it was intentionally developed to treat cancer (Branco *et al*., 2016:4).

2.6.4 Paromomycin

Paromomycin is an antileishmanial active aminoglycoside antibiotic that is naturally obtained as a fermentation product of *Streptomyces rimosus var. paromomycinus* (Bhargava & Singh, 2012:5). It is a white, amorphous, water-soluble and stable drug (Wiwanitkit, 2012:325). It also has milder side effects, cost effective and reduces resistance in *Leishmania* spp. through combinational therapy (Sundar & Chakravarty, 2008:792).

Table 2-2: Properties of antileishmanial compounds.

Drug	Mechanism of action	Administration route	Dose interval and duration	Adverse effects	Cost effective (Y/N)
Pentavalent Antimonials	Inhibits DNA replication [1]. Stimulates ROS and NOS production by macrophages. Reduces Sb^{5+} to a highly toxic Sb^{3+} [2].	Intramuscular (IM) or intravenous (IV) [3]	Half-life ($t_{1/2}$) = 76 hours [9] for 20–30 days [1]	Cardiotoxicity, hepatotoxicity [4], musculoskeletal pain, headache, nausea, asthenia [5], and pancreatitis [6].	No [3]
AMB	Damages the cell membrane [7,8].	IM infusions [9]	Every 2 days for eight weeks [9]	Nephrotoxicity, fever, bone pain, hypotension, anorexia, cardiac arrest, nausea, headache [10,11].	No [11,12]
Miltefosine	Stimulates macrophage NO production by inducible nitric oxide synthetase 2 (iNOS2) [11].	Oral [13]	$t_{1/2}$ of 150 hours [14], for 28 days [13,15]	Permanent abnormal foetal growth and embryo death [16], nausea, diarrhoea, hepatotoxicity and renal insufficiency [10, 17].	No [18]
Pentamidine	Inhibits kinetoplast DNA (kDNA) and mitochondrial topoisomerase II [11,19]. Inhibits protein synthesis [20].	IV and IM [21]	$t_{1/2}$=6-9 hours [21]. 4 doses x7 days for CL, 15 doses x3 days for MCL and 30 doses for x3 days [15, 20, 17].	Insulin-dependent diabetes mellitus, nausea, diarrhoea, cardiotoxicity, hypoglyceamia, hypotension and pancreatitis [1,10, 21].	Yes [22]
Paromomycin	Inhibits parasites ribosomal recycling and protein synthesis [23, 11].	Ointment and a sulphate-based IM or IV injection during CL and VL treatment, respectively [10, 11].	$t_{1/2}$=24 hours [24]. IV=21 days, CL=21-30 days [25].	Injections cause mild nephrotoxicity [26], cochlear and renal toxicity [27]. Topical treatment causes itchiness, erythema, and oedema [27].	Yes, for CL, No for VL treatment [11, 24, 28]

[1] Frézard *et al*., 2009:2318-2322; [2] Haldar *et al.*, 2011:3-5; [3] Frézard *et al.,*2017:421; [4] Kato *et al*., 2014:481; [5] Marques *et al*., 2019:355; [6] Akbari *et al*., 2017:87; [7] Kip *et al*., 2018:166; [8] Laniado-Laborín & Cabrales-Vargas, 2009:224, [9] Kuhlmann & Fleckenstein, 2017:1348; [10] Bhargava & Singh, 2012:5&7; [11] Sangshetti *et al*., 2015:32380-32381;[12] Gutiérrez *et al*., 2016:154; [13] Dorlo *et al*., 2012:2577-2578; [14] Branco *et al*., 2016:4; [15] McCarthy *et al*., 2015:511-513; [16] Gilbert-Barness, 2010:99; [17] Boelaert & Sundar, 2014:646-647; [18] Sunyoto *et al*., 2018:2; [19] Yang *et al*., 2016:6828-6829; [20] Waller & Sampson, 2018:615; [21] Bloom & Ryan, 2013:1104; [22] Roussel *et al*.,

2006;314; [23] Davidson *et al.*, 2009: 654; [24] Sundar & Chakravarty, 2008:789; [25] Faghihi & Tavakolikia, 2003:13; [26] Davidson, 2017:1064; [27] Davidson *et al.*, 2009:658; [28] Wiwanitkit, 2012:325.

2.7 Antiprotozoal Schiff bases

The previous section has identified problem areas for available anti-leishmanial drugs, which include invasive administration routes, severe side-effects, long administration periods with high dosages, and high costs. These highlight the need for tolerable and affordable novel antileishmanial drugs. Therefore, the aim of this section is to review the progress that has been made towards the development of various antileishmanial active compounds over the years, particularly antiprotozoal Schiff bases. The Schiff base pharmacophore (Figure 2-4) has immensely contributed to the development of novel lead compounds where its chemistry played crucial roles on reducing compound side effects (Dzeikala, 2018:990; Kajal, 2013:12) and improving potency (Dzeikala, 2018:990).

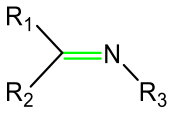


Figure 2-4: General structure of Schiff bases, where R_1 and R_2 and/or R_3=alkyl or aryl.

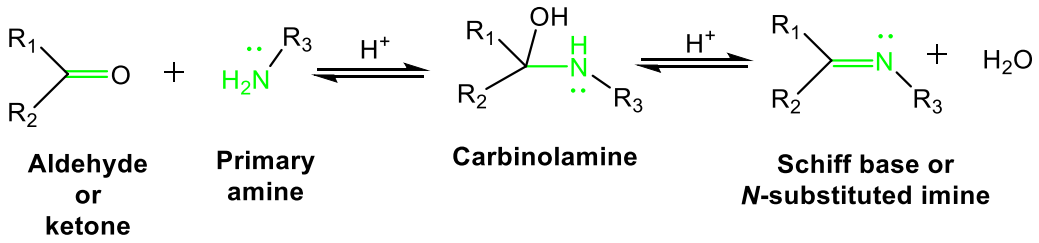


Figure 2-5: Reaction scheme for Schiff base formation.

Schiff bases are products of condensation reactions (Figure 2-5) between primary amines and carbonyl-compounds (Dzeikala, 2018:989). The reaction scheme in Figure 2-5 depicts the formation of an azomethine bond, indicated by a functionality RC=NR (Figure 2-7, green). It is an electrophilic reaction, where a nucleophilic nitrogen group from the primary amine initiates an electrophilic attack on the carbon from a carbonyl (C=O) group present in ketones and/ or aldehydes. An amine nitrogen uses an electron lone pair from its sp^2 hybridised orbital to exert an electrostatic pushing force towards electrons on the oxygen (O) group. Thus, breaking the C=O bond to enable formation of the C=N bond gives a carbinolamine intermediate and an *N*-substituted imine product.

Nitrogen and carbon chemistry play important roles on biological activities of Schiff bases. A nitrogen group imparts its highly nucleophilic and basic character on Schiff base-derived compound, important for conferring biological activity (Dzeikala, 2018:989; Roberts &Caserio, 1977:1096; Verma *et al.*, 2014:69). The nitrogen in the azomethine bond (Figure 2-5, green) has been reported to disturb normal cell functions by forming hydrogen bonds with active centres in cell organelles (Kajal, 2013:1). The carbon group, on the other hand, increased nucleophilicity of a compound and imparts electrophilicity on the C=N bond (Verma *et al.*, 2014:69). This section discusses roles played by electronegative groups on the biological activities of various antiprotozoal Schiff base scaffolds and their derivatives.

2.7.1 8-Aminoquinolines

This is a class of drugs derived from primaquine (PQ) (Figure 2-6), a parent compound for all 8-aminoquinolines that are well-known for their antiprotozoal activity, particularly as antimalarials (Yardley *et al*., 2010:5356). Tafenoquine (TFQ) and sitamaquine (SQ) (Figure 2-6) are members of this class and have shown potent activity against *L. donovani* (Yardley *et al*., 2010:5356; Carvalho *et al.*, 2010:5344). They both confer antileishmanial activity by targeting the mannose-rich glycoconjugate synthesis pathway that produces glycoprotein 63 (Gp63). Gp63 is the main leishmanial glycoprotein found on a promastigote surface. It is coated with high-mannose type *N*-linked glycans used by promastigotes for attaching and invading macrophages. Therefore, SQ and TFQ block the Gp63 synthetic pathway by inhibiting an enzyme called glucose diphosphate (GDP)-pyrophosphorylase (Green, 1994:320; Sangshetti *et al*., 2015:32389). Furthermore, SQ and TFQ disrupt leishmanial replication by inhibiting the mitochondrial respiratory chain (Carvalho *et al*., 2010:5344) (Figure 2-7). The mitochondrion is often referred to as the “energy production hub” of a cell because it has a membrane-bound respiratory chain that operates to produce energy in the form of adenosine triphosphate (ATP) (Carvalho *et al*., 2010:5344). However, SQ and TFQ block the process at different protein complexes in a *Leishmania* cell. TFQ binds to cytochrome C reductase (complex III) while SQ binds to succinate dehydrogenase (complex II) (Figure 2-7). This reduces ATP production within a cell, thus triggering the overproduction of ROS, which causes apoptosis.

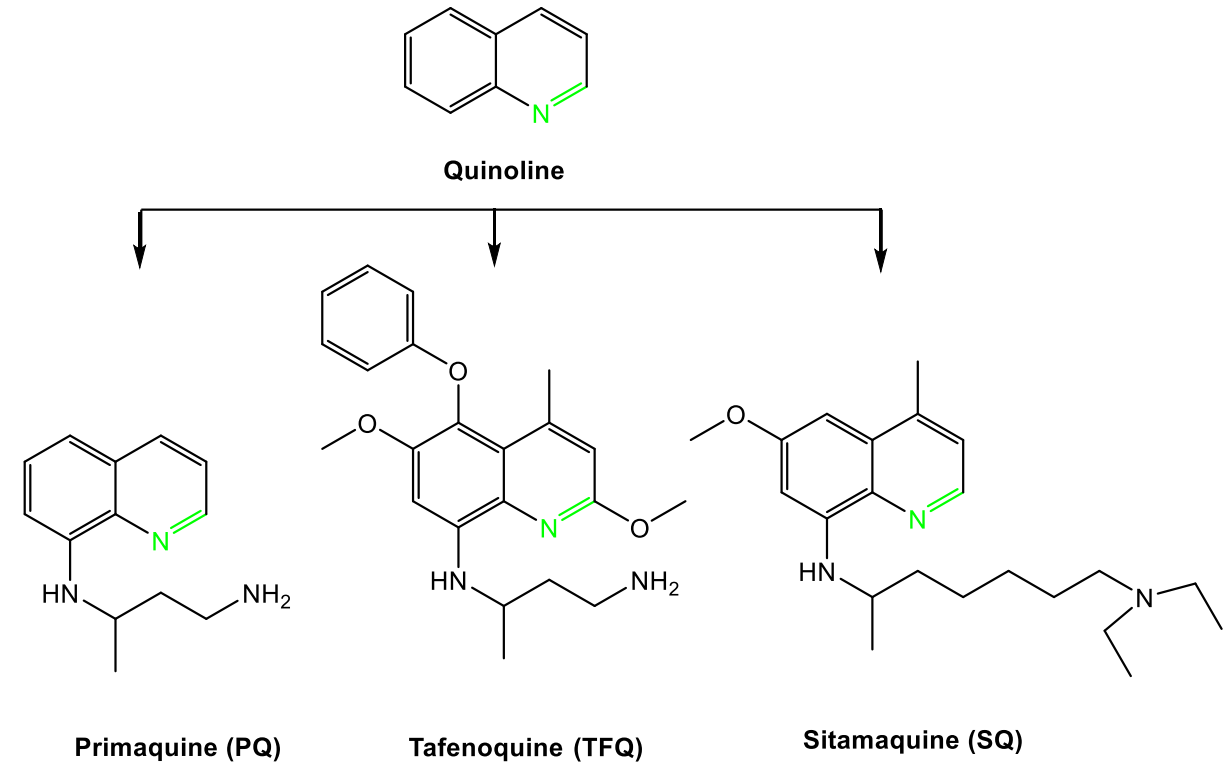


Figure 2-6: Molecular structures of antileishmanial active 8-aminoquinolines.

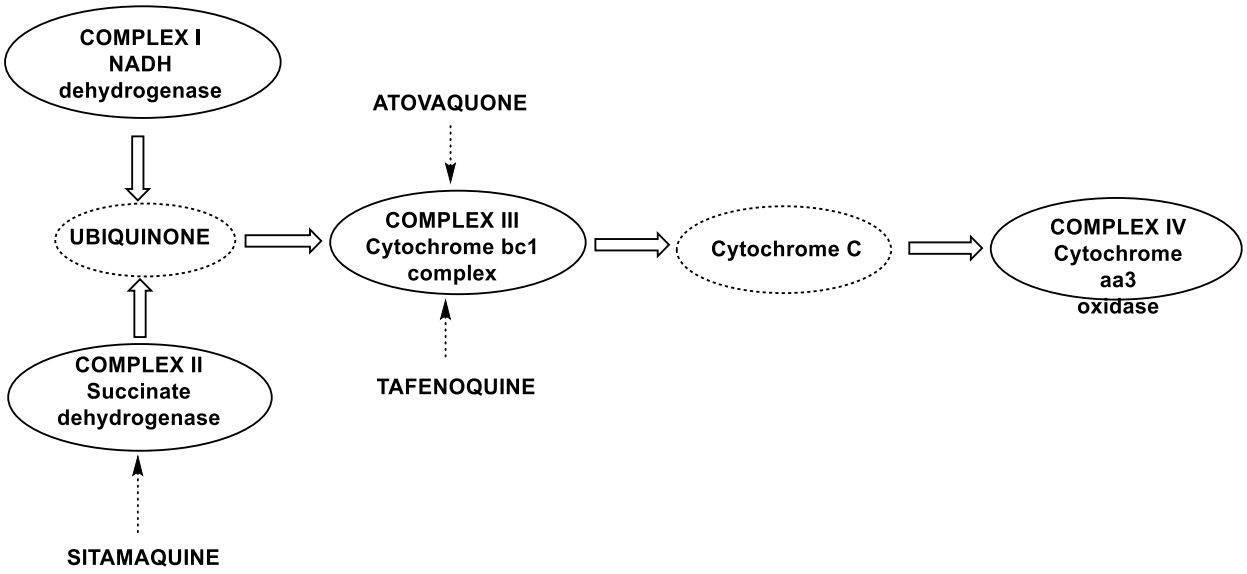
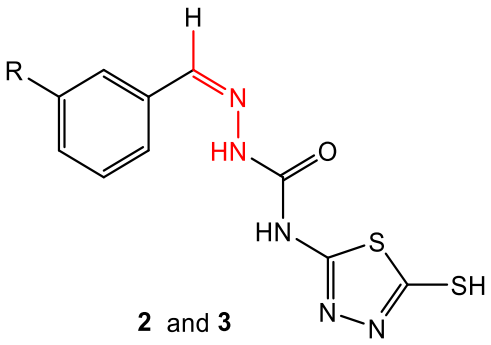


Figure 2-7: A simplified mechanism of action for atovaquone, sitamaquine and tafenoquine.

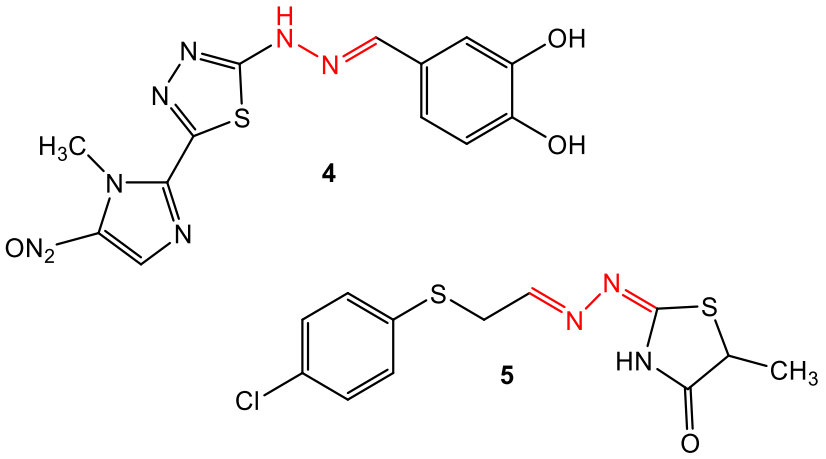
2.7.2 Hydrazones

Hydrazones (Figure 2-8) are a class of organic compounds easily distinguishable by the functional group, R_1R_2=NNH_2 (Figure 2-8, red) (Verma *et al*., 2014:69). They have broad protozoal activity against *Toxoplasma gondii* (*T. gondii*) (Verma *et al*., 2014:75), *Plasmodium* spp. (Verma *et al*., 2014:78), *Trypanosoma cruzi* (*T. cruzi*) (Figure 2-9) (Verma *et al*., 2014:75) and *Leishmania* spp. (Figure 2-8) (Syrjänen *et al*., 2013:7376-7377).



2 and **3**

Figure 2-8: Molecular structure of antileishmanial active hydrazone derivatives. Compound **2**: R=Cl, compound **3**: R=Br.



4

5

Figure 2-9: Molecular structures of antitrypanosomal hydrazones.

Trypanosoma spp. and *Leishmania* spp. belong to the order *Kinetoplastida*. Both parasite spp. have different life cycles and species-specific genes. However, their house-keeping genes are organised in a similar manner, they have a kinetoplast and similar energy production pathways (Donelson *et al*., 1999:2579-2580). They also degrade cellular contents through a lysosomal process called autophagy which is activated in kinetoplasts during nutrient deprivation (Besteiro,

2014:190; Thorburn, 2014:1). *Trypanosoma* spp. and *Leishmania* spp*.* use this process to trigger metacyclogenesis (Besteiro, 2014:190)–an important process in the transmission of leishmaniasis (Ramalho-Ortigao *et al*., 2010:196; Serafim *et al*., 2012:1). *Trypanosoma* spp. and *Leishmania* spp. share important biological characteristics, therefore, it is important to understand the chemistry of potent antitrypanosomal and antileishmanial hydrazone derivatives because this could aid the process of deriving novel antileishmanial hydrazones. Compounds **4** and **5** (Figure 2-9) were reported most active against trypanosomiasis in their respective series. Structural activity relationship (SAR) studies indicated the importance of sulphur, nitrogen and oxygen groups on a heterocyclic ring (Narang *et al*., 2012:580-581). Therefore, the presence of these groups on compound **2** and **3** (Figure 2-8) could be used to justify potent inhibition of carbonic anhydrase (CA) *in L. donovani chagasi* promastigotes (Syrjänen *et al*., 2013:7376-7377). *Leishmania* spp. use carbonic anhydrase (CA) enzymes to interconvert bicarbonate ions (HCO^-_3) and carbon dioxide (CO_2) (Equation 2-1). The reaction reduces acidity effects aimed at parasites following their phagocytosis by macrophages. Chlorine and bromine substitution are important for antileishmanial activity– substitution with bromine on compound **7** (Figure 2-10) completely inhibited parasites *in vivo.*

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \qquad \textbf{Equation 2-1}$$

Furthermore*, Leishmania* spp*.* also share pathophysiological pathways with *Plasmodium* spp*.,* because they both significantly depend on the heme/iron of their host cells for their metabolic processes (Ortalli *et al*., 2019:134). Two antimalarial drugs, namely artemisinins (ARTs) and atovaquone (ATQ), are not classified as hydrazones, but they could be used to support a hypothesis that *Leishmania* spp*.* and *Plasmodium* spp*.* share drug targets. Artemisinin uses its endoperoxides to react with heme, thus producing ROS capable of causing oxidative damage to cellular respiration proteins in the parasite. Atovaquone inhibits plasmodial growth by specifically targeting one of these respiratory proteins called Cytochrome bc1 (Figure 2-6) (Meier *et al*., 2018:10; Ortalli *et al*., 2019:134). In addition, artemisinin employs its antileishmanial activity by resulting in a loss of mitochondrial membrane potential (Verma et. al., 2019;2705). This information leads to the following hypotheses, (i) the mitochondrion is the common internal biological drug target for both *Leishmania* spp. and *Plasmodium* spp. since mitochondrial proteins are similar in all species, and (ii) antimalarial hydrazones (Figure 2-10) (Narang *et al*., 2012:579-580) could also have activity against leishmaniasis (Meier *et al*., 2018:10).

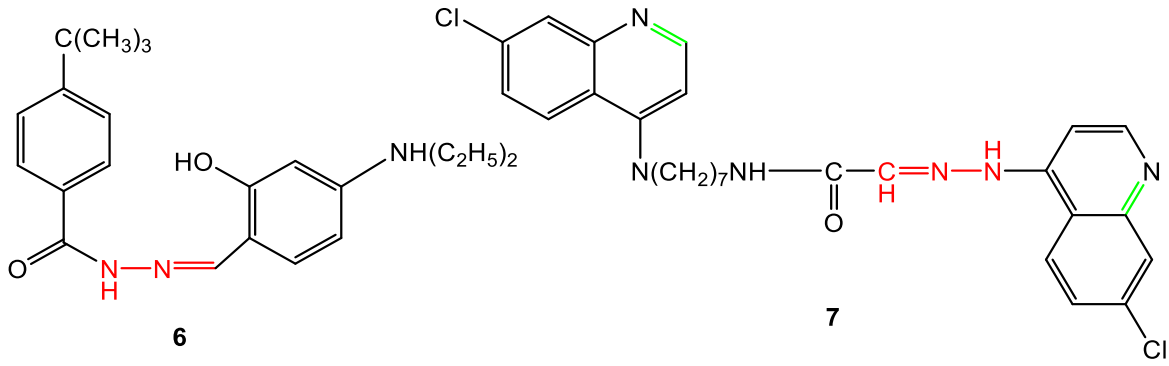


Figure 2-10: Molecular structures of antimalarial hydrazone derivatives.

2.7.3 Quinazolines

Quinazoline (Figure 2-11) is a bicyclic molecule with two non-equivalent nitrogen atoms (Gupta *et al*., 2018:1100). It exists as a yellow, water-soluble crystalline solid formed by the fusion of a benzene and a pyrimidine ring. Quinazoline derivatives, namely tetra- and dihydroquinazolines (Figure 2-11), have been reported as *in vitro* inhibitors dihydrofolate reductase (DHFR) in *L. donovani* with 14.7-94.7% and 100% inhibition, respectively (Agarwal *et al*., 2009:5476). DHFR functions to activate dihydrofolate for the synthesis of nitrogenous bases (purines) and amino acids (Raimondi *et al*., 2019:1-2). Dihydrofolate synthetase (DHFS) catalyses the *de novo* synthesis of folic acid (FA) vitamins as an inactive dihydrofolate, which is later activated by DHFR through an electron transfer reaction from nicotinamide adenine dinucleotide phosphate (NADPH).

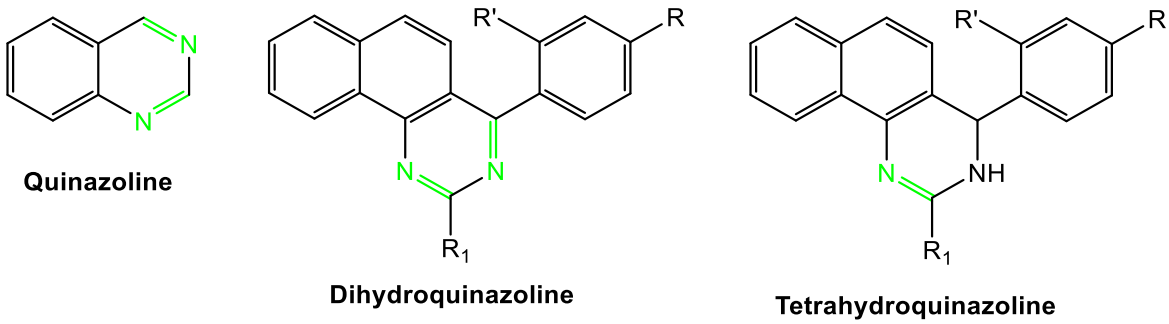


Figure 2-11: Molecular structures of quinazoline and its antileishmanial derivatives.

Various studies have revealed electronegativity and positions of halogen groups on aromatic compounds as vital determinants of antileishmanial activity. Para-substitution (1,4) with chlorine

(Cl) causes more activity, while meta-substitution (1,3) leads to poor to no effect on antileishmanial activity (Sharma *et al*., 2013:4379-4380; Agarwal *et al*., 2009:5475-5476). On the other hand, para-substitution with fluorine (F) causes poorer activity in comparison to hydrogen (H) (Agarwal et al., 2009:5475-5476). Since both Cl and F are electronegative groups, they deactivate aromatic rings at ortho (1,2) and para-positions by withdrawing electrons from the ring. However, F has greater electronegative potential, which suggests that it has the highest deactivation capability at these positions (Ouellette & Rawn, 2015;143-148). This explains poor activities in the presence of F because compounds are less likely to participate in biochemical oxidation reactions within parasitic cells.

2.7.4 Quinazolinones

Quinazolinone (Figure 2-12) is a naturally existing alkaloid used as a building block for animal, microbial and plant molecules. The position of the carbonyl (C=O) group on the quinazolinone scaffold is used to categorise quinazolinones into 3 classes, namely quinazolin-4(3*H*)-one, quinazolin-(2*H*)-one and quinazolinone-2,4(1*H*, 3H)-dione. This section draws more attention on the quinazolin-4(*3H*)-one parent scaffold that is further used to derive three daughter scaffold classes, namely 2-substituted, 3-substituted and 2, 3-disubstituted quinazolin-4-ones (Figure 2- 14) (Mhaske & Argade, 2006:9788-9789; Rajput & Mishra, 2012:66).

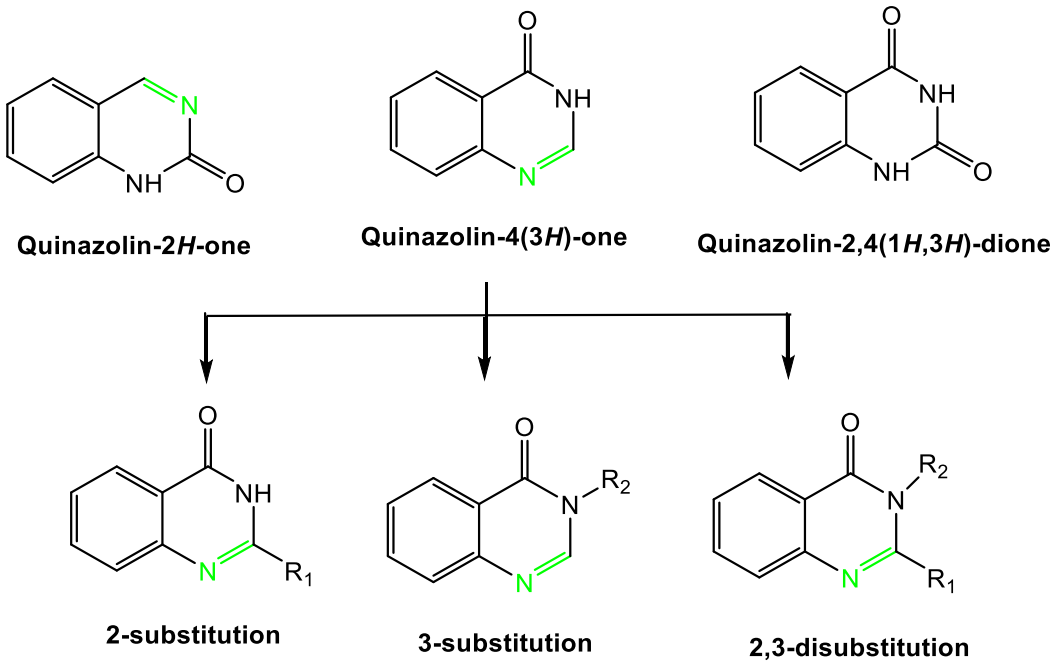


Figure 2-12: Molecular structures of quinazolinone classes and derivatives.

The chemistry of a quinazolin-4(3*H*)-one scaffold qualifies it is an important compound for drug discovery (Tesfahunegn *et al*.,2015;154). The scaffold has electron lone pairs that contribute to its high nucleophilic character at nitrogen atoms N-1 and N-3 (Figure 2-12). These non-bonding electrons increase reactivity and reduce stability within the bicyclic compound (Roberts & Caserio, 1977: 1096; Dzeikala & Sykula.,2018:990). Quinazolin-4(3*H*)-one scaffolds use the N-H bond to participate in hydrogen bonding, promoting water solubility, a factor required for the distribution of compounds and cellular absorption (Roberts & Caserio, 1977:1103). However, the quinazolin-4(3*H*)-one scaffold cannot be directly used for drug development, because it is unstable and may participate in unwanted reactions. A nucleophilic substitution reaction, defined as a chemical reaction where electron-rich nucleophilic groups donate electrons to electron-deficient electrophilic groups (Ikuo *et al*., 2017:556), can be used to form derivatives. The N-H bond is broken and an aromatic ring bearing an electron withdrawing group (EWG) is linked to the quinazolin-4(3*H*)-one scaffold. After successful linkage, the EWG withdraws two electron lone pairs from the nitrogen atoms, thus stabilising the final compound (Roberts & Caserio, 1977:1122; Ouellette & Rawn, 2015;143-148). Stability is also attributed to the conversion of a secondary amine (R_2NH) at nitrogen atoms N-3 to a tertiary amine (R_3N) upon elimination of a hydrogen (H) group (Roberts & Caserio, 1977:1103). However, it is worth noting that increasing stability in quinazolin-4(3*H*)-one derivatives limits hydrophilicity, since there is no hydrogen bonding in tertiary amines (Roberts & Caserio, 1977:1103).

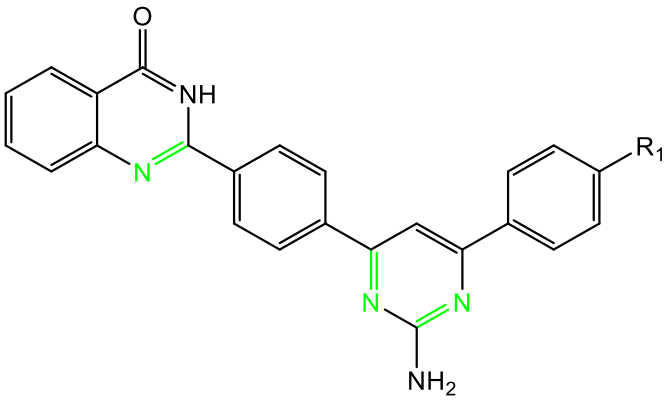


Figure 2-13: Molecular structure of quinazolinone-pyrimidine scaffold.

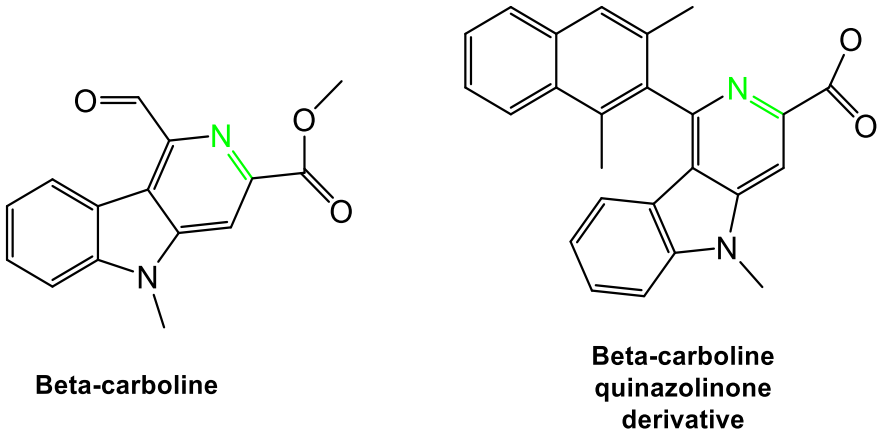


Figure 2-14: Molecular structures of β-carboline-quinazolinone derivatives.

Quinazolin-*4(3H)*-one derivatives with antileishmanial activity include quinazolinone-pyrimidine, 2,3-disubstituted-4(*3H*)-quinazolinone (Figure 2-13) and β-carboline-quinazolinone (Figure 2-14). Sharma *et al*. (2013) synthesised a series of quinazolinone-pyrimidine derivatives (Figure 2-13) and screened them *in vitro* against *L. donovani* amastigotes as part of efforts to improve quinazolinone drug delivery. A SAR study suggested that para-substitution with a chlorine (Cl) group increased activity, while substitution with a fluorine (F) group resulted in loss of activity (Sharma *et al*., 2013:4379-4380). Furthermore, linking the quinazolin-*4(3H)*-one scaffold to biologically recognisable functionalities, such as a β-carboline alkaloid, has been shown to improve antileishmanial activity (Chauhan *et al*., 2015:351). A β-carboline alkaloid confers activity against trypanothione reductase (TR), a *Leishmanial* spp. antioxidant responsible for reducing trypanothione disulphide dithiol to trypanothione. Therefore, it maintains adequate levels of

trypanothione within parasites. In contrast, this study suggested that attaching a para-substituted aryl substituent with a Cl or F group at position 3 of a β-carboline-quinazolinone derivative increased antileishmanial activity and inhibited TR in *L. donovani* amastigotes, with activity <10 µM (IC_{50} range 4.3-6.0 µM) (Chauhan *et al*., 2015:351-353). These compounds also showed toxicities lower than miltefosine and stibogluconate against Vero cells (103-400 µM), while miltefosine (56.5 µM) and sodium stibogluconate (400 µM) had higher cytotoxicity.

2.7.5 Thiazines

Thiazines are six-membered heterocyclic compounds with at least one nitrogen (N) and sulphur (S) atoms (Figure 2-15, pink). They are biologically active against viruses and fungi (Badshah & Naeem, 2016:1), hypertension (Cherepakha *et al*., 2011:6233), and microbes (Porcs-Makkay *et al*., 2014:2169). There are five thiazine classes (Figure 2-15), namely 1,2-thiazines, 1,3-thiazines, 1,4-thiazines, benzothiazines, thiomorpholine and phenthiazines (Choudhary & Silakari, 2018:248). However, this section draws more attention to anti-diuretic benzothiazine-1,1-dioxide derivatives, such as hydrochlorothiazide (HCT, Figure 2-16) (Vongpatanasin, 2015:2).

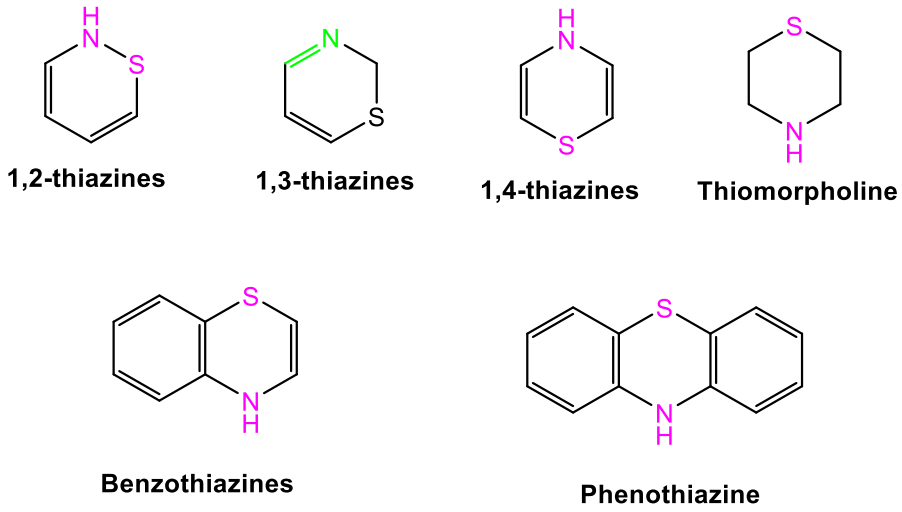


Figure 2-15: Molecular structure of thiazine classes.

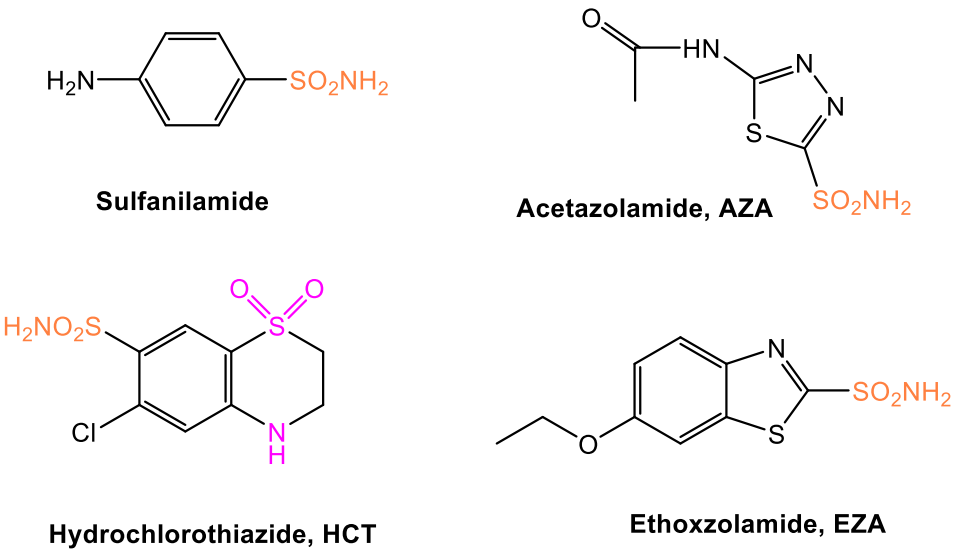


Figure 2-16: Molecular structures of sulphonamides.

Hydrochlorothiazide is a benzothiadiazine-1,1-dioxide that may also be classified as sulphonamides due to the presence of a -SO_2NHR functional group (Figure 2-16 and 2-17, orange), where R is an aromatic, heterocyclic or aliphatic group (Supuran, 2017:1). The presence of N and S atoms in heterocyclic rings of these compounds previously increased pharmacological activities and reduced toxicity (Gupta *et al.*, 2018:1099). HCT and other sulphonamides, including acetazolamide (AZA) and ethoxzolamide (EZA) (Figure 2-16), confer antileishmanial activity by inhibiting carbonic anhydrase (CA), a pH monitoring enzyme in human red blood cells (Frost, 2014:9). HCT and EZA showed potent *in vitro* inhibition of CA in *L. donovani chagasi* promastigotes, while AZA showed weak inhibition (Syrjänen *et al*., 2013:7375). Since HCT is an oral CA-inhibiting diuretic, this raises a hypothesis that it is well distributed to the bloodstream and absorbed by infected macrophages where it inhibits *Leishmania* growth by preventing bicarbonate (HCO^-_3) ions from converting back to CO_2 (Equation 2-1) (Herman & Bashir, 2019). In this process, the release of HCO^-_3 and potassium (K^+) salts from parasitic cells is promoted. Ultimately, parasitic cells are predisposed to macrophage digestion through acid production.

Quinazolinone **Benzothiadiazine-1, 1-dioxide**

Figure 2-17: Molecular structures of quinazolinone and benzothiadiazine-1, 1-dioxide scaffolds.

In order to improve the activity of quinazolinone compounds against *Leishmania* spp., it can be hypothesised that introducing the sulfonamide functionality by replacing a carbonyl group (-C=O) with a sulfonyl group (-SO_2) (Cohen *et a*l., 1960:2731) (Figure 2-17), could impart CA-inhibiting activity (Krungkrai et al.,2005:484). Since benzothiadiazine is multifunctional and a highly specialised, the mechanism of action could also be more complex.

The importance of substituting a C=O group with a -SO_2 group could be clearly understood by separately studying the influence of each group on compound reactivity and stability. Both -SO_2 and -C=O groups are active participants of nucleophilic substitution reactions. They can deactivate and protect the highly basic and nucleophilic nitrogen atoms N-1 and N-3 from unwanted reactions (Figure 2-16 and 2-17) (Roberts & Caserio, 1977:1159-1161). They influence reactivity to different extents and achieve this by changing electron densities within their aromatic rings (Ouellette & Rawn, 2015:145-146). For instance, a -C=O group has a partial positive charge for enhancing inductive effects and withdrawing electrons from the ring and nitrogen N-3 (Ouellette & Rawn, 2015:145-146). Since one of the requirements for nucleophilic substitution reactions for these compounds is that the N-H bond must break to avail nitrogen atom N-3 electrons for bond creation with other atoms. Bond formation can either occur when N-3 donates or accepts electron lone pairs (Roberts & Caserio, 1977:1159; Ikuo *et al.*, 2017:556). Therefore, this suggests that a -C=O group ultimately reduces acidity within a quinazolinone moiety which limits the ability of the nitrogen atom N-3 to form new bonds (Roberts & Caserio, 1977:1175-1176).

On the other hand, a -SO_2 group has been noted to have higher electron withdrawing effects than a -C=O group (Chataigner *et al*., 2007:3289). However, due to poor inductive effects, it is less efficacious on decreasing electron density on nitrogen atom N-3. Therefore, the -SO_2 group

increases stability within the benzothiadiazine ring (Roberts & Caserio, 1977:1159-1161) without compromising acidity of the N-H proton (Roberts & Caserio, 1977:1123).

In theory, developing antileishmanial compounds requires a thorough understanding of properties that reduce compound stability (Mwene-Mbeja, 2019:14). An unstable compound participates in unwanted reactions to form by-products that result in poor drug efficacy and cause undesirable side effects. Quinazolin-*4(3H)*-one derivatives are poor candidates for leishmanial drug discovery because the -C=O group is easily cleaved due to easy breakage of the -C-N- bond under mild acidic conditions (Roberts & Caserio, 1977:1160). An acidic environment enhances the delocalisation of electrons on the -C=O group, ultimately promoting cleavage via acid-catalysed hydrolysis and loss of stability (Chataigner *et al*., 2007: 3288; Roberts & Caserio, 1977:1159-1160). It can be hypothesised that quinazolin-*4(3H)*-one derivatives would destabilise during *in vitro* studies in (i) macrophages and phagolysosomes (pH = 4.8) (Haka, 2009:4935), and (ii) stomach cells (pH = 2) (Kinoshita *et al.*, 2018:182). This is unlikely to occur in benzothiadiazine derivatives. The process of removing a $-SO_2$ group can only be achieved by hydrolysis during a β elimination reaction, termed the *sulphur dioxide extrusion process* (Alonso & Ájera, 2009:369-370). This reaction involves the breakage of a strong S-N bond under strenuous conditions, such as excess concentrated mineral acids and extremely high temperatures or strong bases, such as organolithium or Grignard reagents (Javorskis & Orentas, 2017:13423). In conclusion, it can be hypothesised that fusing quinazolin-*4(3H)*-one and benzothiadiazine-1,1-dioxide moieties through a direct no-linker technique will form stable novel antileishmanial compounds with greater potency (Pawelczyk *et al*., 2016:1).

Thus, the goal of this research project is to synthesise novel benzothiadiazine-1,1-dioxide derivatives through two-step nucleophilic substitution reactions and evaluate their antileishmanial potential through determination of their activities against promastigotes of various Leishmania strains.

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CHAPTER 3

SUBMITTED ARTICLE

Chapter 3 includes the manuscript of an article submitted to the *Archiv der Pharmazie* - Chemistry Life Sciences journal. The manuscript includes a graphical abstract, an abstract, introduction, results (chemistry and biological evaluations), discussions and a conclusion. The experimental section has a materials and method section plus the syntheses of compounds that were studied. This manuscript is prepared using the author's guidelines obtained and available in the Author Information pack on the journal's homepage (Annexure B): https://onlinelibrary.wiley.com/pb-assets/assets/15214184/ArchPharm_Guidelines_2020-1583143819340.pdf

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Archiv der Pharmazie

Synthesis and *in vitro* antileishmanial efficacy of novel benzothiadiazine-1,1-dioxide derivatives

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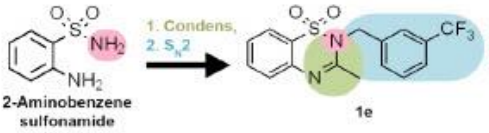
ABSTRACT

Leishmaniasis is a major vector-borne parasitic disease that affects thousands of people in tropical and sub-tropical developing countries. In 2019 alone, it killed 26,000 to 65,000 individuals. Leishmaniasis is curable, yet its eradication and elimination are hampered by major hurdles, such as the availability of only a handful of clinical toxic drugs and the emergence of pathogenic resistance against them. This underscores the imperative need for new and effective antileishmanial drugs. In search for such agents, we synthesized and evaluated the *in vitro* antileishmanial potential of a small library of benzothiadiazine derivatives, by assessing their activity against the promastigotes of three strains of *Leishmania (L.)* and toxicity in healthy cells. The derivatives were found with no toxicity to the mammalian cells and were in general active against all parasites. The benzothiadiazine derivative **1e**, 3-methyl-2-[3-(trifluoromethyl)benzyl]-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, was found to be the most active (IC_{50} 0.2 μM) against *L. major*, responsible for the most prevalent disease form, cutaneous leishmaniasis. Conversely, benozothiadiazine **2c**, 2-(4-bromobenzyl)-3-phenyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, was the most potent (IC_{50} 6.5 μM) against *L. donovani*, a causative strain of the lethal visceral leishmaniasis. Both compounds stand as anti-promastigote hits for further lead investigation into their potential to act as new antileishmanial agents.

KEYWORDS Leishmaniasis, promastigote, benzothiadiazine-1,1-dioxide, cytotoxicity

Graphical abstract



1 INTRODUCTION

Leishmaniasis is a parasitic infectious disease that constitutes a major health concern in the tropical and sub-tropical developing countries in Africa, Asia and Latino-America[1], where it impacts the lives of thousands of people annually. The disease is endemic to ninety-eight countries[1] and is caused by species of the genus *Leishmania*, which are transmitted to humans through the bite of infected female phlebotomine sandflies. There are over twenty *Leishmania* species[2], of which three are predominantly responsible for the three clinical forms of the disease in humans.[3] They include: (i) *L. major*, which communicates the most common form of the disease, i.e. cutaneous leishmaniasis (CL)[1]; (ii) *L. donovani*, responsible for visceral leishmaniasis (VL), the lethal form with a 95% fatality rate if left untreated[1,3]; and (iii) *L. braziliensis*, which causes mucocutaneous leishmaniasis (MCL).[1,3] In 2019 alone, around 600,000-1 million new cases of CL and 50,000-90,000 new cases of VL, were reported to have occurred worldwide.[1]

Like most protozoa, the complexity of *Leishmania* parasites impairs the development of vaccines to prevent leishmaniasis. Thus, to date, the control and fight against this infection rely mainly on chemotherapy. However, only a few clinical drugs are available for the treatment of the various forms of the disease,[4] whose usage is restricted by toxicity and pathogenic resistance .[5] In this context, there is an urgent need for new, effective and inexpensive therapies to combat leishmaniasis.

Taxonomic similarities among parasites of the apicomplexan genera[6] allow effective agents or combinations of drugs against one pathogen to be active against others. The basis for this shared susceptibility to drugs among these protozoa is better illustrated with the therapeutic agents, such as the antifolates and the anti-respiratory drug, atovaquone. Indeed, the current first line treatment of toxoplasmosis is the combination of pyrimethamine and sulfadiazine, with pyrimethamine-atovaquone as an alternative, all these drugs being repurposed antimalarials.

Benzothiadiazine is a bicyclic heterocyclic benzene derivative used in clinics as *inter alia* antihypertensives, *e.g.* bendroflumethiazide (Aprinox) and chlorothiazide (Diuril), and antihypoglycemic drugs, *e.g.* diazoxide (Proglycem) (Figure 1). They are structurally related to the 1,1-dioxothiazine scaffold that is derived from a thiazine core. Aside from the aforementioned therapeutic features, thiazine derivatives have also been found to have other biological properties, *e.g.* the antiviral[7], antimicrobial[8] and antimalarial[9] properties of thiaplakortone A (Figure 1) and some of its derivatives.[10]

Furthermore, benzothiadiazine-1,1-dioxide derivatives bearing other heterocyclic substituents have been synthesized in high yields; however, antileishmanial activity has yet to be reported.[11]

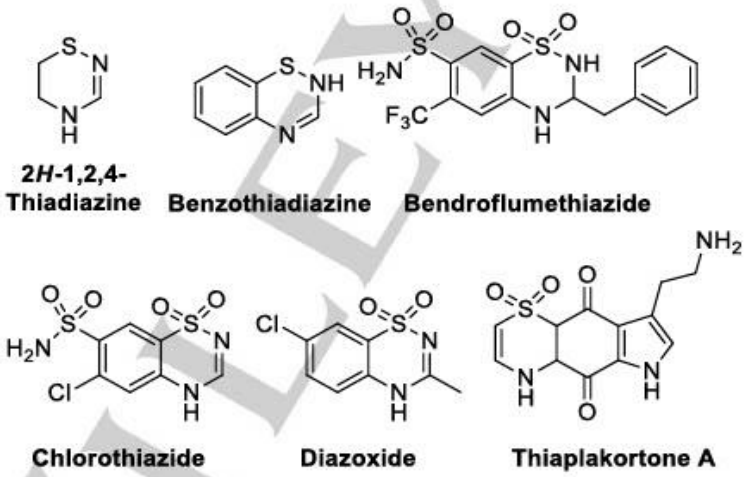


FIGURE 1. Clinical thiazine derivatives with benzothiadiazine-1,1-dioxide scaffold and antimalarial active thiaplakortone A with 1,1-dioxothiazine moiety

Altogether, the taxonomic similarities between *Plasmodium* and *Leishmania* parasites, and the reported promising antimalarial activity of thiaplakortone A and its derivatives, motivated the investigation into the antileishmanial activity of novel 2,3-disubstituted benzothiadiazine-1,1-dioxides. We herein report the synthesis and the biological activities thereof.

2 RESULTS AND DISCUSSION

2.1 Chemistry

The target compounds were synthesized in a two-step process involving 2-aminobenzenesulfonamide, triethyl orthoacetate/benzoate and alkyl/halide benzyl bromide (Scheme 1). First, the condensation of commercial 2-aminobenzenesulfonamide with triethyl orthoacetate/benzoate, acting as both reagent and solvent, resulted in Schiff bases that underwent further *in situ* ring closure to provide the benzothiaziadine-1,1-dioxide **1** (3-Me) and **2** (3-Phe) substituted intermediates[12] (Scheme 1, step i) in excellent (95-98%) yields. The sp^3 hybridized nitrogen atom in each intermediate bore a moderately acidic proton (H-2) with a pKa value 7 - 9, as calculated on MarvinSketch Version 19.4 (ChemAxon Software, www.Chemaxon.com). Thus, in the second step, the intermediates were deprotonated with potassium carbonate (K_2CO_3) and were subjected to *N*-alkylation through nucleophilic substitution (S_N2), with the reaction of alkyl/halide benzyl bromide (Scheme 1, step ii), to afford the target 2,3-disubstituted benzothiaziadine-1,1-dioxide compounds. These *N*-alkylated, or more precisely *N*-benzylated compounds, were isolated in poor to moderate yields (5-41%) after purification by either recrystallization or silica gel column chromatography. Several experimental conditions, including an increase in stoichiometric ratio of reagents, increased temperature and use of N_2 to deoxygenize to the reactional medium, were attempted to no avail to improve the yields. This could plausibly be attributed to unfavorable chemistry between the intermediate and the benzyl bromide reagents as a result of the inaccessibility (steric hindrance) of the nucleophilic site, *i.e.* nitrogen N-2 on the benzothiaziadine-1,1-dioxide moiety, that may have been caused by to the V-shaped sulfone ($-SO_2$) functional group, wherein the O=S=O bond angle varies in the 116-120º region.[13]

SCHEME 1 Synthesis of substituted benzothiadiazine-1,1-dioxide derivatives
Reagents and conditions: a. triethylorthoacetate/benzoate (5 eq.), reflux at 100 °C, 24-36 h.; II. 1 or 2 (1 eq.), alkyl/halide benzyl bromide (2 eq.), K_2CO_3 (2 eq.), N_2, DMF (3-4 mL), 90 ºC, 24-36 h.

Routine spectroscopic techniques were used to confirm the structures of the compounds. The spectra were thoroughly inspected to confirm the nuclear magnetic resonance of key protons (^{1}H NMR), carbons (^{13}C NMR), functional groups (infrared/FTIR spectra) and molecular ions (high-resolution mass spectrometry/HRMS spectra).

The ^{1}H spectra of intermediates **1** and **2** displayed a singlet downfield at 12 ppm which was assigned to the resonance of the acidic proton H-2 (N-*H*). The disappearance of this singlet from the spectra of compounds **1a -1e** and **2a - 2d** was confirmative of the success of the S_N2 reactions. Furthermore, the spectra of compounds **1** – **1e** commonly possessed a singlet in the 2.5-2.3 ppm region, accounting for the resonance of the 3-methyl (CH_3) protons, while the spectra of **1a -1e** additionally showed another singlet *ca.* 5 ppm, which was also present in those of **2a – 2d**, and was attributed to the signals of methylene (CH_2) protons of the benzyl group. Moreover, the ^{1}H spectra of all synthesized compounds commonly displayed a doublet (d, J = 7.8 Hz) *ca.* 8.0 ppm, attributing to the resonance of the aromatic proton H-8 in close vicinity of the sulfone functional group, indicating the presence of the benzothiaziadine-1,1-dioxide scaffold. Additionally, these spectra contained several overlapping doublets (d) and triplets (t) in the 8.0-7.0 ppm due the resonance aromatic protons of the phenyl rings. All the protons of each structure were accounted for.

The ^{13}C spectra provided further evidence, confirmative of the structures of the synthesized compounds. Indeed, the spectra of all compounds contained a distinctive peak *ca.* 160 ppm that was assigned to the aromatic carbon C-3 of benzothiaziadine-1,1-dioxide. Besides, a singlet found around 50 ppm, characteristic of the methylene carbon (CH_2) of benzyl, was present in the spectra of **1a - 1e** and **2a - 2d**, while another singlet at 25 ppm accounted for the methyl carbon (CH_3) specific to **1** and **1a - 1e** structures.

FTIR spectra of the intermediates **1** and **2** revealed the stretching band of N-H bond *ca.* 3300 cm^{-1}. The disappearance of the band from the spectra of **1a - 1e** and **2a - 2d** provided further proof of the successful alkylation reactions. Additional evidence of the presence of the benzothiaziadine-1,1-dioxide moiety in the structures of the compounds was provided with the absorption bands of C=N and S=O stretching in the 1700-1600 and 1400-1300 cm^{-1}, respectively. Furthermore, the absorption of C-F stretching resulted in a strong bands *ca.*1100 cm^{-1} in the spectra of compounds **1b**, **1e**, **2b** and **2d**, while the vibrations of C-Br bonds were evidenced with the presence of sharp bands in the 780-750 cm^{-1} region in the spectra of **1c**, **1d** and **2c**.

All HRMS spectra showed the presence of $[M+H]^+$ fragments, confirming the presence of the molecular ion of each synthesized compound.

3.2 Predicted physicochemical and pharmacokinetic properties

The druglikeness of the compounds was assessed using Lipinski's rule of five to predict whether a compound may be orally active in humans.[14] Preference is given to oral activity due the fact that miltefosine is currently the only oral antileishmanial drug while its very use is threatened by toxicity and pathogenic resistance. Thus, physicochemical properties, such as lipophilicity and aqueous solubility, which *inter alia*, are impactors of Lipinski's rule, were predicted for all the synthesized compounds by using the SwissADME web tool. The data is summarized in Table 1.

TABLE 1. Predicted physicochemical descriptors and ADME parameters of all synthesized benzothiadiazine derivatives

Cpd.	MW (g/mol)	Log P_{ow}[a]	Log S[b]		HBD[e]	HBA[f]	Lipinski's violation	GI absorption	Druglikeness[g]
			ESOL[c]	All[d]					
1	196.23	0.98	-1.58	-1.26	1	3	0	High	Yes
1a	286.35	2.52	-3.23	-2.91	0	3	0	"	Yes
1b	304.34	2.87	-3.38	-3.01	0	4	0	"	Yes
1c	365.24	3.18	-4.13	-3.63	0	3	0	"	Yes
1d	365.24	3.18	-4.13	-3.63	0	3	0	"	Yes
1e	354.35	3.59	-4.06	-3.82	0	6	0	"	Yes
2	258.30	2.09	-2.99	-2.80	0	3	0	"	Yes
2a	348.42	3.61	-4.68	-4.63	0	3	0	"	Yes
2b	366.41	3.87	-4.83	-4.74	0	4	0	"	Yes
2c	427.31	4.16	-5.58	-5.35	0	3	0	"	Yes
2d	416.42	4.57	-5.52	-5.55	0	6	0	"	Yes

[a]Calculated logP (consensus log P). [b]Predicted aqueous solubility, where log S is the logarithm of the amount of compound (in moles) able to dissolve a liter of water. [c]ESOL = estimated aqueous solubility, calculated using a topological method.[15] [d]Calculated using a topological method[16] with log S scale: insoluble < -10 < poorly < -6 < moderately < -4 < soluble < -2 very soluble < 0 highly <. [e]Number of hydrogen bond donors (NH and OH groups). [f]Number of hydrogen bond acceptors (nitrogen and oxygen atoms). [g]Determined with reference to Lipinski's rule of five: MW ≤ 500 g/mol; Log*P* ≤ 5; HBD ≤ 5; HBA ≤ 10; no more than one violation allowed.[14] All values in this table were calculated using SwissADME web tool, http://www.swissadme.ch[17]

All compounds complied with Lipinski's rule with physicochemical properties well within the target ranges as set by Lipinski *et al*.[14] None of the compounds violated the rule, which suggested all these benzothiaziadine-1,1-dioxide derivatives to be drug-like in nature, suitable for oral administration, and highly absorbed in the gastrointestinal tract through passive diffusion.

2.3 Pharmacology

The antileishmanial activities of the synthesized benzothiaziadine-1,1-dioxide derivatives were assessed against the promastigotes of three different *Leishmania* strains, *L. donovani* (1S and 9515) and *L. major* IR-173. *In vitro* cytoxicity was determined on Vero (African green monkey kidney epithelial) cells. Amphotericin B and emetine acted as reference drugs for the antileishmanial and cytotoxicity assays, respectively.

It is noteworthy to indicate that *Leishmania* spp. have two developmental forms, promastigotes (responsible for the insect and infective stages) and amastigotes (responsible for the clinical manifestations in vertebrate hosts). Amastigotes are regarded as the logical form targeted during the process of antileishmanial drug discovery[18]. However, *Leishmania* parasites within skin lesions have been found to not act as obligatory intracellular parasites, with amastigotes exiting the phagocytes after membrane rupture and transforming into extracellular promastigote-like forms to survive immune responses and promote mobility in the extracellular space[19] and contributing lesion changes and scar formation. Therefore, anti-promastigote drugs may be of benefit in lesion treatment or the prevention of scarring. Moreover, studies have shown that antileishmanial drugs that are effective against amastigotes are not always effective against promastigotes.[20] Therefore, there may be a significant benefit in screening compounds against both parasite forms in the search for novel antileishmanial therapies. Hence, we chose a systematic screening approach, by primarily assessing the synthesized compounds against promastigotes of the various *Leishmania* strains. The resulting hits, if any, would then later undergo further testing for possible anti-amastigote activities. Anti-promastigote activity was evaluated by determining the half-maximal inhibitory concentration (IC_{50}) and related selectivity index (SI) values for each compound. The biological data is reported in Table 2.

A glimpse at the table revealed all the compounds to be nontoxic to Vero cells as evidenced by the (IC_{50}>100 µM), inferring that their observed antileishmanial activities were intrinsic. The compounds presented with different activity profiles against the parasites. On the one hand, *L. major* and *L. donovani* 1S promastigotes were susceptible to all compounds. On the other hand, *L. donovani* 9515 parasites were susceptible to only **1d**, **1e** and **2a-c**. Thus, the antileishmanial activity of this series of benzothiaziadine-1,1-dioxide derivatives was strain-specific. No derivative outperformed the reference, amphotericin B, irrespective of the strain considered, *albeit* being safer.

In the 3-methyl substituted sub-series **1-1e**, the trifluoromethyl (CF_3) containing derivative, **1e** was the best performer with IC_{50} 0.2 and 22 µM values against *L. major* and *L. donovani* 9515 promastigotes, respectively, while the intermediate **1** outperformed all with IC_{50} 7 µM against the *L. donovani* 1S parasite. In the 3-phenyl substituted sub-series **2-2d**, however, **2b** and **2d** were the most active against *L. major*, being equipotent at IC_{50} 0.4 µM, whereas **2a** and **2b** with comparable activities, IC_{50} *ca.* 12 µM, stood out against *L. donovani* 9515 while **2c** with IC_{50} 6.5 µM was the most potent derivative against *L. donovani* 1S promastigotes.

Furthermore, substituting the methyl in sub-series 1 with the phenyl to form sub-series 2 resulted in an enhancement of the antileishmanial activity. Although **2** was less active than **1** against both *L. donovani* strains, it displayed marginal increase in activity against *L. major*. Conversely, 3-phenyl substituted derivatives **2a-c** showed significant increases in activity in comparison to their methyl **1a-c** counterparts, especially against *L. donovani* promastigotes.

Moreover, the electronic effect had no bearing on the biological activity of the compounds. Indeed, considering the electronic strength of *para*-R^2 substituent on derivatives **1a-c**, showed the increasing order; **1a**, **1b** and **1c**. This order was not followed through when comparing the IC_{50} values of the compounds regardless of the strain considered. Derivative **1c** (4-Br), bearing the strongest electron withdrawing group (EWG), was more active than **1b** (4-F) with a weaker EWG against *L. donovani* strains, but less active against *L. major*. A similar trend was observed with **1b** and **1a**. In the sub-series 2, however, the order of increasing EWG strength followed directly that of the activities with derivatives **2a-c** against *L. donovani* 1S, inversely against *L. donovani* 9515, while a mixed profile was observed against *L. major*.

Additionally, bioisosterism (substituents or groups with chemical or physical similarities that produce similar biological properties[21]) was also assessed during this research. Bioisosterism may rationally modify a lead compound into a safer and more clinically effective agent through attenuation of toxicity, modification of activity and/or alteration of pharmacokinetics of a lead.[21] The ability of bioisosteres to elicit similar biological activities has been attributed to common physicochemical properties, such as electronegativity, steric size and lipophilicity, which have been quantitated and correlate their values with observed biological activity values.[22] The substitution of hydrogen by fluorine is one of the most commonly employed monovalent isosteric replacements. Hydrogen and fluorine atoms have similar steric parameters with their van der Waal's radii being 1.2 and 1.35 Å, respectively.[23]

Derivative **1b** (4-F) is a bioisostere of **1a** (4-H). Both compounds have comparable lipophilicity (Log $P_{o/w}$ *ca.* 2.5) and demonstrated equipotency against *L. major*, and inactivity against *L. donovani* 1S promastigotes. However, **1b** possessed a slight superiority over **1a** with a two-fold activity against *L. donovani* 9515. Similarly, **2a** and **2b** are bioisosteres with comparable activities against *L. donovani* 9515, but **2b** displayed significant 3- and 9-fold higher potencies than **2a** against *L. donovani* 1S and *L. major*, respectively. In the latter case, bioisoterism contributed to deliver a safer hit as **2b** was found to be comparatively highly selective (SI 250) in its antiparasitic action.

Overall, all the benzothiaziadine-1,1-dioxide derivatives possessed highly potent (IC_{50}<5 µM) and selective (SI 20-500) antileishmanial actions against *L. major* promastigotes, therefore were identified as potential anti-CL hits with the most promising being the nanomolar active derivative **1e** (IC_{50} 0.2 µM and SI 500). Additionally, derivatives **2b**, **2c** and **2d**, by virtue of their molar (IC_{50}≤10 µM) and moderately selective (SI≥10) activities against *L. donovani* 1S parasites, may stand as potential anti-VL hits for further investigation into anti-amastigote activity determination.

TABLE 2. *In vitro* antipromastigote activity and cytotoxicity of synthesized benzothiadiazine derivatives

Cpd.	Antileishmanial activity, IC_{50} ± SD (µM)[a]			Cytotoxicity, IC_{50} (µM)	Selectivity Index, SI		
	L. donovani 1S	*L. donovani* 9515	*L. major* IR-173	Vero[b]	SI_1[c]	SI_2[d]	SI_3[e]
1	7.33 ± 0.92	>100	3.46 ± 0.81	>100	14	1	29
1a	59.58 ± 6.14	>100	3.15 ± 1.25	>100	1	1	32
1b	27.60 ± 0.23	>100	3.51 ± 0.00	>100	4	1	29
1c	52.67 ± 2.08	>100	1.30 ± 0.00	>100	1	1	77
1d	31.75 ± 1.25	75.00 ± 3.36	3.17 ± 1.21	>100	3	1	32
1e	73.70 ± 10.66	22.39 ± 3.82	0.20 ± 0.00	>100	1	5	500
2	18.83 ± 1.51	>100	2.00 ± 0.85	>100	5	1	50
2a	29.00 ± 2.67	12.03 ± 2.81	3.46 ± 1.01	>100	4	8	29
2b	9.82 ± 2.34	12.48 ± 2.99	0.40 ± 0.10	>100	12	8	250
2c	6.52 ± 0.35	15.67 ± 3.68	4.93 ± 3.43	>100	15	6	20

2d	10.15 ± 2.57	>100	0.41 ± 0.00	>100	10	1	244
AMB	0.02 ± 0.00	0.020 ± 0.003	0.030 ± 0.006	57.77 ± 3.22			
EM	-	-	-	0.05 ± 0.004			

[a]Represented as the mean ± the standard error of the mean (SEM) from the triplicate biological experiments. [b]African green monkey kidney epithelial cells. [c]Selectivity index: SI_1 = IC_{50} Vero/IC_{50} *L. donovani* 1S; [d]Selectivity index: SI_2 = IC_{50} Vero/IC_{50} *L. donovani* 9515. [e]Selectivity index: SI_3 = IC_{50} Vero/IC_{50} *L. major* IR-173. AMB = Amphotericin B; EM = Emetine.

3 CONCLUSION

A series of novel benzothiaziadine-1,1-dioxide derivatives were synthesized in a two-step process, involving commercial reagents, 2-aminobenzenesulfonamide and benzyl bromides using chemical reaction types, condensation and nucleophilic substitution 2. The derivatives were mostly isolated in poor yields, were assayed for antileishmanial activity *against Leishmania* promastigotes and cytotoxicity on Vero cells.

The derivatives were found to be nontoxic, with significant demonstrated and selective activity against *L. major* parasites, the causative pathogens of CL, while being either moderately active or inactive against *L. donovani*. All derivatives were identified as potential anti-CL hits by virtue of their combined features of high potency and selectivity, the most promising derivative being **1e** that contained a 3-(trifluoromethyl)benzyl moiety. No outright structure-activity relationship could be deduced from the study. Future endeavors will focus on the optimization of the synthesis process to deliver derivatives with higher yields, and the assessment of the current hits for anti-amastigote activity.

4 EXPERIMENTAL

4.1 Chemistry

4.1.1 General

Materials. Reagents were purchased from Sigma-Aldrich. N,N-dimethylformamide (DMF) was purchased from Merck. All other solvents and K_2CO_3 were purchased from Associated Chemical Enterprises (ACE). All chemicals and reagents were reagent/analytical grade and were used without further purification.

Procedures. The ^1H and ^{13}C NMR spectra of all compounds (11 compounds) were recorded on a Bruker Advance III 600 spectrometer at frequencies of 600 (^1H NMR) and 150.913 MHz (^{13}C NMR), respectively, in deuterated dimethyl sulfoxide DMSO-*d*$_6$ at room temperature (~25 °C), or alternatively 80 °C where indicated. Chemical shifts in spectra obtained were expressed and reported in parts per million δ (ppm), with the residual protons of the deuterated solvents serving as reference. *J* (coupling constant) values were expressed in Hz. Abbreviations used to indicate splitting patterns were as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), triplet of doublets (td), and multiplet (m).

HRMS spectra were recorded on a micrOTOF-Q II mass spectrometer, using an APCI source type heated at 200 °C. The software used to record the mass spectra was the Bruker Compass Data Analysis 4.0 software package. Scans were performed from 50-1600 m/z at a capillary voltage of 4500 volts (V), an end plate offset voltage of -500 V, with the nebulizer set at 1.6 Bar, and a collision cell radio frequency (RF) voltage of 100 volts peak to peak (Vpp).

High-performance liquid chromatography (HPLC) was carried out on all compounds to determine their purity. Full description of the process can be found in Supplementary Information file.

IR spectra were recorded on a Bruker Alpha-P FTIR instrument system. Thin-layer chromatography (TLC) was performed using aluminium-backed silica gel plates from Merck (Johannesburg, South Africa) with a mean pore size of 60 Å with added fluorescent indicator (F254). TLC plates were visualized at 254 nm. Melting points of all compounds were determined on a Büchi melting point B-545 apparatus and are uncorrected.

4.1.2 General procedure for the synthesis of of 3-substituted benzothiadiazine-1,1-dioxide intermediates 1 - 2

A literature method reported by Bozdag *et al.*[12] was adopted to synthesize these intermediates. It is described as follows: 2-Aminobenzene sulfonamide (29 mmol, 5 g, 1eq.) was suspended in triethyl orthoacetate or orthobenzoate (5 eq.) and refluxed at 100 °C for 24-36 hours. The reaction was monitored by TLC until consumption of the limiting starting material. Upon completion, the reaction mixture was cooled at room temperature, resulting in the formation of a precipitate. After filtration, the precipitate was thoroughly washed with diethyl ether (Et_2O) (3x20 ml) and dried under vacuum to afford the target intermediate.

*3-Methyl-2H-benzo [e][1,2,4]thiadiazine 1,1-dioxide (**1**)*

The reaction of aminobenzene sulphonamide with in triethyl orthoacetate afforded **1**. Cream powder; yield: 5.4 g (95%); m.p. 270 °C. IR ν_{max} (cm^{-1}): 3368 (N-H), 1602 (C=N), 1509 (Ar, C=C), 1397 (C-CH_3), 1372 (S=O), 1158 (C-N). ^{1}H NMR (600 MHz, DMSO) δ 12.01 (s, 1H, H-2, N-H), 7.79 (d, *J* = 7.8 Hz, 1H, Ar-H, H-8), 7.67 (t, *J* = 7.8 Hz, 1H, Ar-H, H-7), 7.43 (t, *J* = 7.6 Hz, 1H, Ar-H, H-5), 7.30 (d, *J* = 7.6 Hz, 1H, H-6, Ar-H), 2.30 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, DMSO) δ 157.7 (C-3, *C*-CH_3), 135.7 (Ar, C-3a), 133.5 (Ar, C-6), 126.6 (Ar, C-8), 123.9 (Ar, C-8a), 121.5 (Ar, C-7), 117.7 (Ar, C-5), 23.1 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 197.0380 (Calc. for $C_8H_9N_2O_2S$: 197.0385). Purity (%): 97.

*3-Phenyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**2**)*

The reaction of aminobenzene sulphonamide with in triethyl orthobenzoate afforded **2**. White powder; yield: 6.7 g (98%); m.p. 310 °C. IR ν_{max} (cm^{-1}): 3297 (N-H), 1639 (C=N), 1433 (Ar, C=C), 1282 (S=O), 1141 (C-N). ^{1}H NMR (600 MHz, DMSO) δ 12.21 (s, 1H, H-2, N-H), 8.05 (d, *J* = 7.5 Hz, 2H, H-2', Ar-H), 7.88 (d, *J* = 7.9 Hz, 1H, H-8, Ar-H), 7.77 – 7.73 (m, 1H, H-5, Ar-H), 7.71 (t, *J* = 7.9 Hz, 1H, H-7, Ar-H), 7.64 (t, *J* = 7.5 Hz, 3H, H-3' & 4', Ar-H), 7.52(t, *J* = 7.3 Hz 1H, H-6, Ar-H). ^{13}C NMR (151 MHz, DMSO) δ 155.3 (Ar, C-3), 136.0 (Ar, C-3a), 133.5 (Ar, C-6), 133.3 (Ar, C-4'), 132.3 (Ar, C-8a), 129.3 (Ar, C-3'), 128.7 (Ar, C-2'), 127.2 (Ar, C-8), 123.8 (Ar, C-1'), 121.9 (Ar, C-7), 119.0 (Ar, C-5). HRMS-APCI *m/z*: $[M+H]^+$ 259.0553 (Calc. for $C_{13}H_{11}N_2O_2S$: 259.0541). Purity (%): 98.

4.1.3 General procedure for the synthesis of of 2,3-substituted benzothiadiazine-1,1-dioxide derivatives 1a-e - 2a-d

A general procedure (Scheme 1 – step ii) was used to prepare benzothiadiazine-1,1-dioxide derivatives, and is detailed as follows:
A mixture of intermediate **1** or **2** (1eq.), alkyl/halide substituted benzyl bromide (2 eq.), K_2CO_3 (2 eq.) and DMF (3-4 mL) was stirred at room temperature or 90 °C for 24 -36 hours, under nitrogen (N_2). The reaction was monitored by TLC and upon completion of the limiting reagents, the mixture was diluted with aqueous NH_4Cl (10 mL) and extracted with DCM (3 x 20 mL). The organic phase was dried over $MgSO_4$ overnight. Afterwards, it was filtered off, concentrated under vacuum to afford a clear crude mixture which was subjected to further purification by either recrystallization or column chromatography afford the title compounds.

*2-Benzyl-3-methyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**1a**)*

The reaction of **1** (2 mmol, 400 mg, 1 eq.) and benzyl bromide afforded **1a** after recrystallization in ethyl acetate – hexane mixture. White powder; yield: 110 mg (19%); m.p. 172 °C. IR ν_{max} (cm^{-1}): 2918 (Ar, C-H), 1628 (C=N), 1582 (Ar, C=C), 1403 (S=O), 1295 (C-N). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 (dd, *J* = 7.8, 1.6 Hz, 1H, H-8, Ar-H), 7.48 (ddd, *J* = 7.8, 7.7, 1.6 Hz, 1H, H-7, Ar-H), 7.42 (ddd, *J* = 7.7, 3.7, 1.6 Hz, 1H, H-5, Ar-H), 7.39 (d, *J* = 7.7 Hz, 1H, H-6, Ar-H), 7.34 (t, *J* = 7.3 Hz, 2H, H-5', Ar-H), 7.14 (d, *J* = 7.3 Hz, 2H, H-4', Ar-H), 7.06 (d, *J* = 8.6 Hz, 1H, H-6', Ar-H), 5.27 (s, 2H, H-2', CH_2), 2.48 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 159.7 (Ar, C-3), 137.4 (Ar, C-3a), 133.4 (Ar, C-4'), 133.1 (Ar, C-6), 129.6 (Ar, C-8), 128.4 (Ar, C-5'), 126.5 (Ar, C-7), 125.2 (Ar, C-6'), 123.7 (Ar, C-4'), 116.3 (Ar, C-5), 51.9 (C-2', CH_2), 24.7 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 287.1117 (Calc. for $C_{15}H_{15}N_2O_2S$: 287.0854). Purity (%): 100.

*2-(4-Fluorobenzyl)-3-methyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**1b**)*

The reaction of **1** (2 mmol, 400 mg, 1 eq.) and 4-fluorobenzyl bromide resulted in **1b** which was purified by recrystallization in ethyl acetate – hexane mixture. White powder; yield: 152 mg (25%); m.p. 181 °C. IR ν_{max} (cm^{-1}): 1634 (C=N), 1646 (C=C), 1397 (S=O), 1293 (C-N), 1165 (C-F). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (d, *J* = 7.8 Hz, 1H, H-8, Ar-H), 7.52 (t, *J* = 7.8 Hz, 1H, H-7, Ar-H), 7.45 (t, *J* = 7.5 Hz, 1H, H-6, Ar-H), 7.25-7.07 (m, 4H, H-4', -5', Ar-H), 7.05 (d, *J* = 7.5 Hz, 1H, H-5, Ar-H), 5.27 (s, 2H, H-2', CH_2), 2.50 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.3 (Ar, C-6', *C*-F), 161.7 (Ar, C-3'), 159.6 (Ar, C-3), 137.2 (Ar, C-3a), 133.1 (Ar, C-6), 129.1 (Ar, C-8a), 127.0 (Ar, C-8), 126.6 (Ar, C-7), 125.2 (Ar, C-4'), 123.8 (Ar, C-5), 116.8 (Ar, C-4'), 116.7 (Ar, C-5'), 51.2 (C-2', CH_2), 24.6 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 305.0778 (Calc. for $C_{15}H_{14}FN_2O_2S$: 305.0760). Purity (%): 96.

*2-(4-Bromobenzyl)-3-methyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**1c**)*

1 (2 mmol, 400 mg, 1 eq.) and 4-bromobenzyl bromide afforded **1c** after recrystallization in ethyl acetate – hexane mixture. Creamy brown powder; yield: 109 mg (15%); m.p. 271 °C. IR ν_{max} (cm^{-1}): 2921 (Aryl, C-H), 2851 (Alkyl, C-H), 1598 (C=N), 1568 (C=C), 1399 (S=O), 1298 (C-N), 755 (C-Br). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (dd, *J* = 7.8, 1.2 Hz, 1H, H-8, Ar-H), 7.55 (d, *J* = 8.4 Hz, 2H, H-5', Ar-H), 7.52 (d, *J* = 8.6 Hz 1H, H-5, Ar-H), 7.46 (t, *J* = 7.6 Hz, 1H, H-7, Ar-H), 7.05 (d, *J* = 8.4 Hz, 2H, H-4', Ar-H), 7.02 (d, *J* = 8.6 Hz, 1H, H-6, Ar-H), 5.24 (s, 2H, H-2', CH_2), 2.49 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 159.5 (Ar, C-3), 137.1 (Ar, C-3a), 133.2 (Ar, C-6), 132.8 (Ar, C-3'), 132.5 (Ar, C-5'), 126.9 (Ar, C-4'), 126.7 (Ar, C-8), 125.3 (Ar, C-7), 123.8 (Ar, C-6'), 116.0 (Ar, C-5), 51.4 (C-2', CH_2), 24.6 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 366.9932 (Calc. for $C_{15}H_{14}BrN_2O2S$: 366.9939). Purity (%): 82.

*2-(3-Bromobenzyl)-3-methyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**1d**)*

The reaction of **1** (2 mmol, 400 mg, 1 eq.) and 3-bromobenzyl bromide afforded **1d** after recrystallization in ethyl acetate – hexane mixture. Cream white powder; yield: 86 mg (12%); m.p. 204 °C. IR ν_{max} (cm^{-1}): 1588 (C=N), 1546 (C=C), 1402 (S=O), 1285 (C-N), 783 (C-Br). ^{1}H NMR (600 MHz, $CDCl_3$) δ

8.06 (dd, *J* = 7.8, 1.1 Hz, 1H, H-8, Ar-H), 7.53 (dd, *J* = 11.4, 4.1 Hz, 1H, H-6', Ar-H), 7.51 (d, *J* = 8.2 Hz, 1H, H-5, Ar-H), 7.47 (t, *J* = 7.8 Hz, 1H, H-7, Ar-H), 7.36 (s, 1H, H-4', Ar-H), 7.29 (dd, *J* = 8.2, 1.1 Hz, 1H, H-6, Ar-H), 7.06 (d, *J* = 8.6 Hz, 1H, H-7', Ar-H), 7.03 (d, *J* = 8.6 Hz, 1H, H-8', Ar-H), 5.26 (s, 2H, H-2', CH_2), 2.50 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 159.4 (C-3), 137.1 (C-3a), 135.9 (C-3'), 133.2 (C-6), 131.8 (C-4'), 131.2 (C-6'), 128.4 (C-8), 126.7 (C-7'), 125.3 (C-8'), 123.9 – 123.6 (Ar-C), 116.0 (C-5), 51.3 (C-2', *C*H_2), 24.6 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 366.9932 (Calc. for $C_{15}H_{14}BrN_2O2S$: 366.9939). Purity (%): 100.

*3-Methyl-2-[3-(trifluoromethyl)benzyl]-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**1e**)*

1 (2 mmol, 400 mg, 1 eq.) and 3-(trifluoromethyl)benzyl bromide (2 eq.) afforded **1e** after recrystallization in ethyl acetate – hexane mixture. Cream white powder; yield: 68 mg (9%); m.p. 122 °C. IR ν_{max} (cm^{-1}): 1581 (C=N), 1511 (C=C), 1328 (S=O), 1111 (C-F). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.9 Hz, 1H, H-8, Ar-H), 7.77 – 7.67 (m, 1H, H-4', Ar-H), 7.63 – 7.54 (m, 2H, H-5 & -7, Ar-H), 7.50 (dt, *J* = 17.9, 6.1 Hz, 4H, H-5, -6', -7' & 8', Ar-H), 5.20 (s, 2H, H-2', CH_2), 2.41 (s, 3H, H-1', CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 154.2 (Ar, C-3), 142.1 (Ar, C-3a,), 136.6 (Ar, C-3'), 133.9 (Ar, C-6), 131.5 (Ar, C-5'), 130.0 (Ar, C-7), 127.4 (Ar, C-8), 125.0 (C-9', *C*-F), 123.3 (Ar, C-6'), 121.5 (Ar, C-5), 46.7 (C-2', *C*H_2), 23.6 (C-1', *C*H_3). HRMS-APCI *m/z*: $[M+H]^+$ 355.0696 (Calc. for $C_{16}H_{14}F_3N_2O_2S$: 355.0728). Purity (%): 81.

*2-Benzyl-3-phenyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide (**2a**)*

The reaction of **2** (1.6 mmol, 400 mg, 1 eq.) and benzyl bromide afforded **2a** after recrystallization in hexane-ethyl acetate – methanol mixture. Cream white powder; yield: 137 mg (25%); m.p. 107 °C. IR ν_{max} (cm^{-1}): 1550 (C=N), 1511 (C=C), 1334 (S=O), 1178 (C-N). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.90 (d, *J* = 7.8 Hz, 1H, H-8, Ar-H), 7.80 (d, *J* = 7.5 Hz, 2H, H-2', Ar-H), 7.66 (t, *J* = 7.8 Hz, 1H, H-7, Ar-H), 7.60 (t, *J* = 7.5 Hz, 2H, H-5 & -6, Ar-H), 7.50 (dd, *J* = 13.0, 6.8 Hz, 3H, H-3' & -4', Ar-H), 7.11 (t, *J* = 7.3 Hz, 1H, H-9', Ar-H), 7.04 (t, *J* = 7.5 Hz, 2H, H-8', Ar-H), 6.74 (d, *J* = 7.5 Hz, 2H, H-7', Ar-H), 5.01 (s, 2H, H-5', CH_2). ^{13}C NMR (151 MHz, $CDCl_3$) δ 155.8 (Ar, C-3), 142.6 (Ar, C-3a), 135.0 (Ar, C-3'), 133.9 (Ar, C-8a), 133.3 (Ar, C-6), 131.4 (Ar, C-4'), 129.3 (Ar, C-3'), 128.9 (Ar, C-8), 128.6 (Ar, C-2'), 128.3 (Ar, C-8'), 128.0 (Ar, C-7), 127.6 (Ar, C-7'), 121.2 (Ar, C-5), 51.4 (C-5', *C*H_2). HRMS-APCI *m/z*: $[M+H]^+$ 349.1030 (Calc. for $C_{20}H_{17}N_2O_2S$: 349.1011). Purity (%): 99.

*2-(4-Fluorobenzyl)-3-phenyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide, **2b***

The reaction of **2** (1.6 mmol, 400 mg, 1 eq.) and 4-fluorobenzyl bromide afforded **2b** after purification by column chromatography on silica gel with hexane-petroleum ether (3:2) as eluent. White powder; yield: 303 mg (41%); m.p. 156 °C. IR ν_{max} (cm^{-1}): 2919 (Aryl, C-H), 1743 (C=N), 1339 (S=O), 1219 (C-N), 1177 (C-F). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07 (d, *J* = 7.8 Hz, 1H, H-8, Ar-H), 7.63 (t, *J* = 13.0 Hz, 2H, H-2', Ar-H), 7.60 – 7.53 (m, 4H, H-5, -7 & 3', Ar-H), 7.49 (t, *J* = 7.5 Hz, 3H, H-4' & 8', Ar-H), 7.27 (d, *J* = 8.7 Hz, 2H, H-7', Ar-H), 7.23 (d, *J* = 8.5 Hz, 1H, H-6, Ar-H), 5.38 (s, 2H, CH_2). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.6 (Ar, C-3), 138.6 (Ar, C-9', *C*-F), 137.2 (Ar, C-3a), 133.2 (C-6), 133.0 (Ar, C-8a), 132.0 (Ar, C-6'), 129.0 (Ar, C-4'), 128.8 (Ar, C-7'), 127.1 (Ar, C-8), 126.5 (Ar, C-3'), 126.2 (Ar, C-7'), 125.0 (Ar, C-2'), 117.2 (Ar, C-6'), 53.6 (C-5', *C*H_2). HRMS-APCI *m/z*: $[M+H]^+$ 367.0935 (Calc. for $C_{20}H_{16}FN_2O_2S$: 367.0917). Purity (%): 96.

*2-(4-Bromobenzyl)-3-phenyl-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide, **2c***

The reaction of **2** (1.55 mmol, 400 mg, 1 eq.) and 4-bromobenzyl bromide (3.1 mmol, 2 eq.) afforded **2c** after recrystallization in hexane-ethyl acetate mixture. Yellow powder; yield: 47 mg (7%); m.p. 160 °C. IR ν_{max} (cm^{-1}): 1585 (C=N), 1515 (C=C), 1386 (S=O), 1168 (C-N), 778 (C-Br). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (d, *J* = 7.8 Hz, 1H, H-8, Ar-H), 7.64 (d, *J* = 7.3 Hz, 2H, H-8', Ar-H), 7.59 – 7.53 (m, 2H, H-2', Ar-H), 7.48 (td, *J* = 8.5, 7.8, 2.7 Hz, 3H, H-7, -5 & -4', Ar-H), 7.43 (d, *J* = 8.4 Hz, 2H, H-3', Ar-H), 7.23 (d, *J* = 8.5 Hz, 1H, H-6, Ar-H), 7.02 (d, *J* = 7.3 Hz, 2H, H-7', Ar-H), 5.27 (s, 2H, H-5', CH_2). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.5 (Ar, C-3), 137.3 (Ar, C-3a), 133.6 (Ar, C-6'), 133.3 (Ar, C-6), 132.9 (Ar, C-8a), 132.4 (Ar, C-8'), 131.9 (Ar, C-4'), 128.9 (Ar, C-7'), 127.8 (Ar, C-3'), 127.0 (Ar, C-8), 125.0 (Ar, C-7), 122.2 (Ar, C-9', *C*-Br), 117.4 (Ar, C-5), 53.6 (C-5', CH_2). HRMS-APCI *m/z*: $[M+H]^+$ 429.0127 (Calc. for $C_{20}H_{16}BrN_2O_2S$: 429.0095). Purity (%): 100.

*3-Phenyl-2-[4-(trifluoromethyl)benzyl]-2H-benzo[e][1,2,4]thiadiazine 1,1-dioxide, **2d***

The reaction of **2** (1.55 mmol, 400 mg, 1 eq.) and 4-(trifluoromethyl)benzyl bromide (3.1 mmol, 2 eq.) afforded **2d** after recrystallization in hexane-ethanol-ethyl acetate mixture. White powder; yield: 50 mg (5%); m.p. 150 °C. IR ν_{max} (cm^{-1}): 2922 (Aryl, C-H), 1724 (C=N), 1567 (C=C), 1314 (S=O), 1167 (C-N), 1113 (C-F). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07 (d, *J* = 7.8 Hz, 1H, H-8, Ar-H), 7.63 (d, *J* = 5.8 Hz, 2H, H-2', Ar-H), 7.60 – 7.53 (m, 4H, H-5, -7, Ar-H), 7.49 (t, *J* = 7.5 Hz, 3H, H-3' & H-4', Ar-H), 7.27 (d, *J* = 8.5 Hz, 2H, H-8', Ar-H), 7.23 (d, *J* = 8.5 Hz, 2H, H-7', Ar-H), 5.38 (s, 2H, H-5', CH_2). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.6 (Ar, C-3), 138.6 (Ar, C-6'), 133.2 (Ar, C-6), 128.8 (Ar, C-8'), 127.1 -126.5 (Ar, C-8, -3', -7') 125.0 (Ar, C-10', C-F), 117.2 (C-5), 53.57 (C-5', CH_2). HRMS-APCI *m/z*: $[M+H]^+$ 417.0923 (Calc. for $C_{21}H_{16}F3N_2O2S$: 417.0885). Purity (%): 82.

4.2 Pharmacological assays

4.2.1 Antileishmanial activity

Anti-promastigote activity was evaluated using the resazurin assay on three *Leishmania* strains. The assay involves the irreversible enzymatic reduction of oxidized blue resazurin dye into a pink, highly fluorescent resorufin, by viable cells.[24] *L. donovani* (strains 1S (MHOM/SD/62/1S) and 9515 (MHOM/IN/95/9515)) and *L. major* (strain IR-173 (MHOM/IR/-173)) promastigotes were cultured in M199 with Hank's salts and 0.68 mM L-glutamine, supplemented with 4.2 mM sodium bicarbonate, 25 mM Hepes (all from Sigma Aldrich), 10% fetal bovine serum (Thermofisher Scientific) and 50 U/mL penicillin/streptomycin solution (Lonza), and the pH adjusted to 7.3-7.4. The promastigotes were maintained at 26 °C. For the resazurin assay, logarithmic phase promastigotes (1.25×10^5 cells/mL, 100 µL/well) were seeded in 96-well plates (Nunc, Thermofisher Scientific) in the presence of twelve x two-fold dilution concentrations of compounds for IC_{50} determinations. Amphotericin B (10 µM) served as the standard drug, while growth medium without any parasites served as the blank. The plates were incubated for 72 h at 26 ºC in a humidified atmosphere.

After incubation, 50 µL of resazurin solution (0.01% in phosphate buffered saline (PBS, Sigma-Aldrich)) was added to each well and the plates were further incubated at 26 °C in the dark for 2 h (1S promastigotes) to 4 h (9515 and IR-173 promastigotes). Absorbances were measured at 570 nm and 600 nm, using the Thermofisher Scientific GO Multiscan plate reader. Data analysis was performed for each biological replicate, using SkanIt 4.0 Research Edition software. The background absorbance of resazurin (600 nm) was subtracted from the absorbance values of resorufin (570 nm). The mean absorbance and cell viability were determined by the following equations:

Cell viability % = (Δ Abs sample – Δ Abs blank) / (Δ Abs neg control – Δ Abs blank) x 100

The IC_{50} and Z-score were determined for each compound's three biological replicates, using GraphPad Prism 5. The mean IC_{50} of the biological replicates served as the final value of each compound.

4.2.2 Cytotoxicity

Cytotoxicity was evaluated using the resazurin assay.[25] Vero cells (Cellonex, South Africa) were cultured in Hyclone Dulbecco's modified Eagle's medium (DMEM) with high glucose (Separations), supplemented with 10% fetal bovine serum (Thermofisher Scientific) and 1% L-glutamine, penicillin-streptomycin, amphotericin B and non-essential amino acids (all from Lonza). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For the resazurin assay, 96-well plates were prepared with 200 µL of cell suspension (30,000 cells/mL) and incubated for 24 hours. The cells were then treated with: (i) 100 µL of emetine dihydrochloride (Sigma Aldrich) solution, diluted with growth medium to the necessary concentrations (positive control); (ii) 80 µL of growth medium and 20 µL of solvent (negative control to compensate for possible solvent effects); (iii) 80 µL of growth medium and 20 µL of diluted experimental compound solutions. The blank controls contained growth medium without cells. The treated plates were incubated for 48 h.

To initiate the resazurin assay, 50 µL of sterile-filtered resazurin sodium salt solution (0.01% in PBS) was added and the plates incubated for 2 hours. Absorbances were measured at 570 nm and 600 nm, using the Thermofisher Scientific GO Multiscan plate reader. Data analysis was performed for each biological replicate, using SkanIt 4.0 Research Edition software. The background absorbance (600 nm) was subtracted from the absorbance values (570 nm), the mean absorbance calculated and the percentage cell viability was determined by the following equation:

Cell viability % = (Δ Abs sample – Δ Abs blank) / (Δ Abs neg control – Δ Abs blank) x 100

The IC_{50} and Z-score were determined for each compound's three biological replicates, using GraphPad Prism 5. The mean IC_{50} of the biological replicates served as the final value with standard error of the means (SEM) of each compound.

4.2.3 Statistical analysis

In vitro antileishmanial activities and cytotoxicity, indicated as IC_{50} values, were derived from non-linear regression analysis. Results were represented as the mean ± the standard error of the mean (SEM) from the triplicate biological experiments. Statistical analysis was performed, using SkanIt 4.0 Research Edition software (Thermofisher Scientific) and Prism V5 software (GraphPad). All reported data was significant at $p < 0.05$.

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Disclaimer

Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

The following reagents were obtained through BEI Resources, NIAID, NIH:

L. donovani, Strain 1S (MHOM/SD/62/1S), NR-48821

L. donovani, Strain 9515 (MHOM/IN/95/9515), NR-48822

L. major, Strain IR173 (MHOM/IR/-173), NR-48816

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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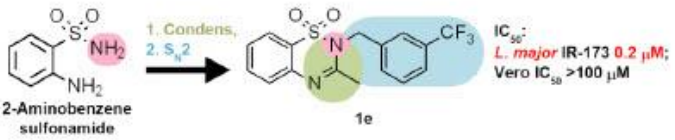

The conversion of 2-aminobenzenesulfonamide, involving condensation and nucleophilic substitution type 2 reactions, resulted in several antileishmanial active benzothisdiazine-1,1-dioxide derivatives. The *meta*-trifluoromethylbenzene moiety-containing derivative, 1e, was the most effective of all against the promastigotes of *Leishmania major*, a *Leishmania* species that causes the most common disease presentation of cutaneous leishmaniasis in humans.

CHAPTER 4

SUMMARY AND CONCLUSION

Leishmaniasis is an emerging category-1 NTD (Deribew *et al*., 2017:3) that continues to progress in poverty-stricken regions of developing countries (Boisson *et al*., 2016:19). Although previously deemed as non-research-worthy disease in many countries (Hailu *et al*., 2016: 142), the fact that leishmaniasis annually claims approximately 20 000 to 30 000 lives (PAHO, 2017) in almost 90 tropical and subtropical countries (Akbari *et al*., 2017: 86; Molyneux *et al*., 2017:312), is an indication that leishmaniasis is deadly.

Pentavalent antimonials (Haldar *et al*., 2011:2-3), amphotericin B (Ouellette *et al.*, 2004:257&261), miltefosine (Branco *et al*., 2016:4), pentamidine (Yang *et al*., 2016:6828-6829) and paromomycin (Bhargava & Singh, 2012:5) are currently the only clinically approved medications for treatment of the various form of leishmanial infection. However, the challenge with these drugs is their toxicity, administration method, expensiveness (Hendrickx *et al*., 2019:2743-2744) and emerging resistance by several *Leishmania* species (spp.) (Bouyahya, *et al*., 2018:51). As part of efforts to contribute towards the research and development of new antileishmanial compounds, various Schiff bases and their mechanisms of actions have been studied. For the purpose of this study, focus was placed on thiazine Schiff base scaffolds, particularly benzothiazine-1,1-dioxide derivatives.

HCT is an orally administered (Herman & Bashir, 2019) benzothiadiazine-1,1-dioxide antidiuretic and a potent inhibitor of CA (Syrjänen *et al*., 2013:7375). It promotes the production of ROS and NOS, which have shown *in vitro* toxicity towards *L. donovani* spp. CA is present in human red blood cells (Frost, 2014;9) and the inhibition thereof has been shown to significantly reduce leishmanial survival by predisposing intracellular parasites to macrophage digestion through acid production (Syrjänen *et al*., 2013:7375). Oral CA inhibitors are, therefore, well distributed to the bloodstream, can be absorbed by infected macrophages and induce parasiticidal effects (Herman & Bashir, 2019). The goal of developing orally administered antileishmanial drugs that inhibit

promastigote replication in human macrophages is, therefore, achievable. HCT is very stable and can withstand an acidic environment resulting from ROS and NOS production within macrophages. The bonding on the sulfonamide group -SO_2NHR allows HCT the ability to remain intact (Javorskis & Orentas, 2017:13423; Alonso & Ájera, 2009:369-370) within stomach cells where the pH is 2 (Kinoshita *et al.*, 2018:182). Since another goal is to also prevent side effects, nitrogen and sulphur atoms within the heterocyclic moiety reduce toxicity (Gupta *et al.*, 2018:1099).

The aims of this study were to synthesise and assess the *in vitro* antileishmanial activities of benzothiadiazine-1,1-dioxide derivatives against *L. donovani* (1S and 9515) and *L. major* (IR-173) strains. In the synthesis of methyl and phenyl substituted benzothiadiazine-1,1-dioxide intermediates (compound **1** and **2**), a modified protocol by Bozdag *et al*. (2017:682) was used. Derivatives (compounds **1a**-**1e** and **2a**-**2d**) were prepared via nucleophilic substitutions with alkyl/ aryl bromides at position N-3 of benzothiadiazine-1,1-dioxide moiety. Monosubstituted compounds **1** and **2** were prepared in good yields, 99% and 98%, respectively, while the 2,3-disubstituted derivatives were obtained in poor yields (5 – 41%) after purification through silica chromatography and/or recrystallisation. The structures of all compounds were confirmed through routine characterization techniques, e.g. ^{1}H NMR ,^{13}C NMR, HRMS and IR.

These compounds were predicted to be druglike, highly absorbed in the gastrointestinal tract following compliance to Lipinski's rule of 5. These properties qualify them for further investigations for their usage as oral antileishmanial drugs. Methyl substituted benzothiadiazine-1,1-dioxide derivatives (sub-series 1) showed improved lipophilicity with clogP varying in the 2.5 to 3.6 range, but resulted in poor activity against *L. donovani* (1S strain) and potency against *L. major* (IR-173 strain) with IC_{50} in the ranges 27.60 -73.70 µM and 0.20-3.51 µM, respectively.

On the other hand, phenyl substituted benzothiadiazine-1,1-dioxide derivatives (sub-series 2) showed high lipophilicity (clogP = 3.61- 4.57) and potency against both 1S and IR-173 with IC_{50} in the ranges 6.52 - 10.15 µM and 0.41- 4.93 µM, respectively for compounds **2b**, **2c** and **2d**. Compound **2a** showed poor activity against 1S but was potent against IR-173. The derivatives **2b** – **2d** para- substituted with Br, F and CF_3, respectively, were shown to possess activity against 1S as previously reported by Chauhan *et al*. (2015:351). Furthermore, all the compounds from both sub-series showed no toxicity towards Vero cells, which is in accordance with the literature (Gupta *et al.*, 2018:1099). Similarly, the activity of the compounds against an *L. major* strain corroborate previous findings by Syrjänen *et al*., (2013) on the antileishmanial activity of the benzothiadiazine scaffold.

In conclusion, the antileishmanial activity of benzothiadiazine-1,1-dioxide ring can successfully be improved through structural modifications at its carbons C-2 and C-3 positions, resulting in promising novel 2,3-disubstituted derivatives. Thus, this derivatisation strategy may pave the way for the development of potential novel orally administrable and non-toxic antileishmanial drugs based on the benzothiadiazine-1,1-dioxide scaffold.

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Supplementary Information

Synthesis and *in vitro* antileishmanial efficacy of novel benzothiadiazine-1,1-dioxide derivatives

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ORCID ID: 0000-0002-2327-0551

3-Methyl-2*H*-benzo [*e*][1,2,4]thiadiazine 1,1-dioxide, **1**

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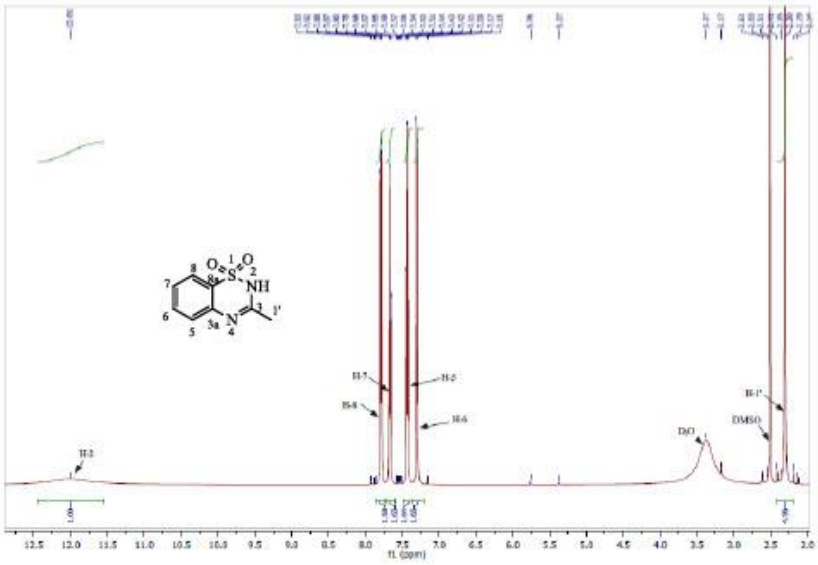

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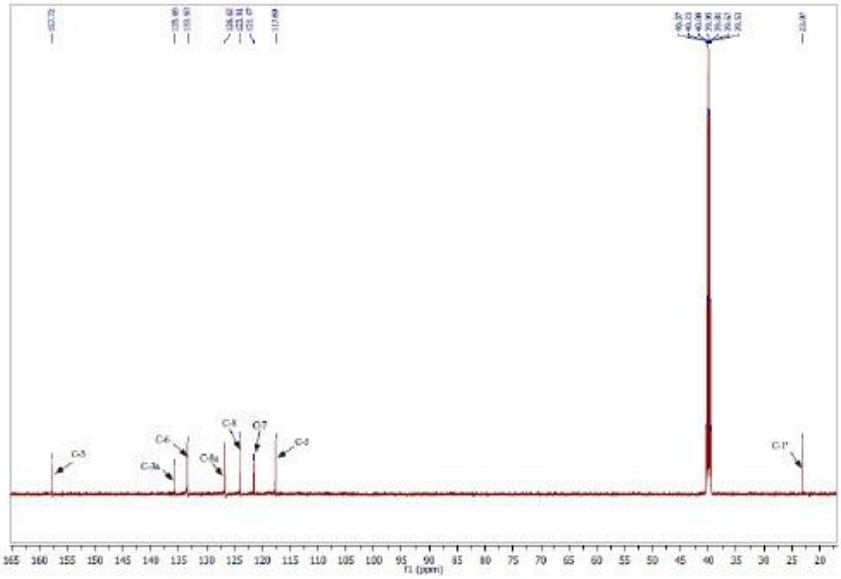

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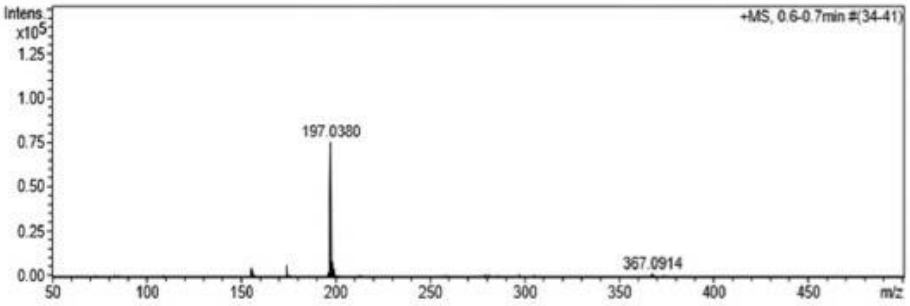

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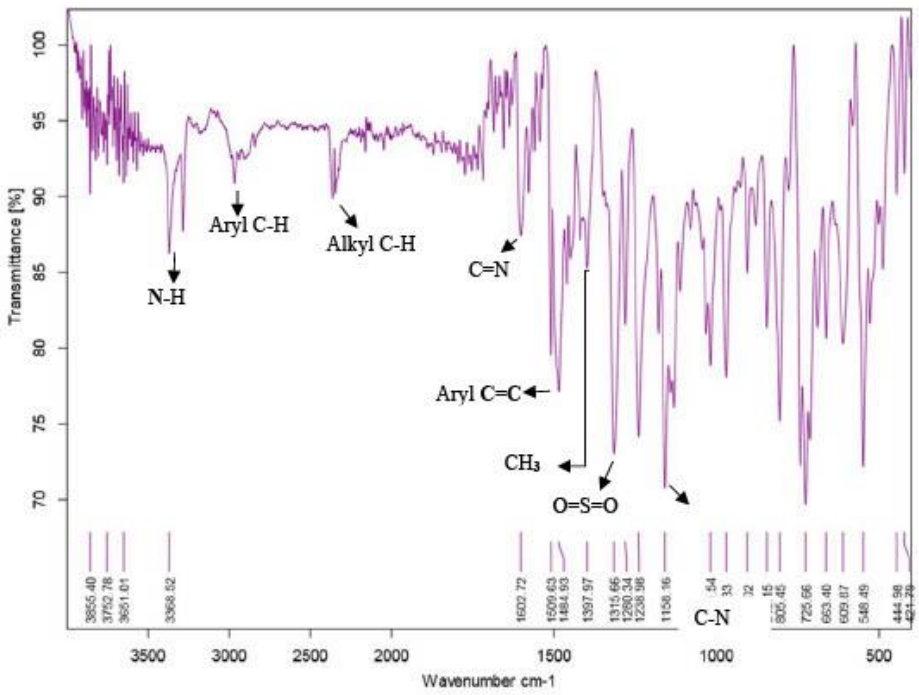

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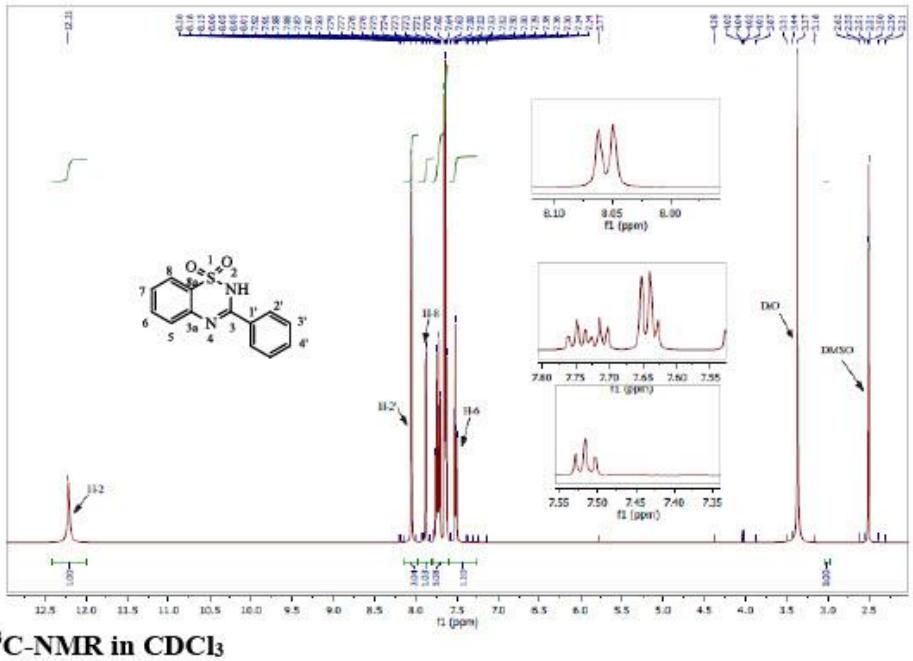

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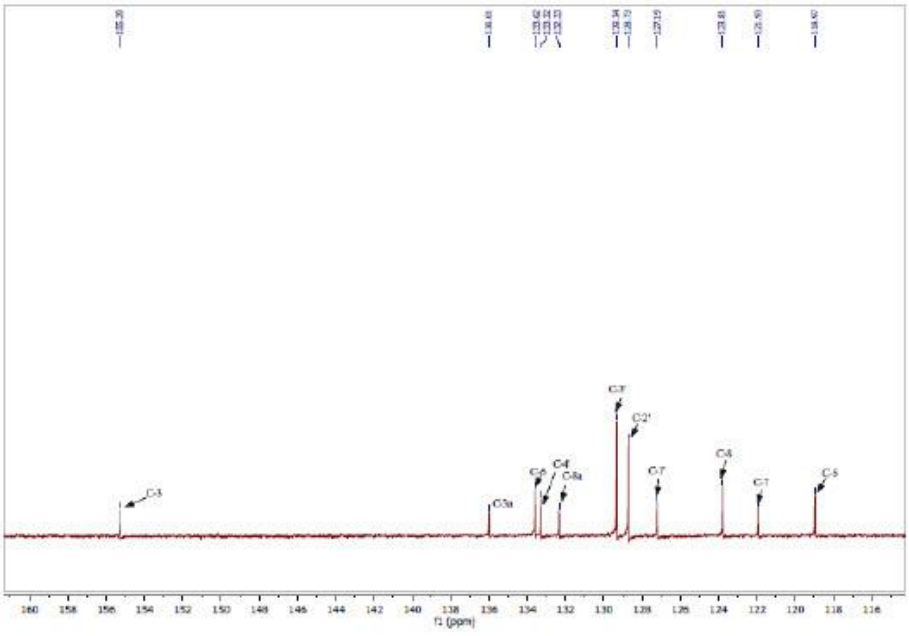

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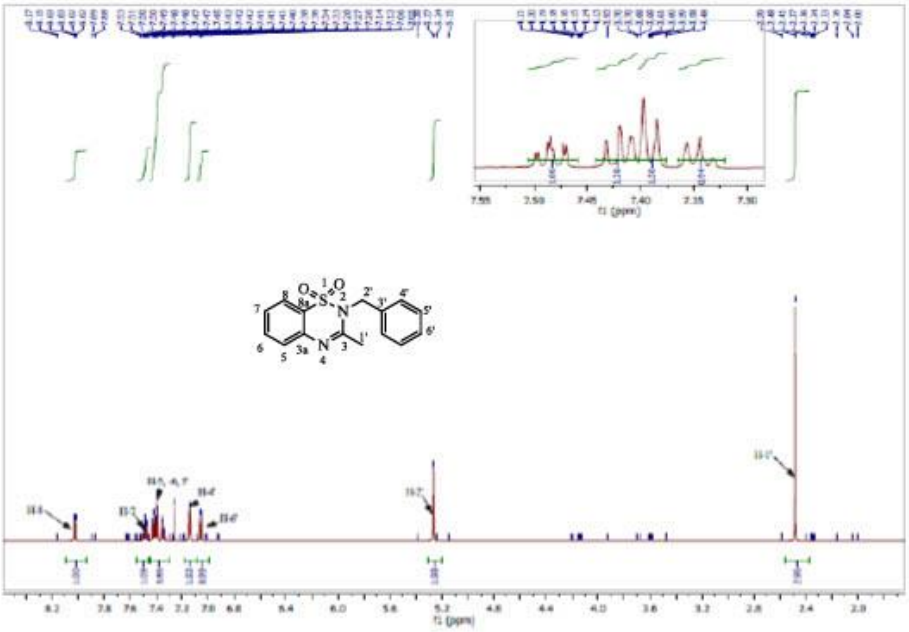

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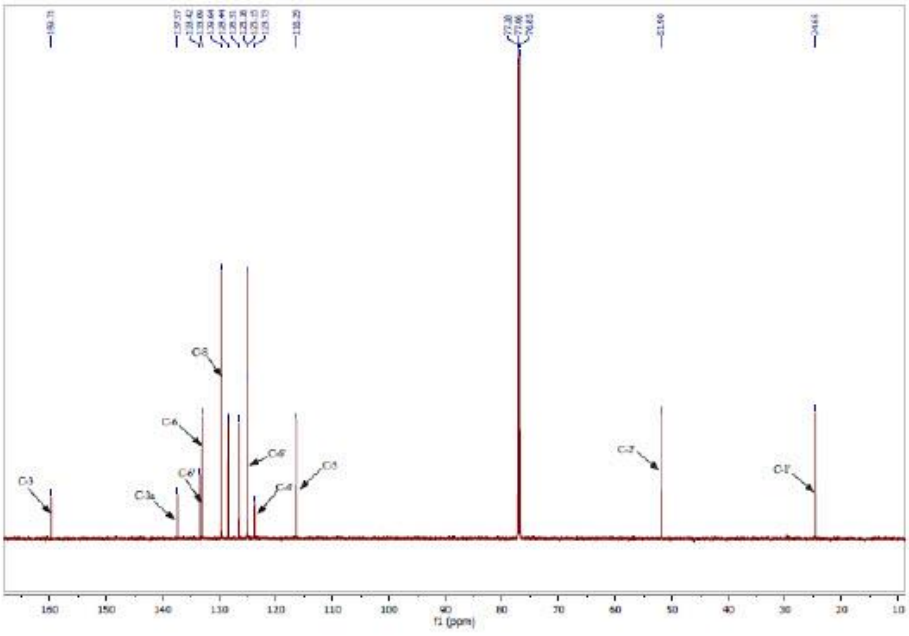

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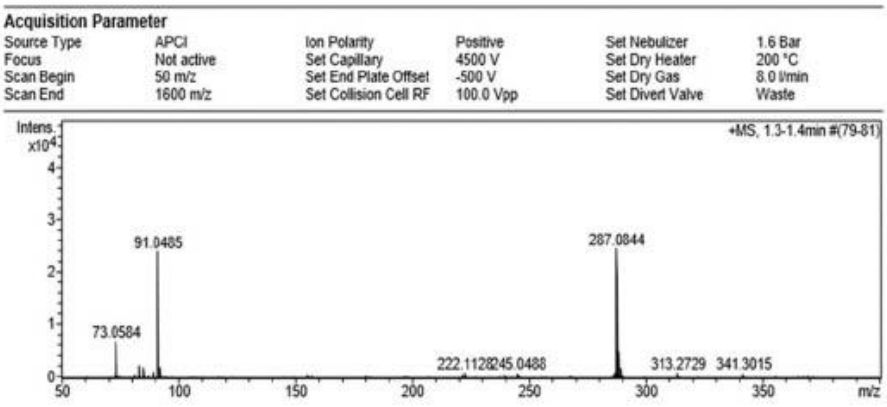
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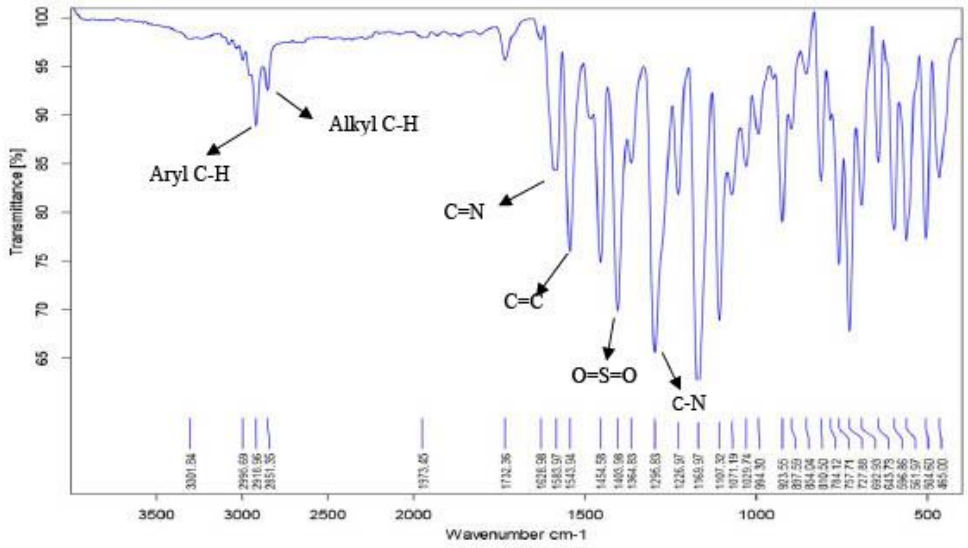
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Wavenumber cm-1

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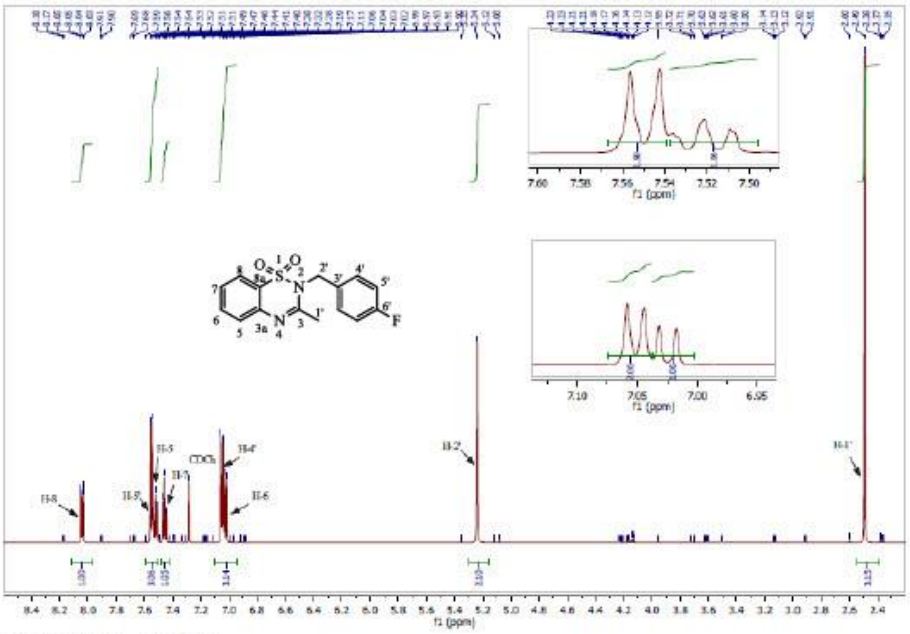

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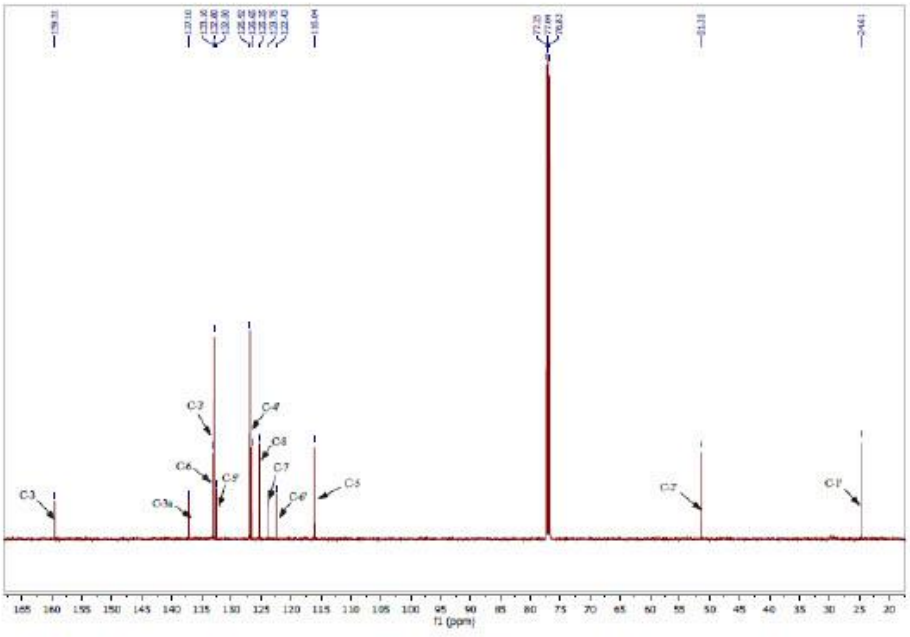

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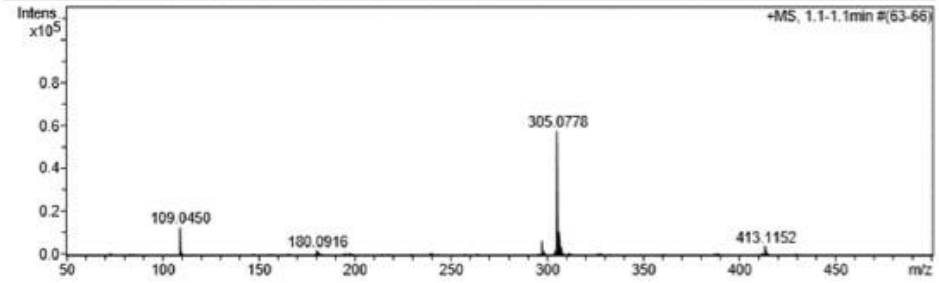

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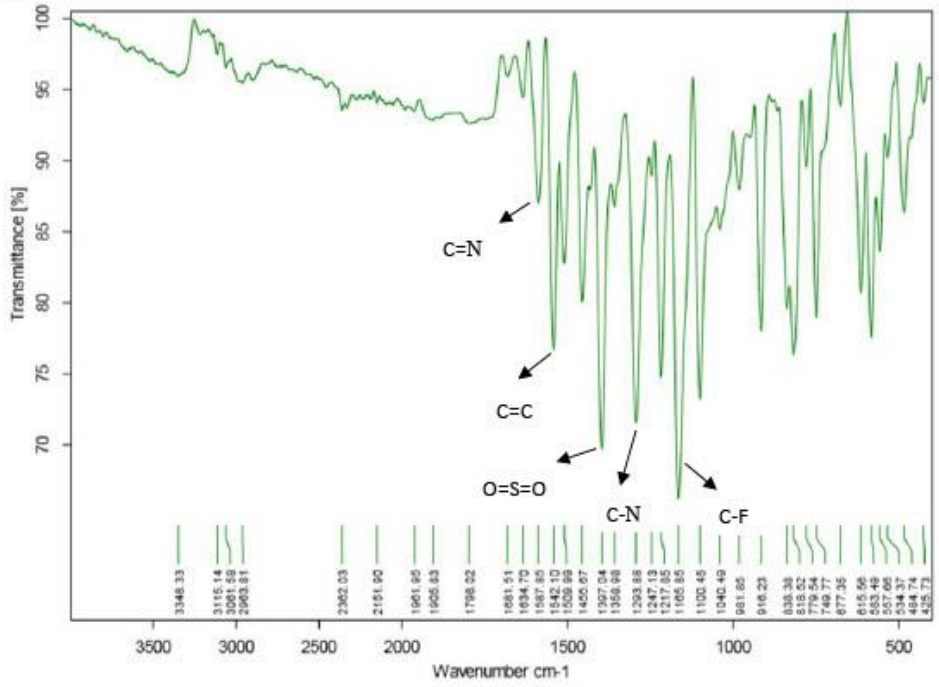

2-(4-Bromobenzyl)-3-methyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **1c**

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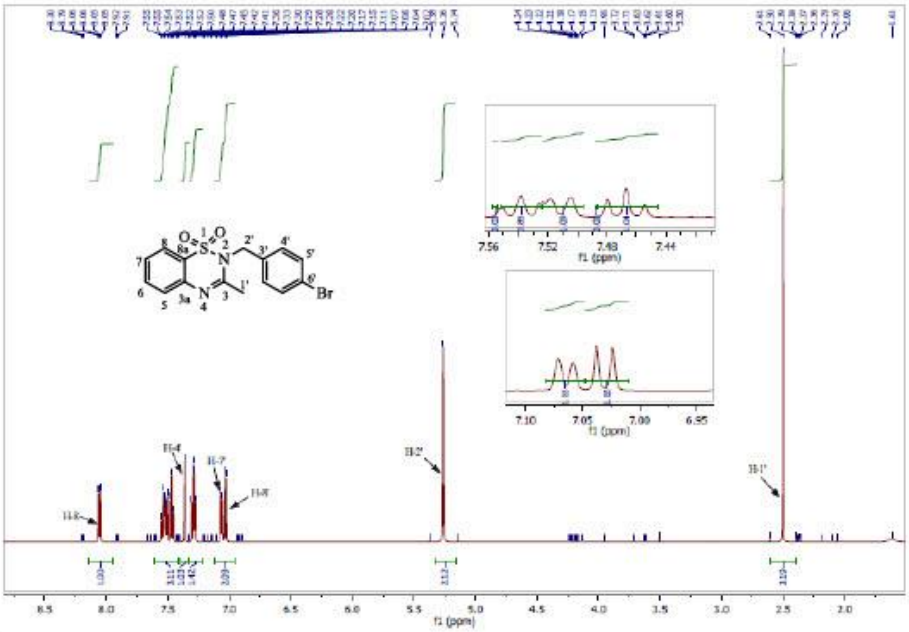

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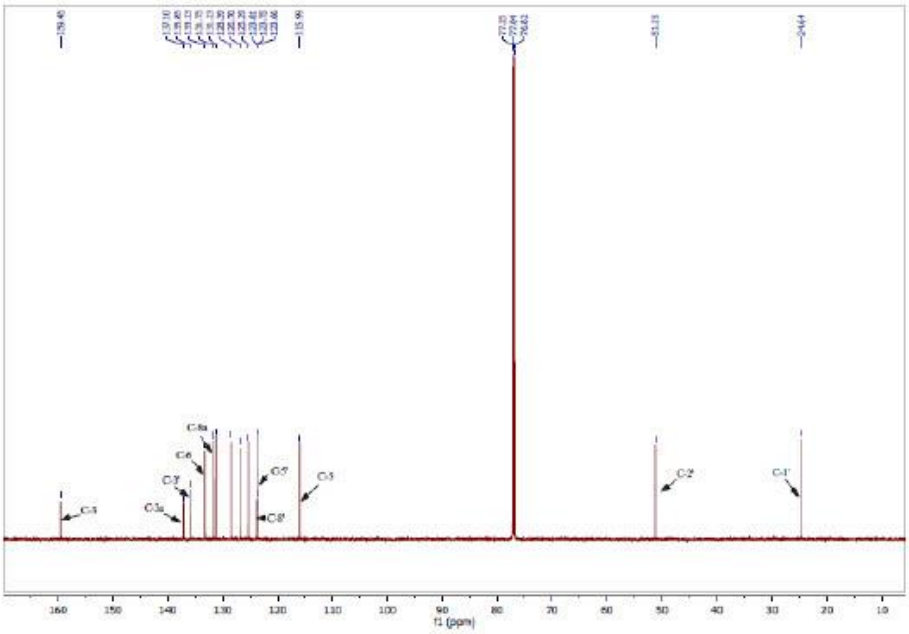

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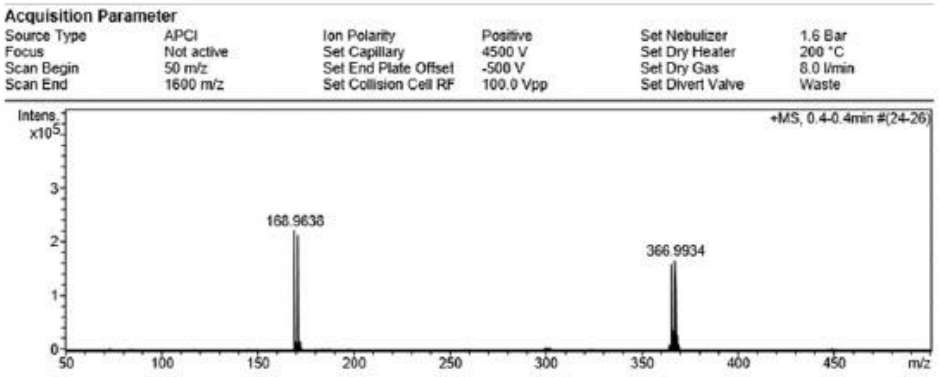
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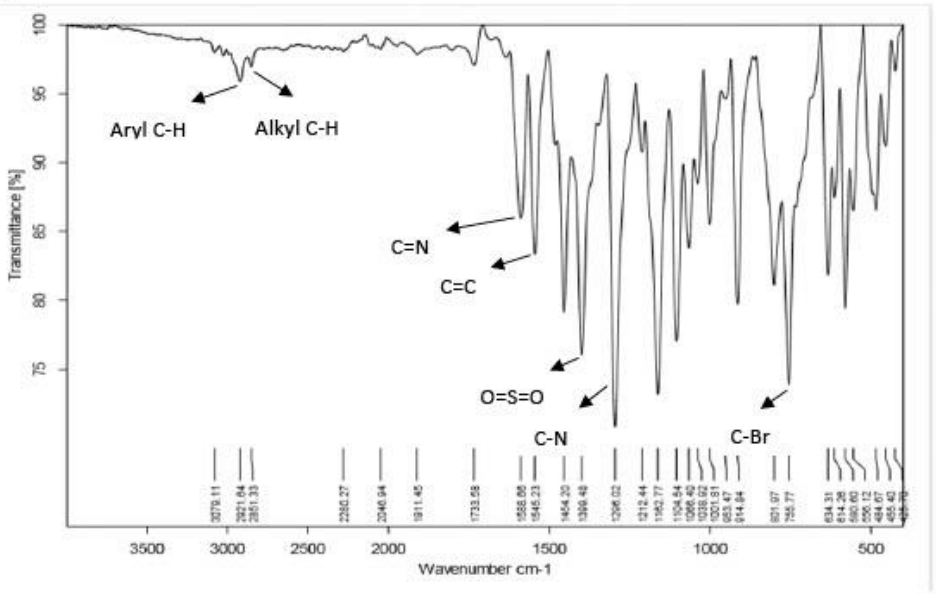
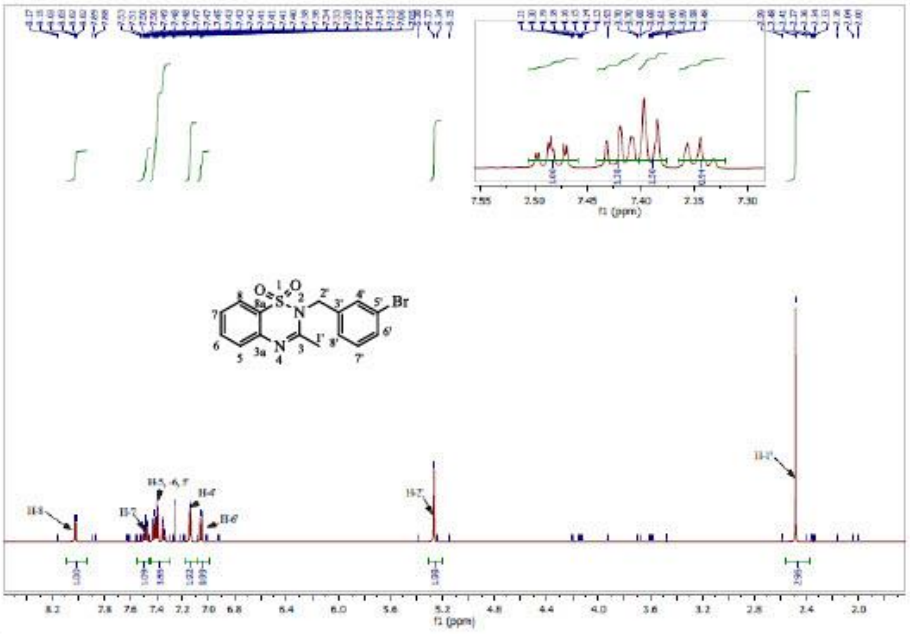
Intens.
x10^5
+MS, 0.4-0.4min #(24-26)
168.9638
366.9934
m/z

IR

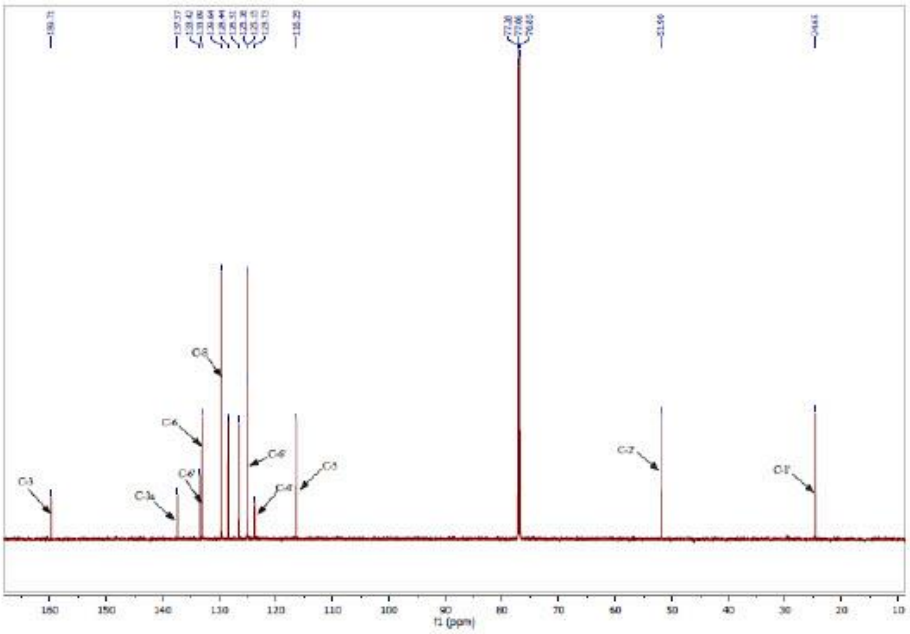

Aryl C-H
Alkyl C-H
C=N
C=C
O=S=O
C-N
C-Br
Transmittance [%]
Wavenumber cm-1

2-(3-Bromobenzyl)-3-methyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **1d**

^{1}H-NMR in $CDCl_3$



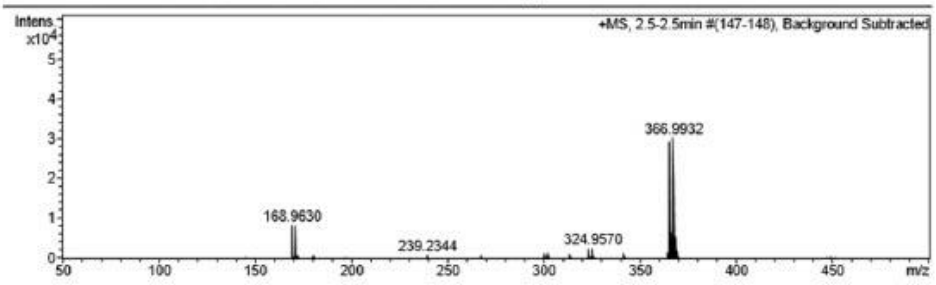
^{13}C-NMR in $CDCl_3$



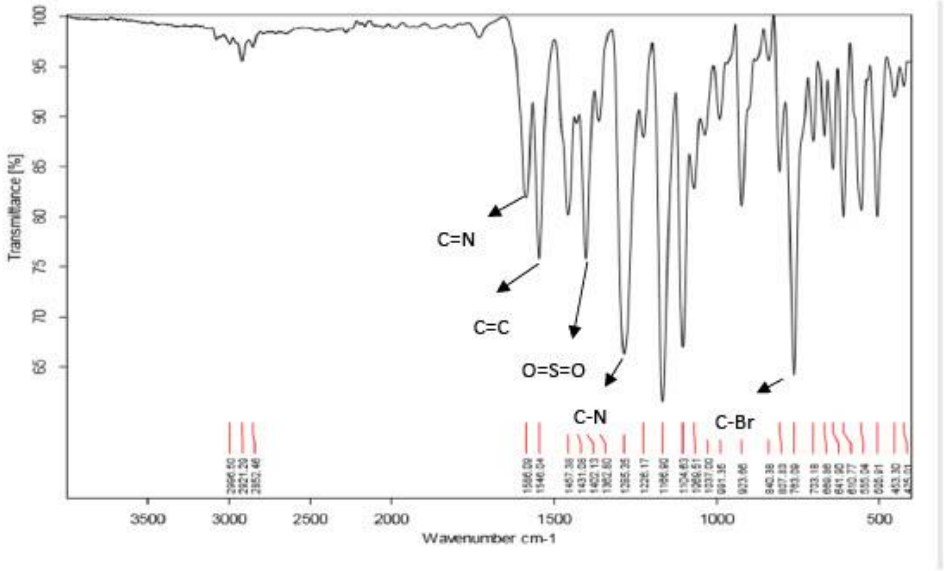
HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

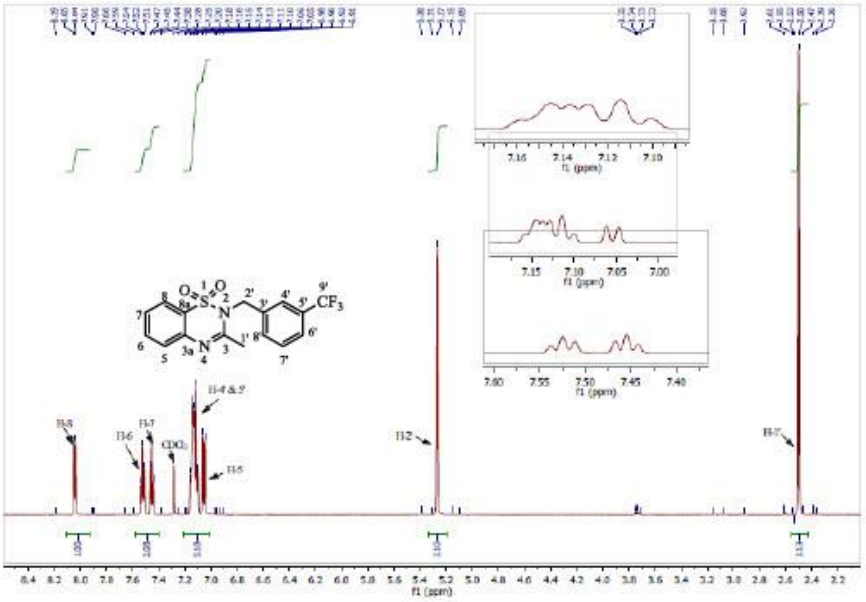


IR

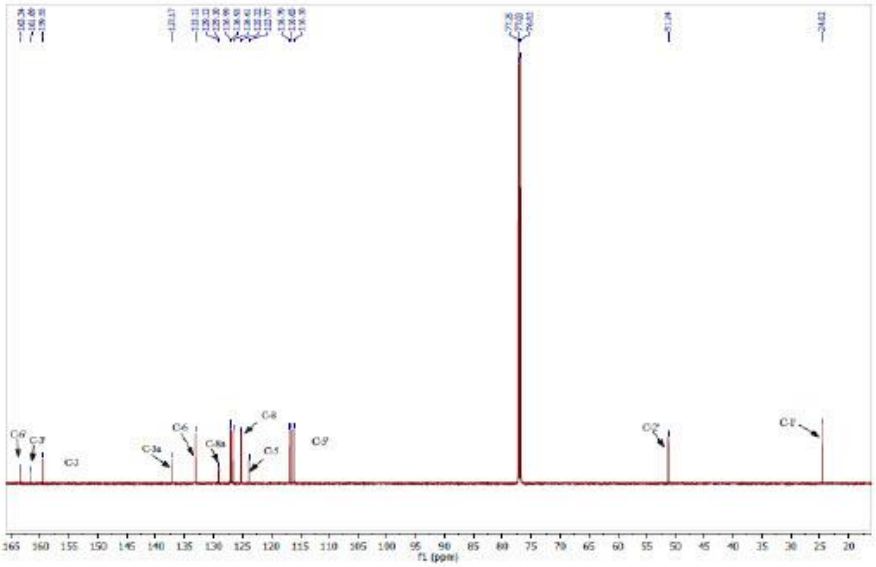


3-Methyl-2-[3-(trifluoromethyl)benzyl]-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **1e**

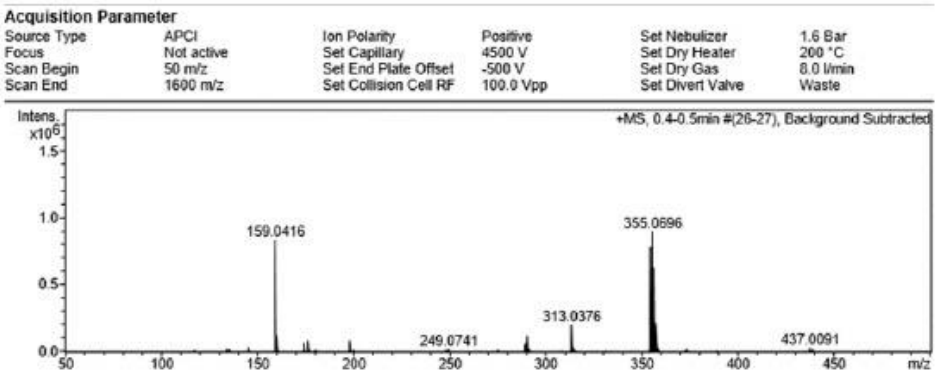
^{1}H-NMR in $CDCl_3$



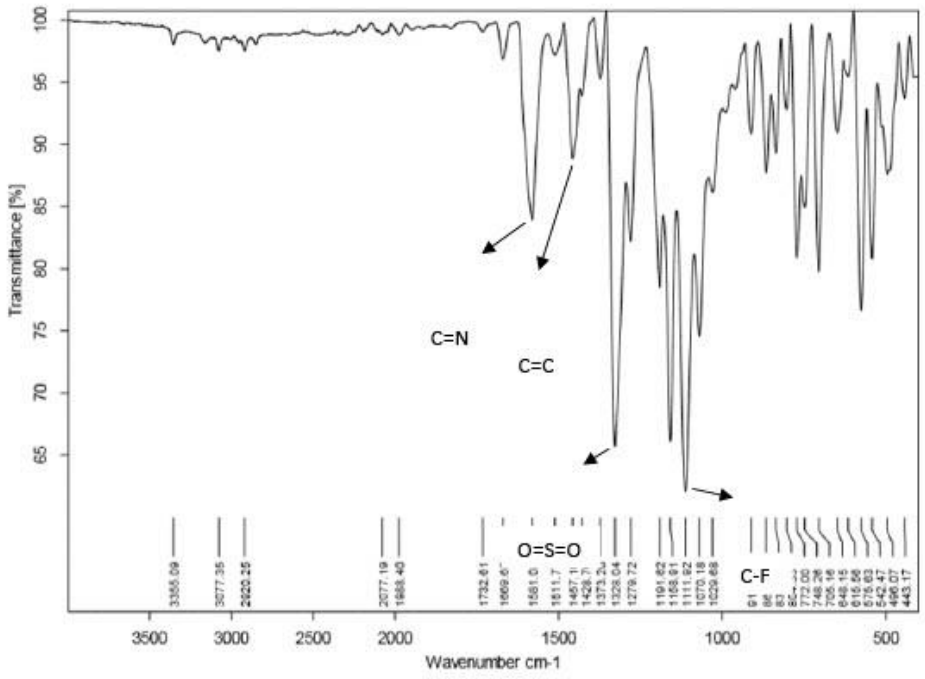
^{13}C-NMR in $CDCl_3$



HRMS

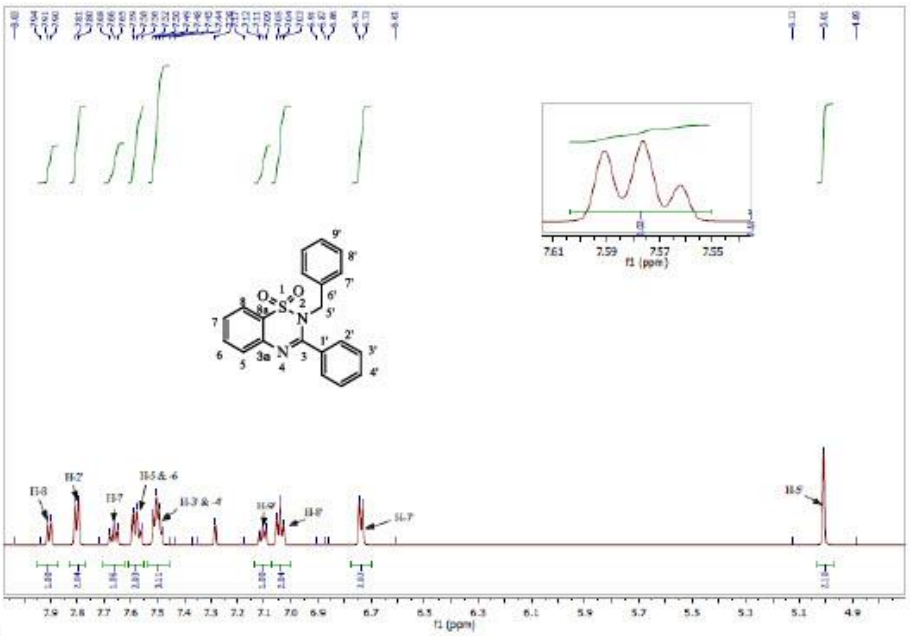

Acquisition Parameter
Source Type APCI
Ion Polarity Positive
Set Nebulizer 1.6 Bar
Focus Not active
Set Capillary 4500 V
Set Dry Heater 200 °C
Scan Begin 50 m/z
Set End Plate Offset -500 V
Set Dry Gas 8.0 l/min
Scan End 1600 m/z
Set Collision Cell RF 100.0 Vpp
Set Divert Valve Waste
Intens.
x10^6
+MS, 0.4-0.5min #(26-27), Background Subtracted
159.0416
355.0696
313.0376
249.0741
437.0091
m/z

IR

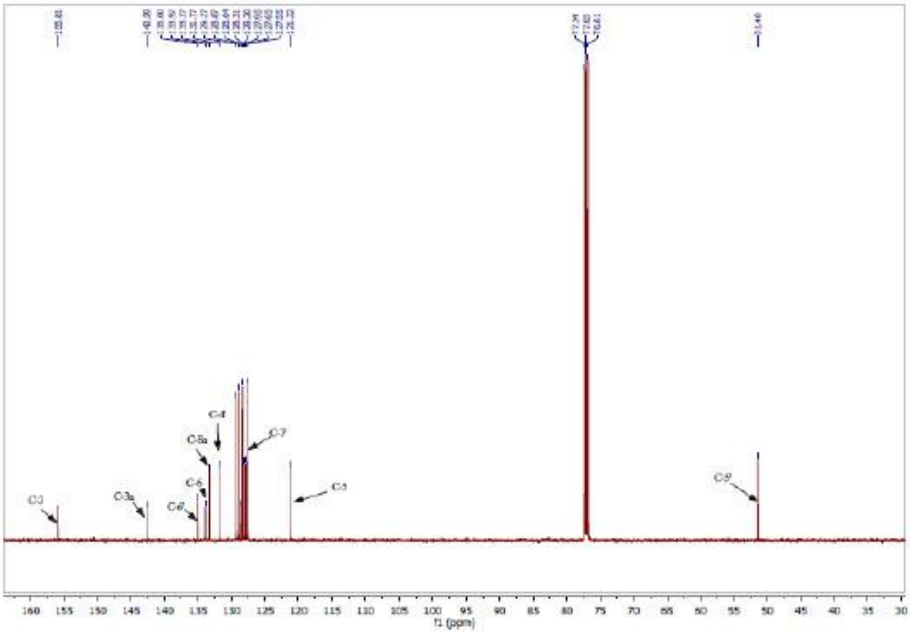

Transmittance [%]
C=N
C=C
O=S=O
C-F
Wavenumber cm-1

2-Benzyl-3-phenyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **2a**

^{1}H-NMR in $CDCl_3$

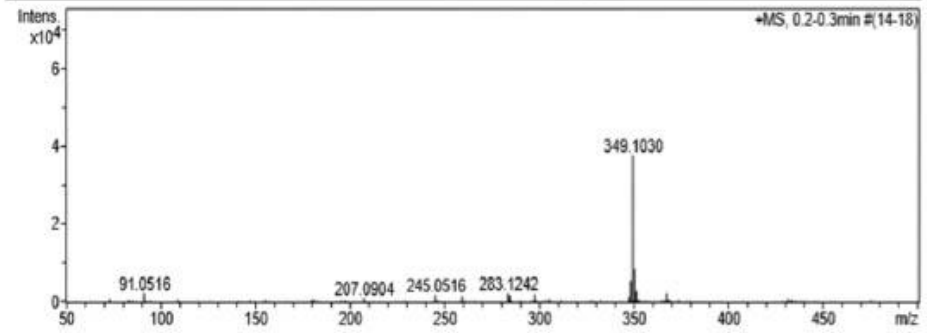


^{13}C-NMR in $CDCl_3$

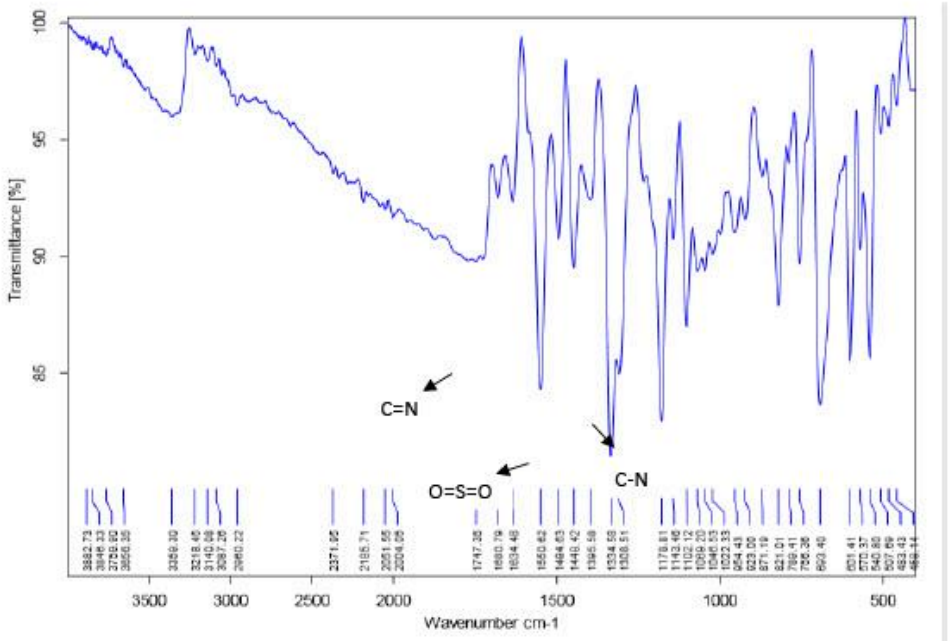


HRMS

Acquisition Parameter					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	80.0 Vpp	Set Divert Valve	Waste

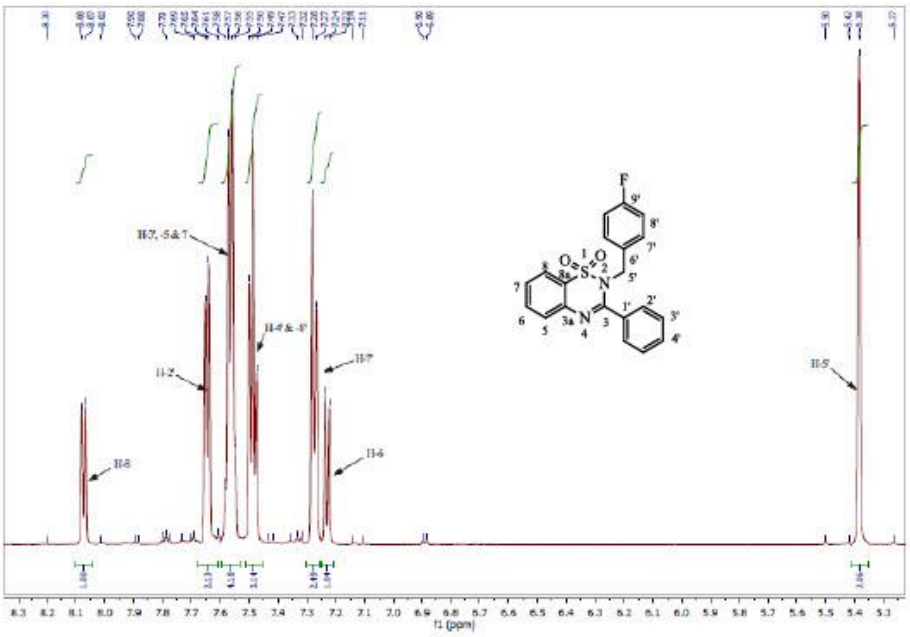


IR

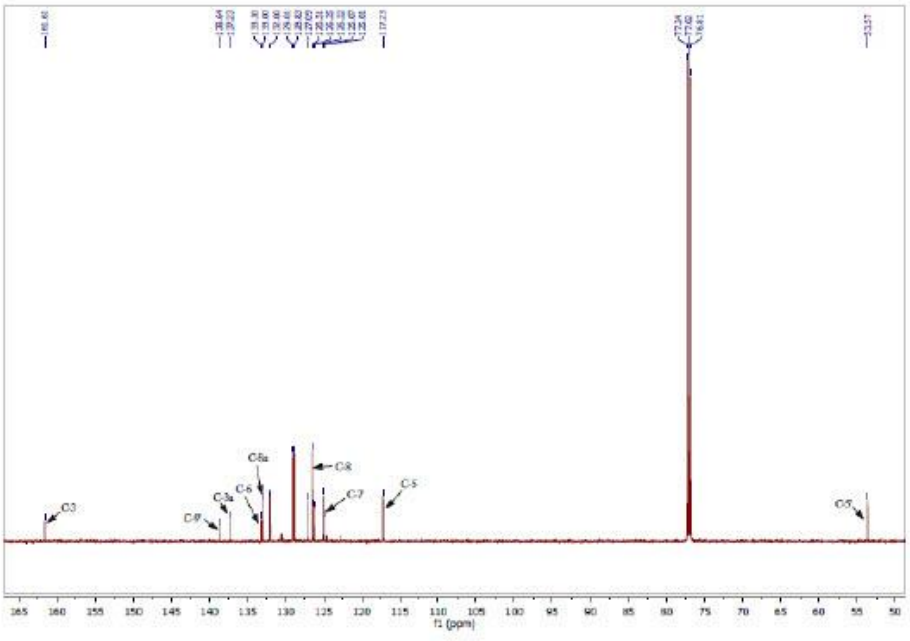


2-(4-Fluorobenzyl)-3-phenyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **2b**

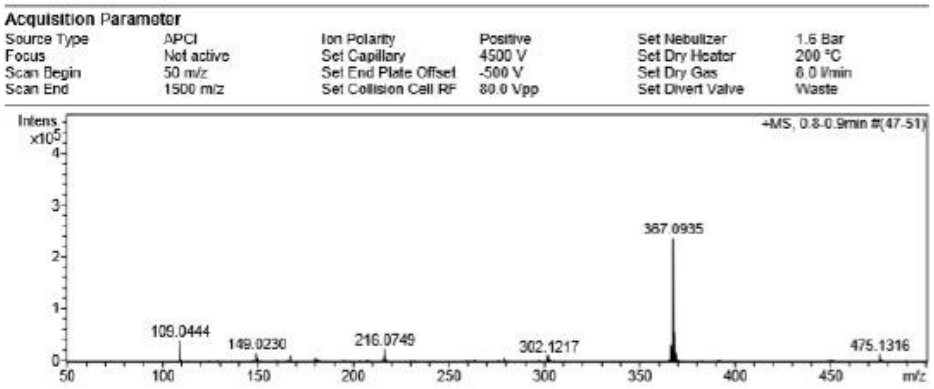
^{1}H-NMR in $CDCl_3$



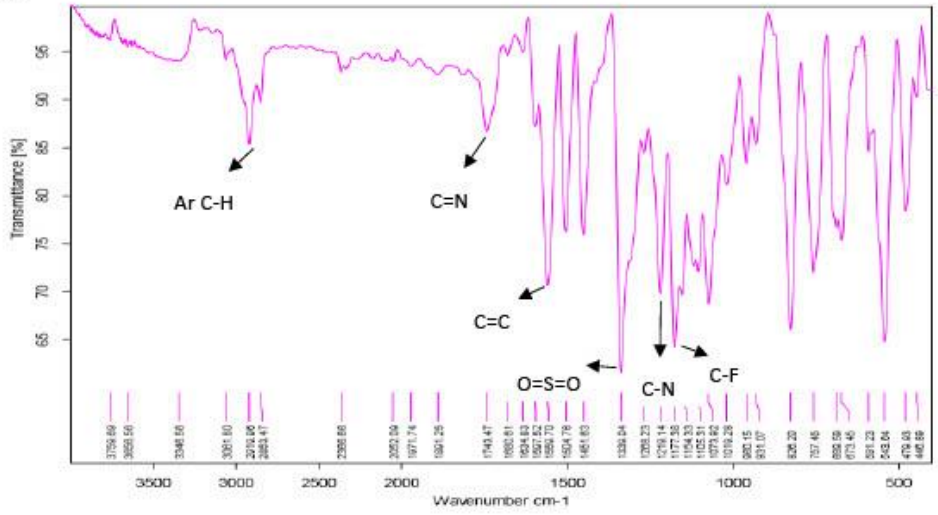
^{13}C-NMR in $CDCl_3$



HRMS

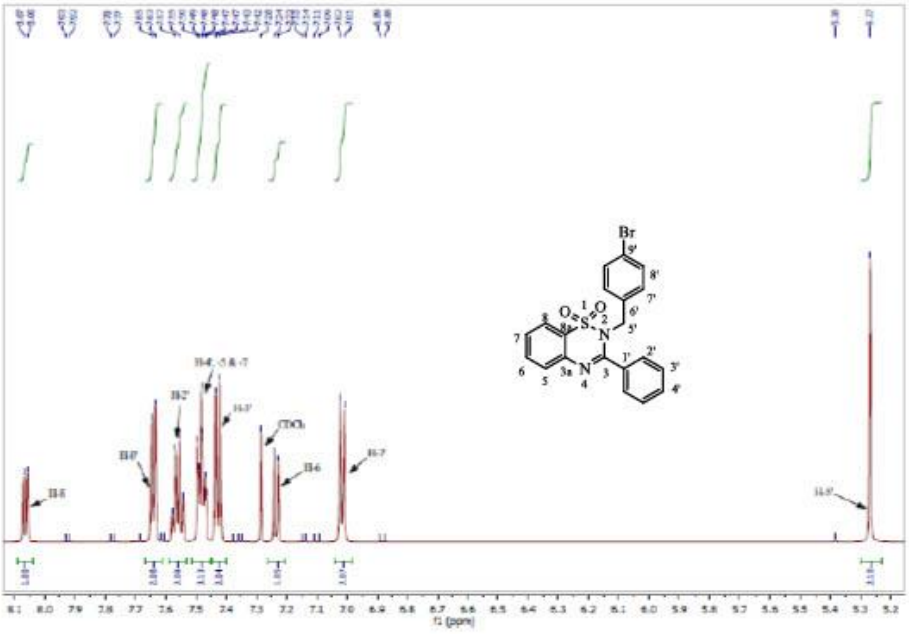

Acquisition Parameter
Source Type APCI
Ion Polarity Positive
Set Nebulizer 1.6 Bar
Focus Not active
Set Capillary 4500 V
Set Dry Heater 200 °C
Scan Begin 50 m/z
Set End Plate Offset -500 V
Set Dry Gas 8.0 l/min
Scan End 1500 m/z
Set Collision Cell RF 80.0 Vpp
Set Divert Valve Waste
Intens.
x10^5
+MS, 0.8-0.9min #(47-51)
367.0935
109.0444
149.0230
216.0749
302.1217
475.1316
m/z

IR

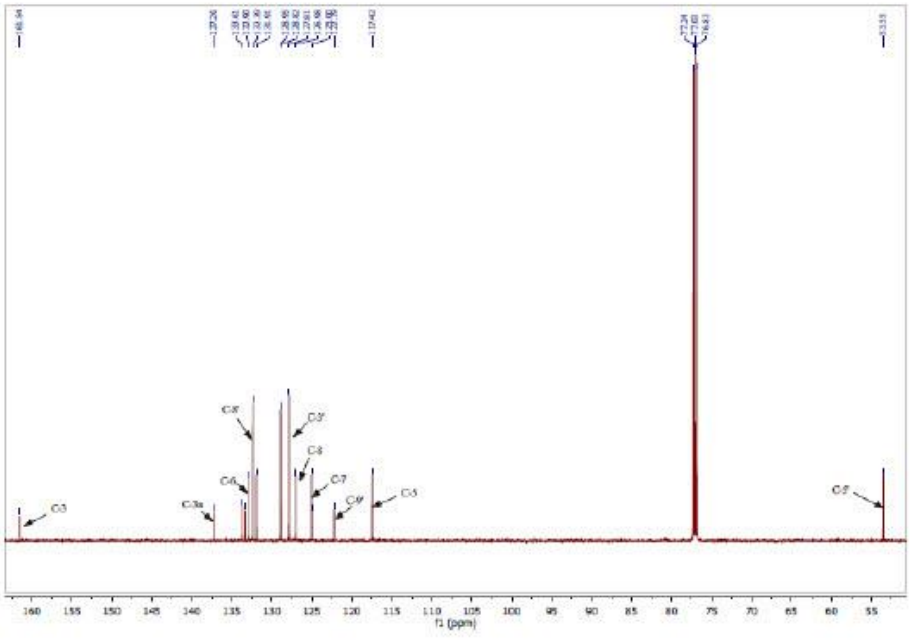

Transmittance [%]
Ar C-H
C=N
C=C
O=S=O
C-N
C-F
Wavenumber cm-1

2-(4-Bromobenzyl)-3-phenyl-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **2c**

^{1}H-NMR in $CDCl_3$



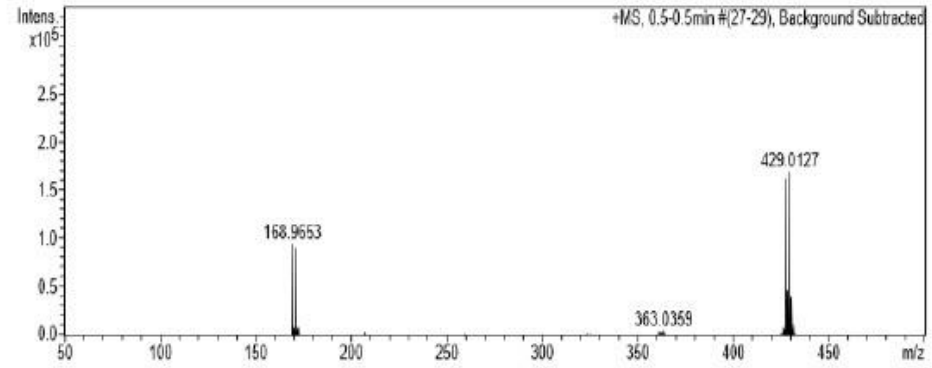
^{13}C-NMR in $CDCl_3$



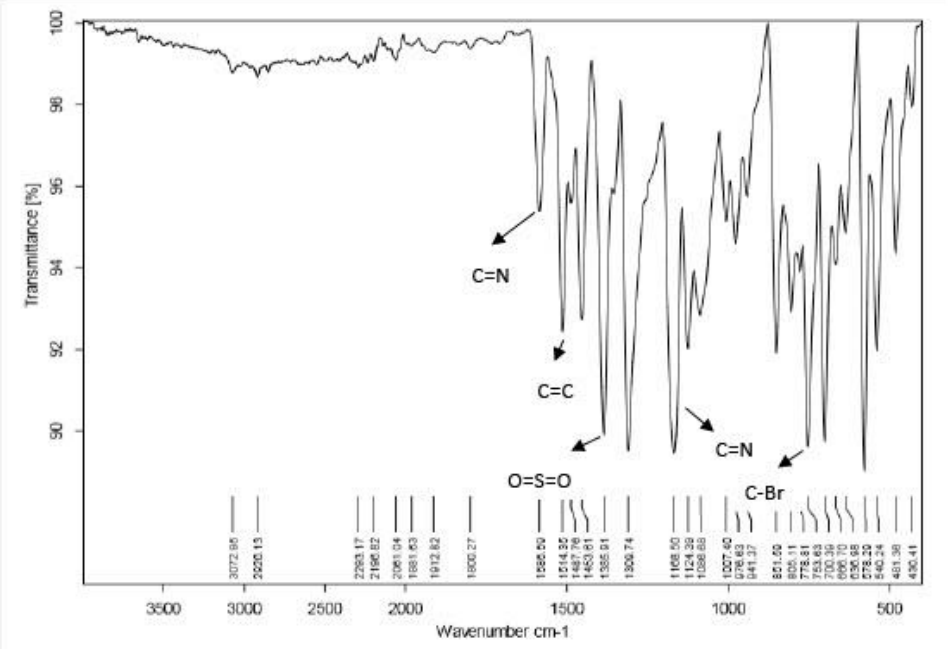
HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

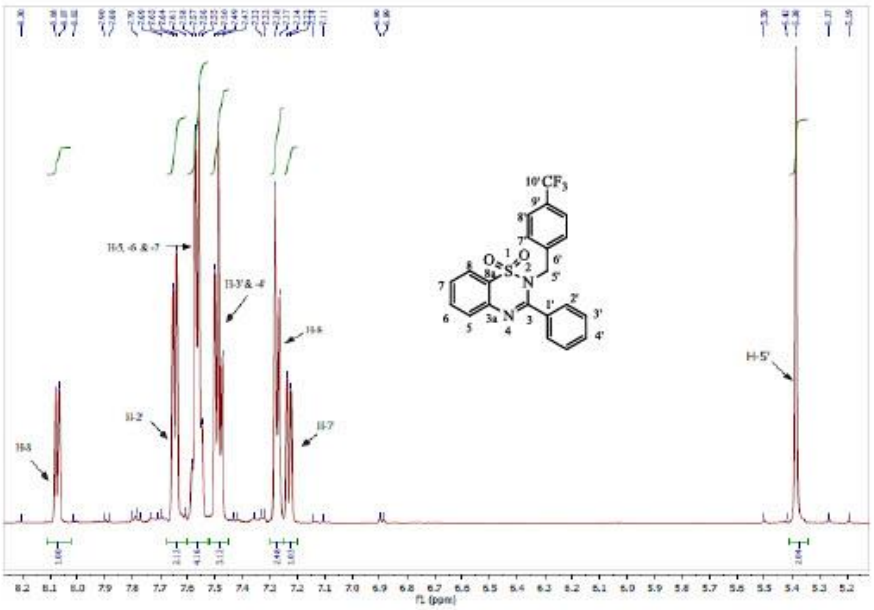


IR

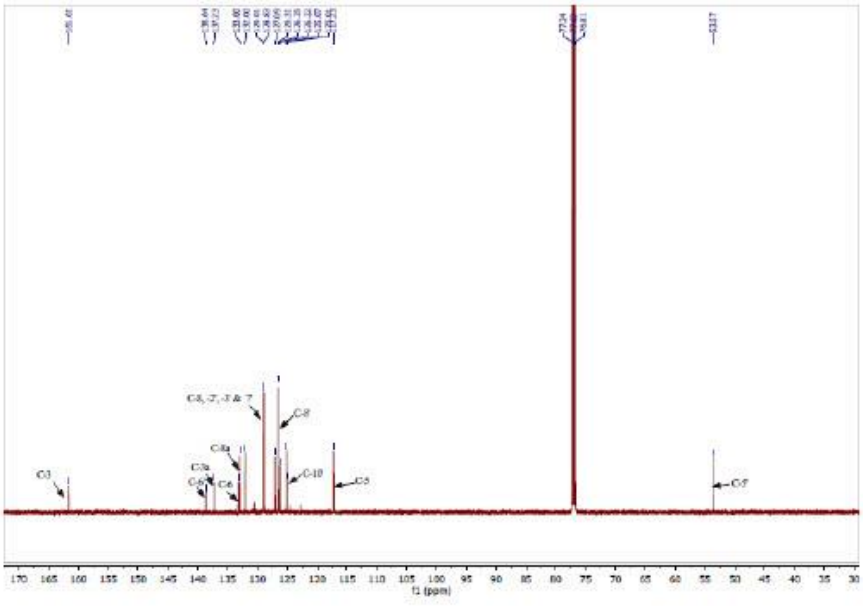


3-Phenyl-2-[4-(trifluoromethyl)benzyl]-2*H*-benzo[*e*][1,2,4]thiadiazine 1,1-dioxide, **2d**

^{1}H-NMR in $CDCl_3$



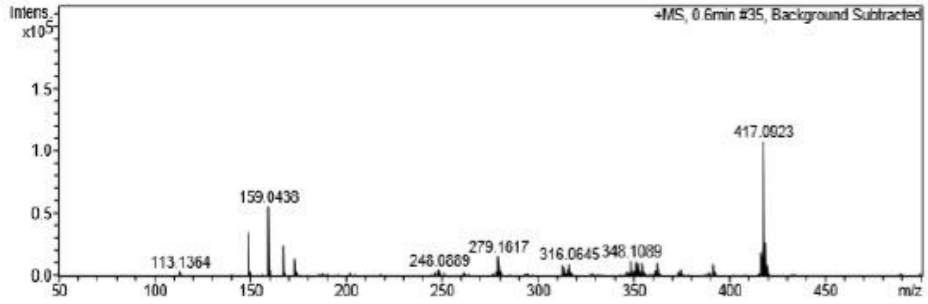
^{13}C-NMR in $CDCl_3$



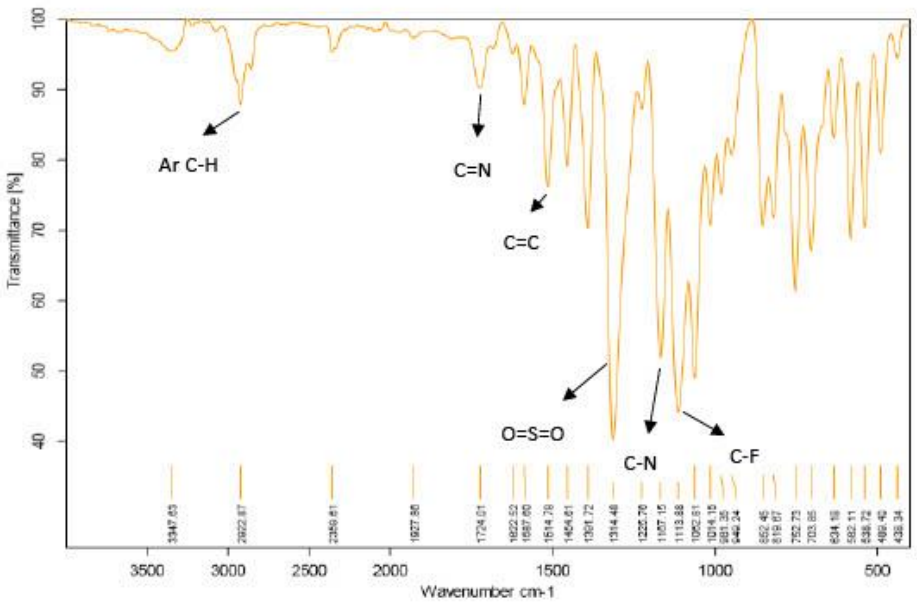
HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	80.0 Vpp	Set Divert Valve	Waste



IR



HPLC purity

General procedure

The system used was an Agilent 1100 HPLC system outfitted with a quaternary pump and an Agilent 1100 series diode array detector. HPLC-grade solvents, such as acetonitrile and deionised or Milli-Q (Millipore) water, were used for chromatographic purity analysis. A Venusil XBP C18 column (measurements: 4.6 mm x 150 mm; particle size: 5 µm) with an initial mobile phase of 70% acetonitrile and 30% deionized water was employed at a flow rate of 1 ml/min. A linear increase in the concentration of the acetonitrile portion of the mobile phase was affected over a period of five minutes to a final concentration of 85%. Equilibration time between runs of the system was five minutes and each run took approximately fifteen minutes to complete (total time: twenty minutes per compound analyzed). Injection volumes varied from 5-10 µl. The eluent was monitored at wavelengths of 210, 230, 254, 280, 300 nm.

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Supplemental Material: Novel Compounds and Biological Screening Results

Synthesis and *in vitro* antileishmanial efficacy of novel benzothiadiazine-1,1-dioxide derivatives

Daisy K. Mangwegape[1], Nonkululeko H. Zuma[2], Janine Aucamp[2], David D. N'Da[2]*

1 Pharmaceutical Chemistry, School of Pharmacy, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa
2 Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa

*Correspondence:
Prof David D. N'Da, Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa
Email: David.nda@nwu.ac.za; ORCID ID: 0000-0002-2327-0551

Compound No.	InChI	Biological Activity (IC_{50})[a]
1	InChI=1S/C8H8N2O2S/c1-6-9-7-4-2-3-5-8(7)13(11,12)10-6/h2-5H,1H3,(H,9,10)	7.33; >100; 3.48
1a	InChI=1S/C15H14N2O2S/c1-12-16-14-9-5-6-10-15(14)20(18,19)17(12)11-13-7-3-2-4-8-13/h2-10H,11H2,1H3	59.58; >100; 3.15
1b	InChI=1S/C15H13FN2O2S/c1-11-17-14-4-2-3-5-15(14)21(19,20)18(11)10-12-6-8-13(16)9-7-12/h2-9H,10H2,1H3	27.60; >100; 3.51
1c	InChI=1S/C15H13BrN2O2S/c1-11-17-14-4-2-3-5-15(14)21(19,20)18(11)10-12-6-8-13(16)9-7-12/h2-9H,10H2,1H3	52.67; >100; 1.30
1d	InChI=1S/C15H13BrN2O2S/c1-11-17-14-7-2-3-8-15(14)21(19,20)18(11)10-12-5-4-6-13(16)9-12/h2-9H,10H2,1H3	31.75; 75.00; 3.17
1e	InChI=1S/C16H13F3N2O2S/c1-11-20-14-7-2-3-8-15(14)24(22,23)21(11)10-12-5-4-6-13(9-12)16(17,18)19/h2-9H,10H2,1H3	73.70; 22.39; 0.2
2	InChI=1S/C13H10N2O2S/c16-18(17)12-9-5-4-8-11(12)14-13(15-18)10-6-2-1-3-7-10/h1-9H,(H,14,15)	18.83; >100; 2.00
2a	InChI=1S/C20H16N2O2S/c23-25(24)19-14-8-7-13-18(19)21-20(17-11-5-2-6-12-17)22(25)15-16-9-3-1-4-10-16/h1-14H,15H2	29.00; 12.03; 3.46
2b	InChI=1S/C20H15FN2O2S/c21-17-12-10-15(11-13-17)14-23-20(16-6-2-1-3-7-16)22-18-8-4-5-9-19(18)26(23,24)25/h1-13H,14H2	9.82; 12.48; 0.4
2c	InChI=1S/C20H15BrN2O2S/c21-17-12-10-15(11-13-17)14-23-20(16-6-2-1-3-7-16)22-18-8-4-5-9-19(18)26(23,24)25/h1-13H,14H2	6.52; 15.67; 4.93
2d	InChI=1S/C21H15F3N2O2S/c22-21(23,24)17-12-10-15(11-13-17)14-26-20(16-6-2-1-3-7-16)25-18-8-4-5-9-19(18)29(26,27)28/h1-13H,14H2	10.15; >100; 0.41

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Short communications should contain novel experimental or theoretical results in need of urgent publication. Please add the term "Short Communication" below the title. A short communication contains a summary and should not exceed 4 printed pages (including Tables, Figures and References). The same manuscript organization as described under Section 3.1 applies.

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Reviews are comprehensive critical descriptions on present knowledge of actual research subjects or technological developments. Please add the term "Review" below the title. Section division is left to the author(s), otherwise criteria of Section 3.1 apply. The length of review articles should not exceed printed 9–10 pages (equivalent to a total of 60 000 characters). Reviews are frequently submitted by invitation. They are, of course, also welcome from other authors.

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25 October 2019

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Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 25/10/2019, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Synthesis and antileishmanial activity of novel benzothiadiazine-1, 1-dioxide derivatives
Principal Investigator/Study Supervisor/Researcher: Prof DD N'Da
Student: DK Mangwegape - 25481819

Ethics number:

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Application Type: Single study
Commencement date: 25/10/2019

Risk: **No Risk**

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Chapter 11

Biomarkers in Leishmaniasis: From Basic Research to Clinical Application

Sofia Esteves, Inês Costa, Célia Amorim,
Nuno Santarem and Anabela Cordeiro-da-Silva

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Abstract

Leishmania is an intracellular protozoan parasite and the etiological agent of a vector-borne disease known as leishmaniasis. This neglected tropical disease exhibits high morbidity and mortality putting at risk people from multiple countries worldwide. It is endemic in 97 countries and 700,000–1 million new cases are estimated to occur each year. Leishmaniasis management is very challenging, the symptoms are non-pathognomonic (in both human and canine populations) and the treatments are associated with significant toxicity. Therefore, the need for detection in symptomatic and asymptomatic hosts is important to tackle the dissemination of infection, increasing the need for highly specific biomarkers. In this complex the available disease biomarkers will be addressed in a retrospective manner, focusing on their development from laboratory to their direct use in clinical settings.

Keywords: leishmaniasis, biomarkers, diagnosis techniques

1. Introduction

1.1. Leishmaniasis as a neglected tropical disease

Neglected tropical diseases are a diverse group of diseases that prevail in tropical and subtropical countries. The geographical distribution of these diseases associated with lack of sanitation, close contact with domestic animals, livestock and infectious vectors contributes to disease dissemination, affecting more than 1 billion people and costs billions each year in developing economies.

Leishmaniasis is a devastating and significantly under-recognized vector-borne disease that causes serious global health burden. After malaria, it is the second parasitic disease with the highest mortality rate [1]. It mainly affects populations in Africa, Asia and Latin America, and is associated with malnutrition, population displacement, poor housing, weak immune system and lack of resources [1].

Although this disease was initially limited to the tropics and subtropics, several factors lead to the spread of leishmaniasis to new locations [2]. Perturbations of the natural vector ecosystem caused by urbanization, deforestation, global warming and natural disasters have a serious impact in disease dissemination. Increased people and animal migration, traveling and military operations contribute to disease spreading. Organ transplantation, lack of *Leishmania* screening in the blood bank or immune suppressive therapies can also contribute to disease perpetuation [2, 3].

This disease can be presented in several ways, the most common ones being: Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL) and Visceral Leishmaniasis (VL), also known as kala-azar. Depending on the specific clinic-pathological form of the disease, symptoms range from cutaneous and mucosal tissue lesions to vital visceral organ damage [4, 5].

1.2. Epidemiology

Out of 200 countries and territories reporting to WHO, 97 are endemic for leishmaniasis (**Figure 1**). This includes 65 countries endemic for both VL and CL, 10 countries endemic only for VL and 22 endemic only for CL (**Figure 1**) [1].

Ninety percent of global VL cases were reported from seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. About 95% of CL cases occur in the Americas, the

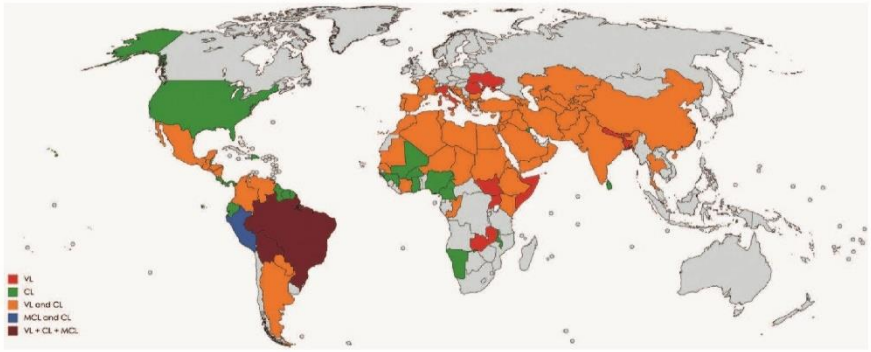

Figure 1. World map: Human leishmaniasis distribution. VL, Visceral Leishmaniasis; CL, Cutaneous Leishmaniasis; MCL, Mucocutaneous Leishmaniasis. Status of endemicity of the different forms of leishmaniasis in 2016, according to the World Health Organization.

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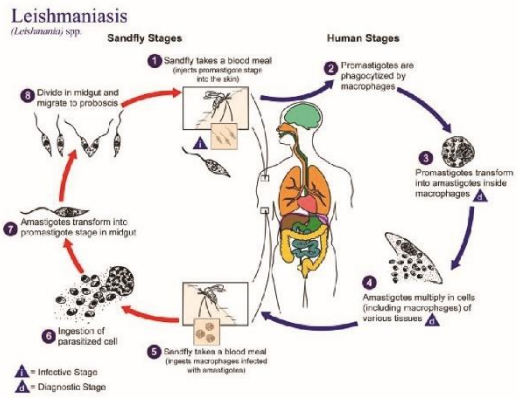

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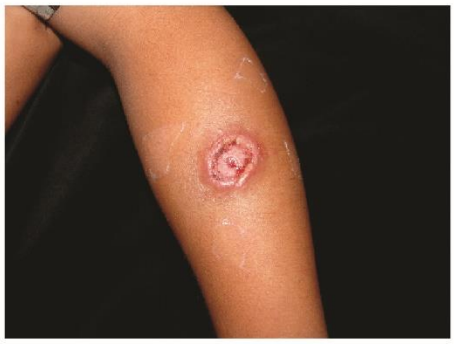

Ulcer on the upper limb with crusts and raised borders.

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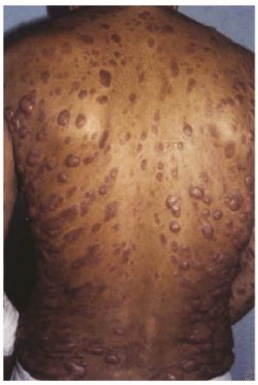
Leishmaniasis: A review

Article Full-text available

May 2017

Edoardo Torres-Guerrero · Marco Romano Quintanilla-Cedillo · Julieta Ruiz-Esmenjaud · Roberto Arenas

Leishmaniasis is caused by an intracellular parasite transmitted to humans by the bite of a sand fly. It is endemic in Asia, Africa, the Americas, and the Mediterranean region. Worldwide, 1.5 to 2 million new cases occur each year, 350 million are at risk of acquiring the disease, and leishmaniasis causes 70,000 deaths per year. Clinical features d...



Diffuse cutaneous leishmaniasis (anergic clinical form).

Source publication

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