DEVELOPMENT OF FLAVONOID COMPOUNDS AS ANTILEISHMANIAL AGENTS

CHAN CHIN FUNG

PhD

The Hong Kong Polytechnic University

2020

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Development of Flavonoid Compounds as Antileishmanial Agents

Chan Chin Fung

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

September 2018

Certificate of Originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has made in the text.

_____ (Signed)

Chan Chin Fung

Abstract

A library of 49 novel flavonoid compounds were designed, synthesized and characterized. Some of them were demonstrated to be potent *in vitro* against *Leishmania* parasites that cause leishmaniasis, an endemic tropical disease prevalent in 80 countries worldwide. Among them, **FM05b** and **FM09h** were selected for further study. **FM05b** was found to be active against promastigotes (IC_{50} = 0.6 – 0.9 µM) and amastigotes (IC_{50} = 0.3 – 0.7 µM), metabolically-stable (89% and 83.1% remained after 30-minute incubation with human liver microsomes (HLM) and rat liver microsomes (RLM)), safe (no toxicity in mice), orally available (F = 32%) and accumulated in the spleen and liver (target organs of visceral *Leishmania*) at a level (4.5 and 3.2 µM respectively) above its *in vitro* IC_{50} of 0.7 µM at 24 hours after oral administration at 50 mg/kg. *In vivo* efficacy study demonstrated that intralesional administration of **FM05b** (10 mg/kg, once every 4 days for 8 times) reduce the footpad lesion thickness in a cutaneous model of Leishmaniasis (*L. amazonensis* LV78) by 49 ± 22% compared with solvent control group. In addition, oral administration of **FM05b** (50 mg/kg; once daily for 14 days) reduce the parasite burden of a visceral model of leishmaniasis (*L. donovani* HU3) in the liver by 32 ± 18% with no toxicity observed. Another potent drug candidate, **FM09h** (IC_{50} = 0.5 – 1.1 µM against promastigotes, IC_{50} = 0.3 µM against amastigotes, metabolic stability in HLM and RLM = 39.4% and 65.7 % respectively and F = 5%) was found effective in reducing the lesion thickness of the footpad in cutaneous leishmaniasis by 72 ± 15% via intralesional injection (10 mg/kg, once every 4 days for 8 times).

To conclude, the antileishmanial activities and pharmacokinetics of synthetic flavonoid monomers had been improved through structural modification. **FM05b** and **FM09h** were generated. Additionally, **FM05b** was found to accumulate in liver and spleen, the target organs of visceral leishmaniasis. It was also demonstrated that both **FM05b** and **FM09h** were effective in treat cutaneous leishmaniasis via intralesional administration. Oral administration of **FM05b** reduce the parasite burden in liver in the visceral leishmaniasis model. This suggests the use of synthetic flavonoids to treat leishmaniasis.

List of Publications and Conference Presentation by the Author

Conference Presentation

Chan, C. F.; Wong, I, L. K.; Zhu, X. Z.; Chan, T. K., Chow, L. M. C.

"Investigation of the Use of Flavonoid Compounds as Antileishmanial Agents" Drug Discovery for Parasitic Diseases, Granlibakken Tahoe, Tahoe City, California, USA, January 24-28, 2016

Acknowledgements

Firstly, I would like to express my deepest gratitude to my supervisor Prof. Larry Ming-Cheung Chow for giving me this great opportunity to participate in this project and his continuous support of my PhD study. In these years, I could feel his passion towards Sciences. His immense knowledge and enlightenment opened my mind and had driven me to be more enthusiastic in scientific research. His guidance, valuable discussions and encouragements were deeply appreciated.

I would also like to thank Prof. Bill Tak-Hang Chan. While he lives in Canada, he gave me opinions about the project via every emails and Skype meetings, which broadened my horizon very much. He is a chemistry expert that advised me a lot on the chemistry part of my research project. And I must thank Dr. Iris Wong for her guidance as she is the only researcher who worked with *Leishmania* in this laboratory, sharing a lot of experience and knowledge in the early study years. Beside her, I also thank Dr. Kin-Fai Chan and Mr. Zhen Liu for synthesizing so many flavonoid compounds for the study.

I thank the rest of labmates, Dr. Clare Yan, Dr. Kit-Ying Choy, Dr. Tsz-Cheung Chong, Dr. Xuesen Hu, Mr. Tsun-Sing Chow, Mr. Xue-Zhen Zhu, Miss Teng Chen, Miss Peng Li, Miss Wen-Qin Sun, Miss Ge-Ge Sun and Miss Xiao-Chun Su, for creating a nice atmosphere for my research, stimulating discussions and all the fun we had during the whole PhD study. Thanks also go to all the technicians and staff in Department of Applied Biology and Chemical Technology and University Research Facility in Life Sciences (ULS), for their generous help throughout the project.

Last but not the least, I would like to express my sincere thanks to my family, my church and my friends for their spiritual love and support throughout these years. Their encouragements had made me perseverant in facing so many obstacles.

Terry Chin-Fung Chan

14th September 2018

Contents

List of Figures

List of Tables

List of Abbreviation

WHO	World Health Organization
DNDi	Drug for Neglected Diseases Initiatives
CL	cutaneous leishmaniasis
VL	visceral leishmaniasis
LCL	localized cutaneous leishmaniasis
MCL	mucocutaneous leishmaniasis
DCL	diffuse cutaneous leishmaniasis
PKDL	post kala-azar dermal leishmaniasis
DALY	Disability-adjusted life years
SSG	sodium stibogluconate
CYP450	cytochrome P450
HLM	human liver microsomes
RLM	rat liver microsomes
NADPH	nicotinamide adenine dinucleotide phosphate
CrEL	CremophorEL
IC_{50}	Half maximal inhibitory concentration
SAR	Structure activity relationship

UPLC	Ultra performance liquid chromatography
QQQ	Triple quadrupole
qTOF	quadrupole time of flight
SD	Standard deviation.
AUC	Area Under Curve
MRT	Mean Residence Time
LDU	Leishman-Donovan Unit

1 Introduction

Parasitic disease is one of major diseases in the world. There are over 3 billion people worldwide infected with parasites. They are mostly neglected because the place affected are mainly in tropical areas with poverty. Leishmaniasis is listed as one of the major parasitic diseases by World Health Organization (WHO) and Drug for Neglected Diseases Initiatives (DNDi). Leishmaniasis can be broadly divided into two sub-categories, cutaneous leishmaniasis and visceral leishmaniasis. Lesion and nodules are developed in cutaneous leishmaniasis that may be self-healed but left with permanent scars. Visceral leishmaniasis develops hepatosplenomegaly in the patient's body and can be fatal if left untreated.

1-1 Global Situation of Leishmaniasis

Leishmaniasis ranks ninth among all the infectious diseases with more than 220,000 cases of cutaneous leishmaniasis and 58,000 cases of visceral leishmaniasis per year officially [1]. A report of the WHO Expert Committee on the Control of *Leishmania*ses issued in 2010 estimated that there would be 2 million new cases, among which 1.5 million would be cutaneous leishmaniasis and 0.5 million of which would be visceral leishmaniasis. Besides, 50,000 deaths caused by visceral leishmaniasis and over 2.3 million disability-adjusted life years (DALY) were estimated [2]. Leishmaniasis is classified as one of the most neglected because of limited resources invested in diagnosis, treatment and control, and is strongly associated with poverty [3]. A technical report about leishmaniasis covering more than 20 years of study was issued by WHO in 2010 that had highlighted the need to update the epidemiological evidence base in order to plan appropriate approaches

to control leishmaniasis and to raise the awareness of leishmaniasis. An epidemiological update was reported in 2014, estimating that there were a total of 399 and 556 million people living in endemic areas at risk of cutaneous and visceral leishmaniasis respectively [4]. However, drugs for treating leishmaniasis are limited and they possess various drawbacks. In this chapter, the cause of leishmaniasis, its geographical distribution, its clinical manifestation and the currently available antileishmanial drugs were reviewed.

1-2 *Leishmania*, the Causative Agent of Leishmaniasis

Leishmania which is a kind of protozoan parasites belong to the order Kinetoplastida and the family *Trypanosomatidae*. It is the causative agent of leishmaniasis. It is a kind of heteroxenous parasite that exists in two morphological stages, namely promastigotes and amastigotes. Promastigotes reside within the intestinal tract of the insect vector whereas amastigotes reside within the host macrophages. Promastigotes have a long and slender cell body (around 15 – 30 μm by 2 – 3 μm) with a central nucleus, a kinetoplast and a long free anterior flagellum (**Figure 1-1**). Amastigotes are round shaped (2 – 6 μm in diameter) without exterior flagellum (**Figure 1-2**) [5]. Recently, approximately 53 *Leishmania* species have been described. *Leishmania* can be classified into different subgenera (**Figure 1-3**). The classification is mainly based on the extrinsic (geographic distribution and developmental features in sandfly gut) and intrinsic (biochemical, immunological, and molecular markers) characteristics [6]. Thirty-one of which are parasites of mammals while 20 of them are pathogenic to human beings. Eighteen of them cause cutaneous leishmaniasis while 6 of them cause visceral leishmaniasis (**Table 1-1**).

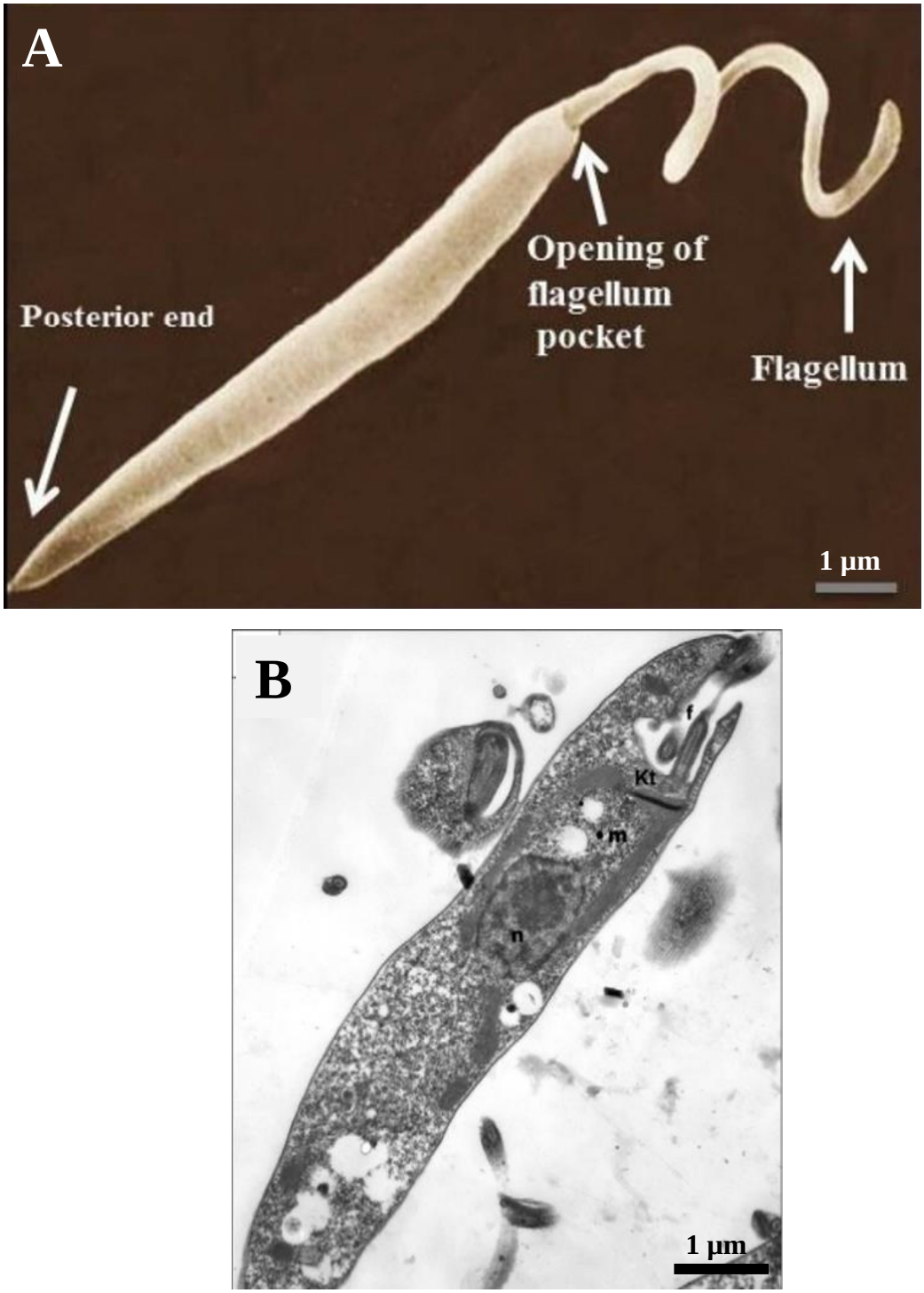


Figure 1-1 Extracellular *Leishmania* promastigotes.

(A) Scanning electron microscopy of an extracellular promastigote. The promastigote is slender and rod-shaped with a long flagellum (scale bar = 1 µm) [7]. (B) Transmission electron microscopy of an extracellular promastigote showing different organelles. Flagellum (f), kinetoplast (kt), mitochondrion (m) and nucleus (n) (scale bar = 1 µm) [8].

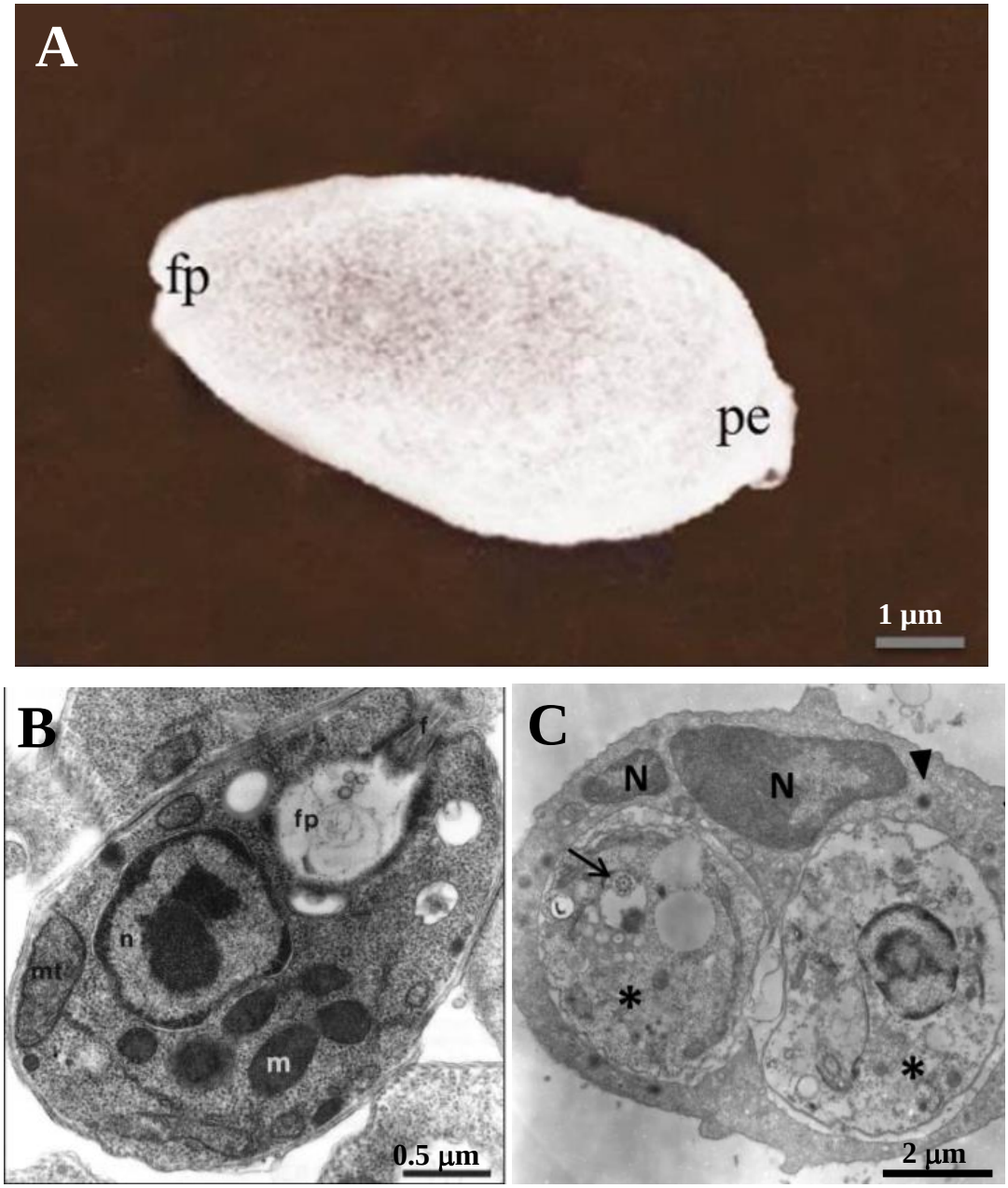


Figure 1-2 Intracellular *Leishmania* amastigotes inside the macrophage.

(A) Scanning electron microscopy of an amastigote. Flagellum pocket (fp), posterior end (pe) (scale bar = 1 µm) [7]. (B) Transmission electron microscopy of an axenic amastigote. The flagellum (f) is enclosed in the flagellar pocket (fp), membrane-bound megasome (m), mitochondrion (mt) and nucleus (n) (scale bar = 0.5 µm) [9]. (C) Transmission electron microscopy of amastigotes engulfed by a neutrophil. Amastigote (*) and neutrophil nucleus (N) and flagellum remnant enclosed in the flagellar pocket (arrowhead). Electron-dense granules (black triangle) (scale bar = 2 µm). Round-shaped amastigotes are transformed from promastigotes upon engulfment by macrophages. The flagellum is enclosed within the flagellar pocket [10].

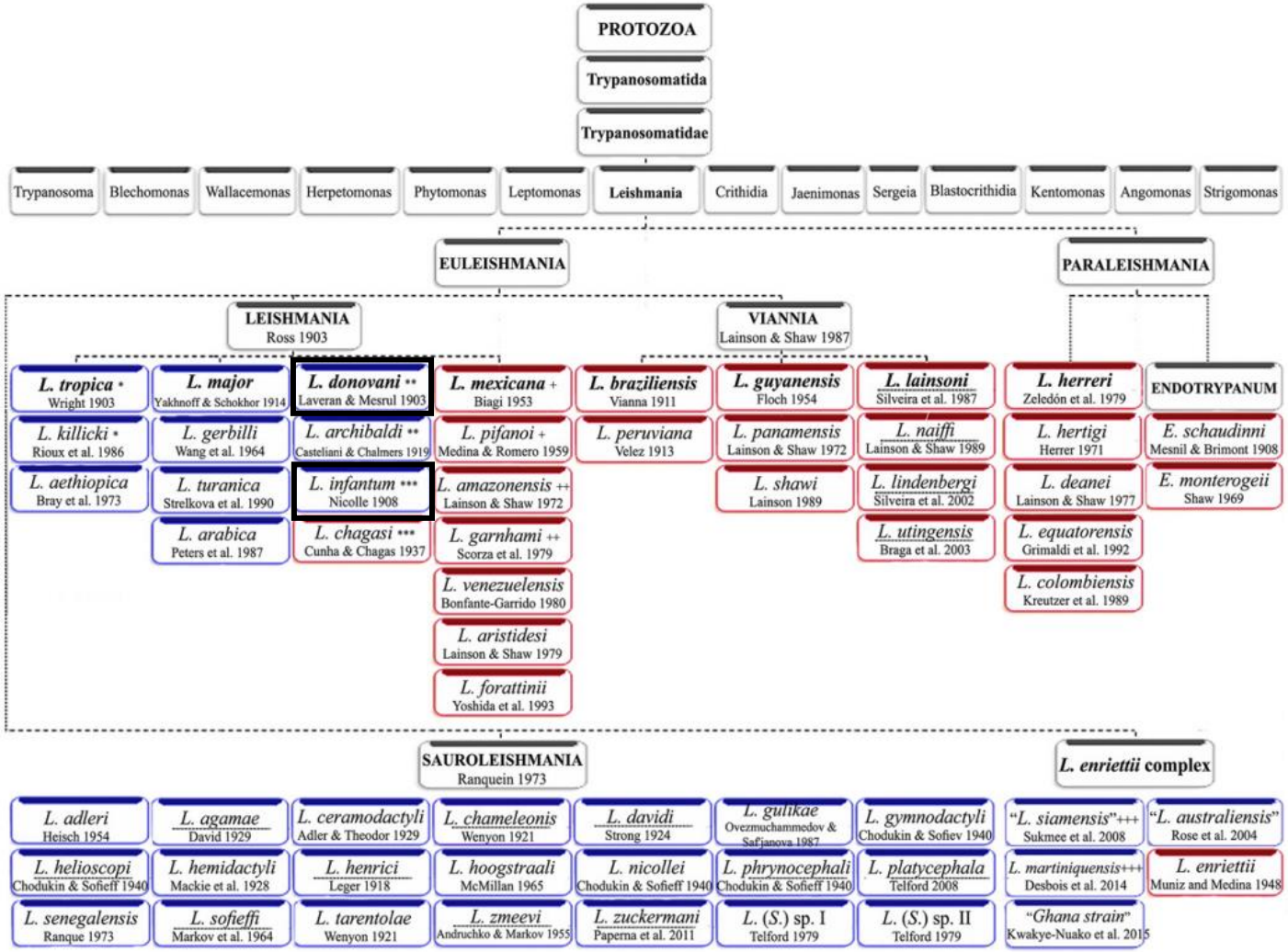


Figure 1-3 Classification of *Leishmania*

Leishmania belonging to *trypanosomatidae* are classified into 5 subtypes, *Leishmania*, *Viannia* and *Sauroleishmania*, *Paraleishmania* and *L. enrietti* complex. Classification is mainly based on the extrinsic (geographic distribution and developmental features in sandfly gut) and intrinsic (biochemical, immunological, and molecular markers) characteristics. Blue: old world species; red: new world species. *L. donovani* and *L. infantum* are old world species parasites that cause visceral leishmaniasis in human that is fatal if left untreated (black box) [6].

Table 1-1 Various species of *Leishmania* and the diseases they caused

Leishmania species	Old World/ New World	Clinical Disease	Distribution
L. aethiopica	OW	CL, DCL	East Africa
L. amazonensis	NW	CL, DCL, MCL	South America
L. donovani	OW	VL, PKDL	Central Africa, south Asia, Middle East, India, China
L. infantum	OW	CL, VL	North Africa, Mediterranean, southeast Europe, Middle East, Central Asia, North, Central and South America
L. major	OW	CL	Central and North Africa, Middle East, Central Asia
L. mexicana	NW	CL, DCL	USA, Ecuador, Peru, Venezuela
L. tropica	OW	CL, VL	Central and North Africa, Middle East, Central Asia, India
L. venezuelensis	NW	CL	Northern South America, Venezuela
L. braziliensis	NW	CL, MCL	Western Amazon Basin, South America, Brazil, Bolivia, Peru Guatemala, Venezuela
L. guyanensis	NW	CL, MCL	Northern South America, Bolivia, Brazil, French Guiana, Suriname
L. lainsoni	NW	CL	Brazil, Bolivia, Peru
L. lindenbergi	NW	CL	Brazil
L. naiffi	NW	CL	Brazil, French Guyana
L. panamensis	NW	CL, MCL	Central and South America, Brazil, Panama, Venezuela, Colombia
L. peruviana	NW	CL, MCL	Peru, Bolivia
L. shawi	NW	CL	Brazil
L. martiniquensis	OW	CL, VL	Martinique, Thailand
L. siamensis	OW	CL, VL	Central Europe, Thailand, USA
L. colombiensis	NW	CL, VL	Colombia

CL: Cutaneous leishmaniasis
DCL: Diffuse cutaneous leishmaniasis
MCL: Mucocutaneous leishmaniasis
VL: Visceral leishmaniasis
PKDL: Post kala-azar dermal leishmaniasis

Various species of *Leishmania* are classified into New-World or Old-World species according to their geographical distribution. Different species can cause different subtypes of leishmaniasis [6].

1-3 Sandfly, Transmission Vector of Leishmaniasis

Sandflies belong to the order of *Diptera,* the family of *Psychodidae,* the sub-family of *Phlebotominae*. They can be further categorized into five genera, namely *Phlebotomus, Sergentomyia, Lutzomyia, Brumptomyia* and *Warileya*. They are small flying insects which are around 2 mm long. Their colour varies from light brown to black (**Figure 1-4)**. Both male and female sandflies usually feed on fruit and plant juice but female sandflies require a blood meal before they lay eggs. Therefore, female sandflies carrying *Leishmania* parasites transmit parasites to animals and humans during blood feeding [11]. Around 800 species of sandflies have been described, among which 70 species from Phlebotomus and Lutzomyia are proven or suspected to be vector of *Leishmania* [5].

The tropical climate favors the growth of sandflies as well as the transmission of leishmaniasis. Leishmaniasis is therefore endemic in tropical and subtropical areas such as Africa, the Mediterranean region and South America [12]. Due to global warming, sighting sandflies and incidence of leishmaniasis has a trend of moving northward in Europe [13, 14] and North America [15].

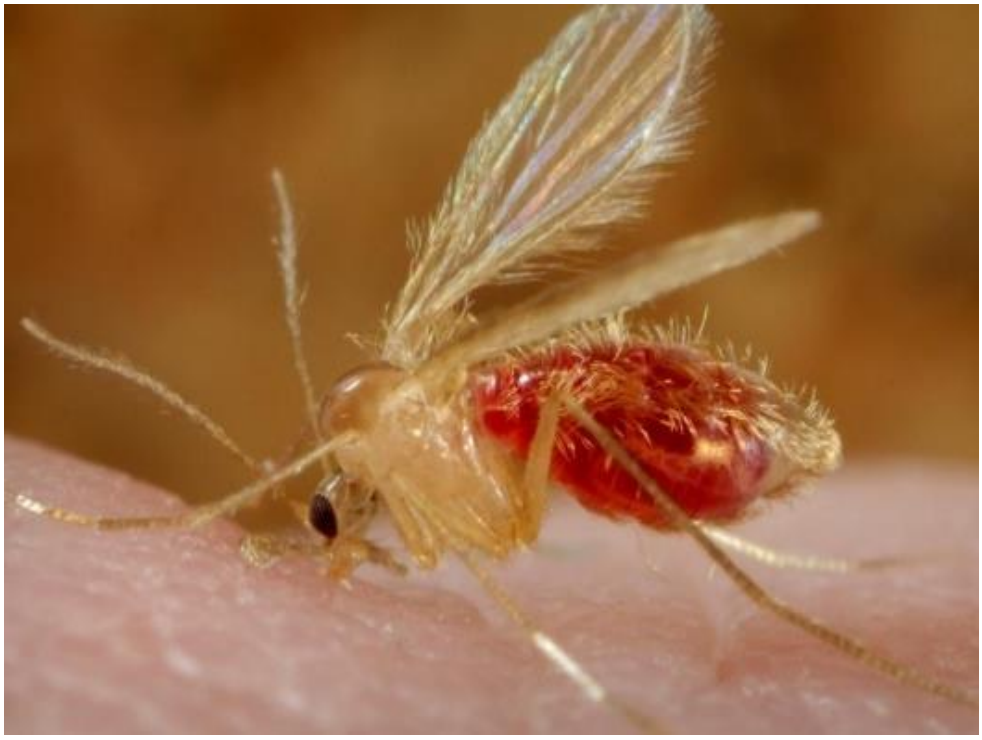


Figure 1-4 Phlebotomine sandfly

Sandflies are around 2 mm long with color ranging from yellow to black. Sandflies transmit leishmaniasis while they take blood meals [16].

1-4 Life Cycle of *Leishmania*

Leishmania replicates and interchanges between insect vector and mammalian hosts throughout its life cycle. To complete this life cycle, phlebotomine sandflies are responsible for transmitting the parasites from sandfly vector to mammalian hosts. When *Leishmania* is carried by the sandfly vector, it lives as extracellular promastigotes inside the mid-gut of the sandfly. When the sandfly bites another mammalian host such as a human, the parasite is transmitted from the mid-gut to the human systemic circulation. Engulfed by macrophages, the promastigotes transform into amastigote and grow intracellularly within macrophages. During the process, the flagellum is internalized, and the promastigotes become round-shaped amastigotes. Inside the macrophage, amastigotes keep multiplying and start to infect other macrophages when being released upon cell burst [17]. The life cycle completes when another sandfly bites the host and take up blood that carries the parasites (**Figure 1-5**).

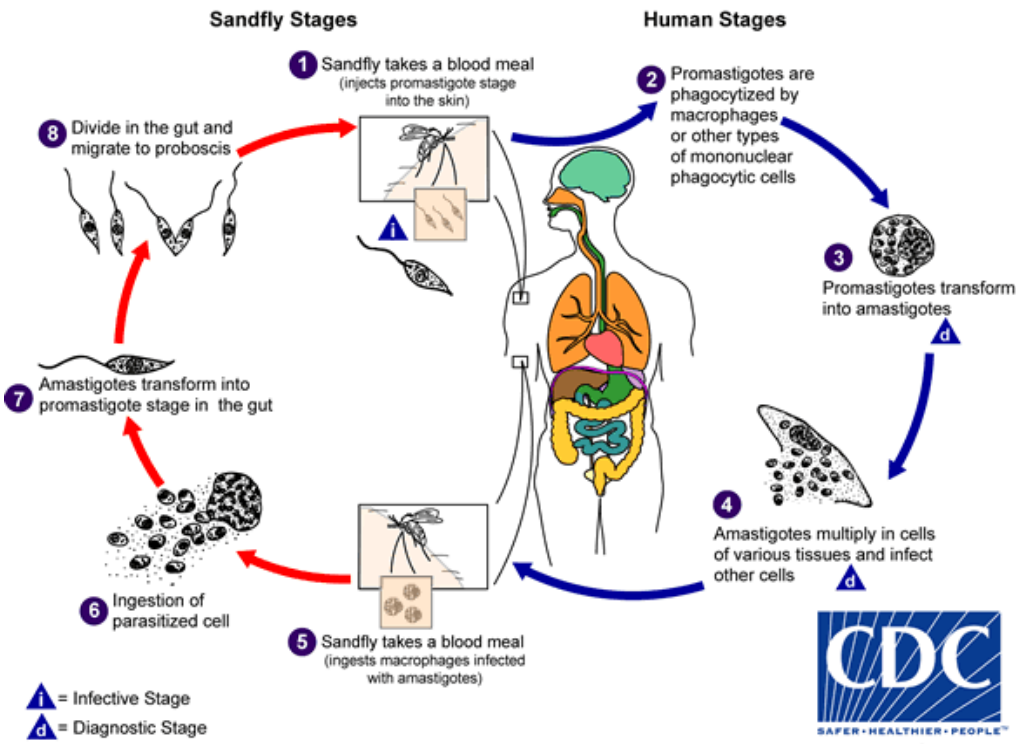


Figure 1-5 Life Cycle of *Leishmania* including human and sandfly vector

Promastigotes carried by the vector (sandfly) are transmitted to mammalian host (human) upon the bite for a blood meal. Promastigotes are transformed into amastigotes in the macrophages after phagocytosis. Amastigotes multiply inside the macrophages and start to infect other macrophages. The life cycle is complete when other sandflies take a blood meal of an infected human and the parasite transforms from amastigote into promastigote form [16].

1-5 Clinical Manifestation

Leishmaniasis can be divided into cutaneous and visceral leishmaniasis. It can also be further classified into different subtypes. They are localized cutaneous leishmaniasis (LCL), diffused cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL), kala-azar (visceral leishmaniasis) and post kala-azar dermal leishmaniasis (PKDL). Different subtypes of leishmaniasis are caused by different species of *Leishmania* and they present different clinical symptoms in patients.

1-5-1 Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis is endemic in more than 70 countries worldwide. The ten countries with highest case counts, namely Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru, accounted for 70 – 75% CL incidence [1]. About 1.5 – 2 million new cases were estimated each year. Although cutaneous leishmaniasis is not fatal to human, its disease burden seriously affects human lives. Cutaneous leishmaniasis starts with a bite by sandflies that the *Leishmania* parasites are transmitted to the host. A papule is developed at the site of infection followed by a nodule. In LCL, the nodules ulcerate and form a lesion. Spontaneous cure with lifelong immunity usually occurs but leaving permanent scars. In DCL, non-ulcerative nodules spread from the initial site of infection and cover the entire body of the patients. MCL is the most serious among the three subtypes. Metastasis of parasites to mucosal tissues causes nasal inflammation followed by lifelong mucosa ulceration, septum perforation and facial disfiguration. Compared with LCL, DCL and MCL are more difficult to treat and they can never be cured spontaneously. Besides, it is potentially fatal when secondary bacterial infection occurs [18].

Different *Leishmania* species can cause different subtypes of cutaneous leishmaniasis. *L. major, L. tropica, L. aethiopica* in North and East Africa, Central Asia and Middle East and *L. mexicana* and *L. amazonensis* in Central and South America, the USA and Mexico cause LCL. *L. aethiopica, L. mexicana* and *L. amazonensis* often develop DCL under certain conditions [19]. MCL usually occurs in patients infected with *L. panamensis, L. guyanensis, L. braziliensis,* and *L. amazonensis*. Patients infected with *L. braziliensis* are more commonly found [18, 19].

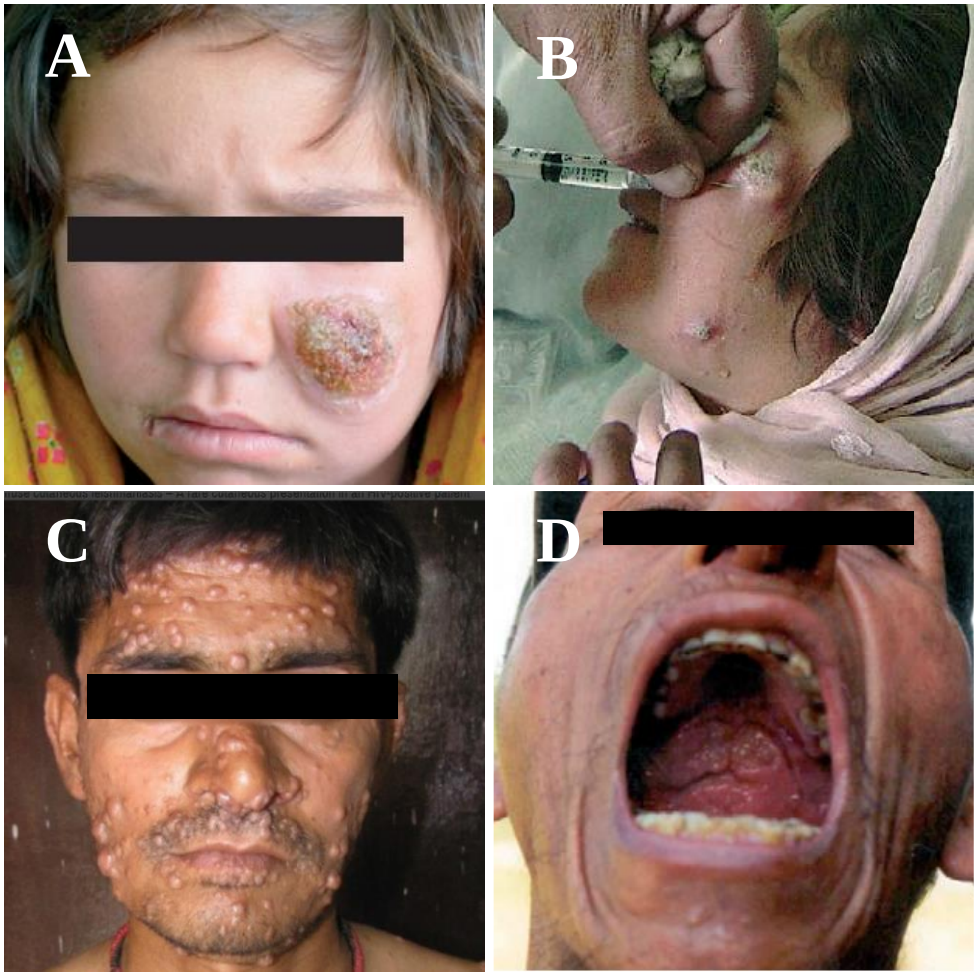


Figure 1-6 Cutaneous leishmaniasis

The pictures show the clinical manifestation of different subtypes of cutaneous leishmaniasis. (A) localized cutaneous leishmaniasis (LCL). Lesion grew on the face of the patients. (B) The patient was receiving pentavalent antimonials by intralesional injection. (C) Diffuse cutaneous leishmaniasis (DCL). Non-ulcerative nodules grew on the skin. (D) Mucocutaneous leishmaniasis (MCL). Mucosal destruction developed in the mouth area [18, 20, 21].

1-5-2 Visceral Leishmaniasis (VL)

Visceral leishmaniasis is endemic in six countries including India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil, which accounts for over 90% incidence worldwide. An estimate of 0.5 million new cases and 20,000 to 40,000 deaths every year was reported [1]. It is fatal if left untreated. Patients suffering from visceral leishmaniasis develop symptoms including fever, fatigue, weakness, loss of appetite, weight loss and signs of parasitic invasion such as enlarged lymph nodes, spleen and liver. When the disease advances, splenomegaly and hepatomegaly are developed along with abdominal distension and pain. However, the symptoms and signs of bacterial co-infections such as diarrhea, pneumonia and tuberculosis may confuse the clinical picture at the time of initial diagnosis. Therefore, the VL symptoms may persist from weeks to months before patients seek medical care [22].

Visceral leishmaniasis is usually caused by two species of *Leishmania*, *L. donovani* and *L. infantum*, depending on the geographical area where it happens. *L. infantum* mostly infects children and immunosuppressed individuals while *L. donovani* infects all age groups [22].

Besides VL, post kala-azar dermal leishmaniasis (PKDL) is a complication of visceral leishmaniasis that usually develops after the treatment of VL. It is immunologically mediated because of increased production of interferon-γ as a reaction to survived parasites in the skin, causing inflammation. [23] PKDL is characterized by macular, maculopapular and nodular rash which starts around the mouth and spread to other parts of the body [23].

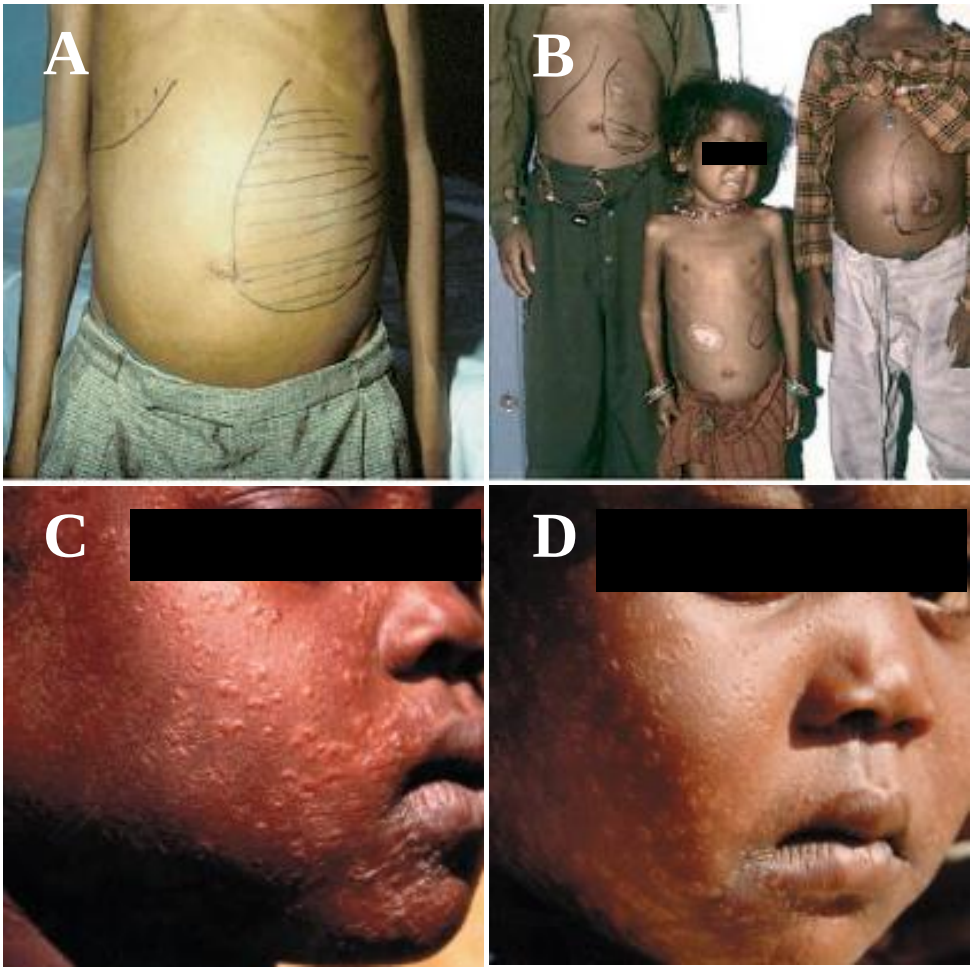


Figure 1-7 Visceral leishmaniasis

The pictures show the clinical manifestation of different subtypes of visceral leishmaniasis (kala-azar). (A & B) Hepatosplenomegaly developed in the patients with visceral leishmaniasis. (C) Post kala-azar dermal leishmaniasis (PKDL). (D) Permanent scars were left on her face after the treatment with pentavalent antimoniate [20, 23].

1-6 Current Antileishmanial Drugs

No vaccines are available for the prevention of leishmaniasis [24]. Apart from chemotherapy, there are many strategies to cure leishmaniasis such as photodynamic therapy and immunotherapy. However, the use of photodynamic therapy is only limited to cutaneous leishmaniasis. Immunotherapy using TLR7 agonist imiquimod combined with pentavalent antimony to treat cutaneous leishmaniasis was tested in clinical trial [25]. Treatment of leishmaniasis still relies on chemotherapy. However, only a few therapeutic options are available, and they are far from satisfactory. The use, limitations and challenges of the current antileishmanial drugs are described below.

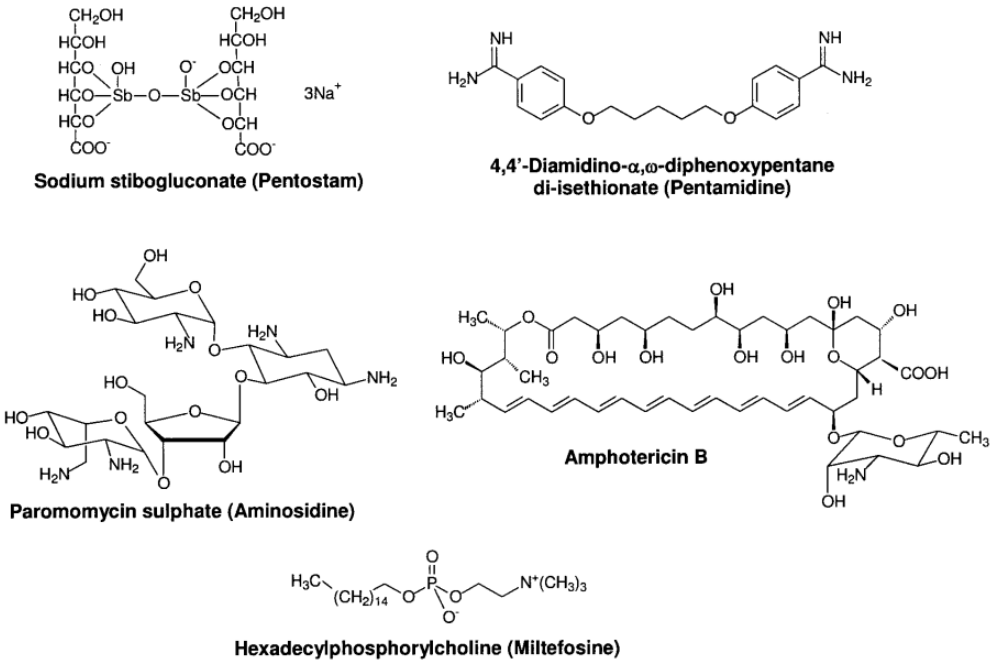


Figure 1-8 Chemical structure of current antileishmanial drugs

Drugs for treating leishmaniasis are limited. Currently available drugs are sodium stibogluconate, pentamidine, paromomycin, amphotericin B and miltefosine. [26]

1-6-1 Pentavalent Antimonials

Pentavalent antimonials were discovered in 1946 [27] and have been used for five to six decades as the first-line antileishmanial drug. The compounds clinically available are sodium stibogluconate (SSG, brand name: Pentostam) and meglumine antimoniate (Brand name: Glucantime).

The mechanism of action of pentavalent antimonials against *Leishmania* is not clearly understood. It is generally accepted that Sb(V) is a prodrug and Sb(III) is the active form. However, there are still no answers to the questions that whether Sb(V) is enzymatically converted and where the reduction takes place. It was proposed that antimony reduction is mediated by thiol-dependent glutathione S-transferase [28] or glutaredoxin-dependent arsenate reductase [29], that subsequently cause a rapid disappearance of trypanothione and glutathione by thiol efflux out from the parasites. Sb(III) was also shown as a reversible inhibitor of trypanothione reductase *in vitro* that inhibits the recovery of intracellular thiols. The Sb(III)-mediated loss of thiols results in redox potential decrease and parasite death [26].

SSG is recommended to be administered via intramuscular or intravenous injection for visceral and mucosal cutaneous leishmaniasis. Intralesional injection is preferred in the treatment of cutaneous leishmaniasis. However, intramuscular and intralesional injections cause pain to patients. SSG at 20 mg/kg/day for 20 days is recommended for cutaneous leishmaniasis. The same dosage for 28 days is recommended for visceral and mucosal cutaneous leishmaniasis [30].

Intramuscular injection of antimonials often causes local pain during intramuscular injections and systemic side effects. Typical side effects include nausea, vomiting,

weakness and myalgia, abdominal colic, diarrhea, skin rashes, hepatotoxicity and cardiotoxicity [31]. Besides, antimonials as the first-line therapeutic option are becoming ineffective in many endemic areas. The main reason for the emergence of pentavalent antimonial resistance is the easy accessibility and inappropriate prescription of drugs by unqualified medical practitioners in India [26]. Some common practices were used to prevent renal toxicity such as introducing drug-free interval and splitting daily doses. However, such practices expose the parasites to lower drug pressure that eventually leads to drug resistance [32]. It has been reported that reduced expression of aquaglyceroporin1 (AQP1) resulted in reduced accumulation of Sb(III) in SSG-resistant *Leishmania* [33]. Besides, increased synthesis of trypanothione and glutathione was found in resistant cell lines that might replenish intracellular thiols [26]. The use of sodium stibogluconate in Bihar, India was abandoned in 1985. Its use is also becoming limited in other countries [34].

1-6-2 Pentamidine

Pentamidine is an aromatic diamine primarily used for treating pneumonia. It has become a second-line drug to treat leishmaniasis since its introduction in 1952. It is normally used to treat CL, DCL and VL in pentavalent antimony-resistant cases [35].

The mechanism of action of pentamidine against *Leishmania* is still not clearly understood. It was reported that pentamidine enters the cell membrane of parasites through polyamine and arginine transporter [36]. It was thought to target kinetoplast DNA and inhibit its function [37]. Other proposed mechanisms include

inhibition of polyamine biosynthesis, DNA minor groove binding and disruption on mitochondrial membrane potential [26].

Between late 1970s and early 1980s, pentamidine was successfully used in treating leishmaniasis. A high cure rate of 98.8% without any relapses was reported when patients was given 4 mg/kg pentamidine intravenously three times a week for 3 – 4 weeks (10 to 12 injections). However, a decline of cure rate started in the 1980s with only 75.2% cure rate even when 20 injections were used. Dose optimization to 3 mg/kg pentamidine administered intramuscularly for 4 alternate days could achieve a 96% cure rate [37].

The major limitation of using pentamidine is its toxicity. Myalgias, pain at the injection site, nausea, headache and less commonly metallic taste, burning sensation, numbness and hypotension were reported [37]. Some literatures reported severe toxicity including insulin-dependent diabetes mellitus, nephrotoxicity, tachycardia, hypoglycemia and hyperglycemia [36-38]. Besides, clinical resistance of pentamidine had been reported since 1980. A quick drop in response rate from > 95% to < 70% was observed when used in Sb-refractory patients in India [26]. The resistance was associated with ATP-binding cassette (ABC) protein PRP1 [36], reducing pentamidine accumulation in parasite mitochondria [39]. Therefore, pentamidine can only be used in combination with other antileishmanial drugs [36]. Currently, pentamidine is seldom used and it is mainly used in antimony-refractory cases.

1-6-3 Amphotericin B

Amphotericin B, primarily used as a polyene antifungal agent, is a potent drug to treat leishmaniasis since the 1960s. Ergosterol, which is the target of amphotericin B, is first identified in fungi. Amphotericin B, when bound to ergosterol on the cell surface of *Leishmania*, can disrupt the membrane function and cause a leakage of cellular content. Amphotericin B has many solid forms. Amphotericin B deoxycholate is used most clinically. A clinical study was conducted in Bihar, India to demonstrate the efficacy of amphotericin B deoxycholate. It was administered to 938 patients at 1 mg/kg via a 2-hour intravenous infusion every day for 20 days to achieve a 99% cure in VL [40].

Although amphotericin B binds human cholesterol to a lesser extent, patients still suffer from adverse side effects such as fever, chills, bone pain, hypokalemia, bronchopneumonia, acute renal failure and heart failure [37, 40]. Some formulations of amphotericin B were made to solve this problem such as amphotericin B lipid complex (ABLC, Abelcet), amphotericin B colloidal dispersion (ABCD, Amphocil), and liposomal amphotericin B (L-AmB, Ambisome). The most famous one is the liposomal form. L-AmB contains three major components, hydrogenated soy phosphatidylcholine, distearoylphosphatidyl glycerol and cholesterol. Hydrogenated soy phosphatidylcholine serves as the majority in the liposome which is not easily hydrolyzed inside the body. Distearoylphosphatidyl glycerol can facilitate the retention of amphotericin B inside the liposomes because of its electrostatic interaction with the positively-charged amino group in amphotericin B. Cholesterol in the liposome formulation can bind amphotericin B to facilitate further the retention of amphotericin B inside the liposome bilayer [41]. After the liposome use, amphotericin B had an extended

terminal half-life (152 hr), higher plasma concentration and lower urine clearance (4.5%) [42]. The effectiveness of liposomal amphotericin B over the conventional amphotericin B deoxycholate was demonstrated in 13 clinical trials [43]. Many reports have shown very promising results. Some research groups compared the effectiveness between the liposomal form and the conventional amphotericin B. A high cure rate was achieved with shorter duration of treatment but higher costs. It was suggested that a 60% price reduction was required to make liposomal amphotericin B more competitive in the market [44].

Although the liposomal formulation can prolong the residence time of amphotericin B, it will also result in toxicity. Short course dosing or single shot injection of liposomal amphotericin B might be needed to decrease toxicity, as proposed and supported by Drugs for Neglected Diseases Initiative (DNDi) [42]. Some clinical studies have demonstrated its effectiveness. In a clinical study of VL conducted in Bihar, India, a single dose of liposomal amphotericin B (7.5 mg/kg) achieved a 96% cure rate (195/203, 12 relapsed) 6 months after the treatment [45]. In another study, a higher dosage of liposomal amphotericin B (10 mg/kg) achieved 100% cure of VL (304/304, 13 relapsed) [46]. In these two cases, fewer adverse effects were reported. Based on the observations, single dose of liposomal amphotericin B will be more acceptable afterwards.

Although amphotericin B was found to eliminate *Leishmania* parasites effectively, some amphotericin B-resistant cases were found in patients who suffered from visceral leishmaniasis/HIV co-infection. Down-regulation of S-adenosyl-L-methionine:C-24-Δ-sterol methyltransferase (SCMT) was revealed and confirmed in both laboratory selected promastigotes [26] and clinical isolates [47]. This results in the absence of ergosterol and higher membrane fluidity to decrease the affinity

for amphotericin B. Besides, elevated level of multidrug resistant protein (MDR1) was found that causes drug efflux of amphotericin B from the cells [47].

The oral use of liposomal amphotericin B in mice was also discussed. It was demonstrated that oral administration of liposomal amphotericin B at 10 or 20 mg/kg twice daily achieved at least 90% reduction in parasitemia. However, no related studies in humans were found until now [48]. Although this drug is very effective, and its liposomal form can lower the toxicity, the high cost for liposome preparation is still an obstacle for its widespread use.

1-6-4 Paromomycin

Paromomycin is aminoglycoside aminocyclitol antibiotics which is active against Gram-positive and Gram-negative bacteria. Then it was also found active in some protozoa as well as *Leishmania* parasites. Paromomycin is not orally-available and can only be given in parenteral route [49]. The mechanism of action of paromomycin is to inhibit protein translation by binding to 16S ribosomal RNA [50].

In 2007, this compound was licensed to cure leishmaniasis in India [51] because of the high cure rate (94.6%) in treating visceral leishmaniasis (474/501, 22 relapsed) when administered intramuscularly at 11 mg/kg for 21 days [52]. In treating cutaneous leishmaniasis, due to its inefficacy in parenteral route, paromomycin is given in the form of cream ointment to treat cutaneous leishmaniasis. But the efficacy varied with the formulation and the species that caused cutaneous leishmaniasis [51, 53].

Clinical resistance of paromomycin is seldom reported. There is only one literature reporting paromomycin resistance in patients. Isolates of *L. aethiopica* taken from relapsed patients were 3 to 5-fold less sensitive to paromomycin [26]. The mechanism of resistance to paromomycin in bacteria was known to be related to decreased drug uptake, alteration of ribosomal binding and modification of amino groups or hydroxyl groups by inactivation of N-acetyl transferases, O-phosphotransferase or O-nucleotidyl transferases [26]. However, paromomycin resistance mechanism in *Leishmania* was seldom discussed. Only decrease uptake of paromomycin in promastigotes was found in laboratory-resistant cell line [54]. No enzymatic modification or mutation to small-subunit of rRNA gene was observed, implying paromomycin resistance in *Leishmania* may be due to other mechanisms [55].

Paromomycin, being a low-cost but effective drug, compared with amphotericin B deoxycholate, has fewer adverse effects. The most frequently-reported side effects are injection site pain, elevation in hepatic transaminases, ototoxicity and renal dysfunction [56]. However, in treating visceral leishmaniasis, the requirement of hygienic places for drug administration is the major drawback [56].

1-6-5 Miltefosine

Miltefosine, originally used as an anticancer agent to treat melanoma, is now repurposed to become an antileishmanial agent to treat leishmaniasis since the 1990s. It is also the first orally-available drug in treating leishmaniasis. Miltefosine is a phosphorylcholine ester of hexadecanol, membrane-active alkylphospholipid. Mechanisms of action are still limited. It is now known that miltefosine can

modulate effects on phospholipid metabolism and parasite membrane composition [57] and induce cell apoptosis [58]. Miltefosine targets the key enzymes responsible for metabolism of ether phospholipids (present on the cell surface of parasites) including sn-l-acyl-2-lyso-glycerophosphocholine and sn-l-alkyl-2-lyso-glycero-3-phosphocholine acyltransferase and diethylacetonephosphate acetyltransferase and glycosomal alky-specific-acyl CoA acyltransferase (**Figure 1-9**) [59]. Miltefosine is highly orally-available, with an oral bioavailability over 80% in rats and dogs. Besides, it is metabolically-stable and resistant to modifications by CYP450s except phospholipase D [57]. Due to its high oral bioavailability, miltefosine is the first approved oral drug to treat leishmaniasis. It has a successful cure rate of 94% in visceral leishmaniasis using miltefosine at 2.5 mg/kg/day orally for 28 days [35].

Since approval, the efficacy of miltefosine has been studied for more than 20 years. In 1999, the *in vivo* efficacy of miltefosine in humans suffering from visceral leishmaniasis was evaluated by S. Sundar. A high cure rate of 95% (114/120) was measured with 6 relapsed cases recorded 6 months after oral administration of miltefosine with different regimens [60]. Another study conducted in 2002 also demonstrated the effectiveness of miltefosine at 50 or 100 mg/day (94% cure rate (282/299), 9 (3%) relapses) [61]. Sundar further published in 2012 to evaluate the safety and efficacy of this drug for treating visceral leishmaniasis. A high rate (90.3%) can still be maintained. However, the relapse rate was 6.8% (39/567) which doubled the figure recorded ten years previously. The substantial increase in failure rate implied the emergence of clinical miltefosine resistance [62].

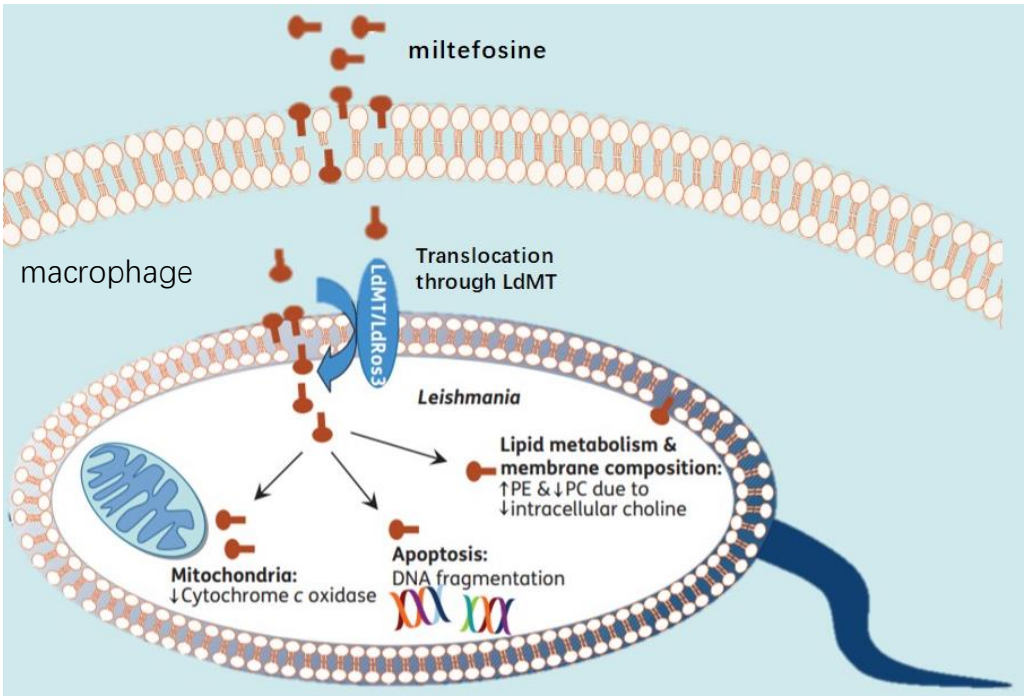


Figure 1-9 Mechanism of action of miltefosine against *Leishmania*

Miltefosine entering the macrophages is translocated through the *L. donovani* miltefosine transporter (LdMT) to target the key enzymes involved in ether phospholipid metabolism, inhibit the cytochrome c oxidase and subsequent induce apoptosis (modified from [57]).

The reason for the emergence of miltefosine resistance in *Leishmania* may be associated with its slow elimination. A long terminal half-life of 30.9 days was observed in CL-infected patients who received 150 mg/day miltefosine for 28 days. Miltefosine at subtherapeutic concentration may contribute to the selection of drug-resistant parasites [63]. The resistance mechanism to miltefosine was also studied. Novel plasma membrane P-type transporter, *L. donovani* miltefosine transporter (LdMT), which is responsible for the uptake of both miltefosine and glycerophospholipid, was identified. Inactivation of LdMT resulting in reduced uptake of miltefosine was accomplished by single point mutation on the transporter [26].

The safety of miltefosine was also assessed and evaluated. Severe vomiting, nausea, diarrhea, nephron- and hepatotoxicity were found in patients during the treatment. Teratogenicity potential of miltefosine limits its use in pregnant women [64].

Although miltefosine is the first orally-available drug and is effective in treating cutaneous and visceral leishmaniasis, the ease of resistance emergence and side effects make miltefosine not a promising drug in treating leishmaniasis.

1-7 Flavonoids as Novel Antileishmanial Drugs

Without available vaccines, the treatment of leishmaniasis solely relies on chemotherapy, which suffered from clinical drug resistance, relapse, high toxicity and high cost. Novel drug developments with less toxic and cost-effective drugs with greater efficacy will be needed. The use of natural products as potential antileishmanial agents has been widely studied because of wide variety of species and large availability worldwide. Besides, the secondary metabolites produced by plants are found to be active against pathogens such as fungi, virus and bacteria. There are 250,000 plant species worldwide while only a small fraction has been tested for their potency. [65] Many of them were moderately active towards *Leishmania* parasites *in vitro*, however, only some of them were tested *in vivo* [7, 65].

1-7-1 Natural Flavonoids

Recently, some secondary metabolites in the plants had been isolated, purified, identified and found to be active against *Leishmania*. One of the categories was

flavonoids. Flavonoids are phenolic compounds which are commonly found in wine, tea and vegetables and other medicinal plants. They are present in high abundance in our human diet and therefore believed to be non-toxic to humans. Flavonoids are found to have anti-inflammatory, antioxidant, free-radical scavenging and anti-cancer properties [66]. The role of flavonoids in preventing different diseases has been studied. Flavonoids were demonstrated to significantly improve cognitive capabilities and related neurodegenerative disorders including Alzheimer's disease via inhibition of cholinesterase, β-secretase, free radicals, and modulation of signaling pathways [67]. In animal models, flavonoids nobiletin [68] and luteolin [68] could reduce the β-amyloid formation in brain tissues. Flavonoids have also been shown to have anti-inflammatory and anti-cancer activities by inhibiting reactive oxygen species (ROS), xanthine oxidase and cyclooxygenase-2 (COX-2) that are important in tumor initiation and progression [69]. Quercetin, which is one of flavonoids abundant in vegetables could downregulate Bcl-2 and Bcl-xL but upregulate the proapoptotic proteins, Bid, Bad and Bax, caspase-3, caspase-9 and cytochrome c in growth inhibition of metastatic ovarian cancer PA-1 cells [70]. Flavonoids were also reported with antibacterial, antifungal and antiviral activity. They also have anti-bacterial activity possibly by inhibiting DNA gyrase, nucleic acid synthesis, cytoplasmic membrane function and energy metabolism [71].

Natural flavonoids have been studied for antileishmanial activity including luteolin, quercetin and apigenin (**Figure 1-10**). Luteolin isolated from *Vitex negundo* (Verbenaceae) and quercetin isolated from *Fagopyrum esculentum* (Polygonaceae) were potent against *L. donovani* promastigotes with IC_{50} of 12.5 and 45.5 μM, respectively. They can reduce the parasite load by 70% in intracellular amastigotes

at such concentration [72] while apigenin was active against *L. amazonensis* promastigotes and intracellular amastigotes with IC_{50} of 23.7 and 4.3 μM, respectively [73, 74]. *In vivo* study also showed that luteolin at 3.5 mg/kg and quercetin at 14 mg/kg could reduce the splenic parasite load by 80% and 90% in VL-hamster model when administered orally twice a day [72]. On the other hand, oral apigenin at 2 mg/kg once daily for 38 days could reduce the lesion size effectively in CL-mouse model [74]. The above results demonstrated the effectiveness of flavonoids in treating leishmaniasis.

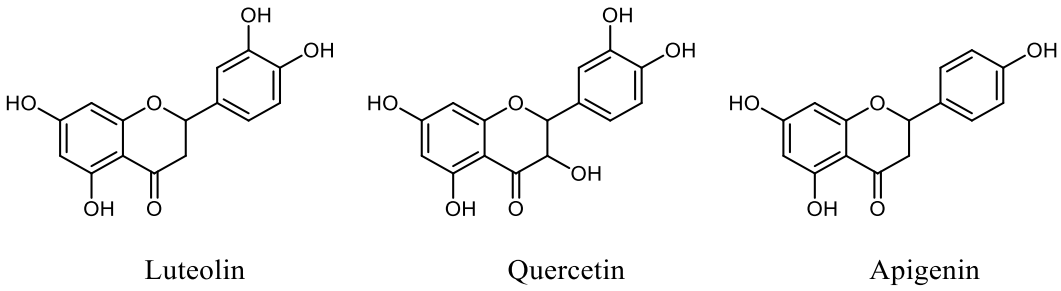


Figure 1-10 Chemical structure of three natural flavonoids, namely luteolin, quercetin and apigenin.

The mechanism of action of these compounds was also studied. Luteolin and quercetin were found to inhibit parasite DNA synthesis via inhibition of DNA topoisomerase II. Such inhibition induced kinetoplastid DNA linearization and kDNA minicircle cleavage that led to cell cycle arrest at G0/G1 phase and subsequent apoptosis [65, 72]. In addition, they are also specific inhibitors of topoisomerase I in *Leishmania*. On the other hand, quercetin and apigenin were associated with the reactive oxygen species (ROS) generation in both promastigotes [75] and intracellular amastigotes [74, 76], leading to mitochondrial dysfunction [73, 75]. Besides, quercetin and its analogs were found to inhibit manganese-dependent *Leishmania* arginase via the interaction with the substrate arginine and the cofactor Mn^{2+}. Moreover, by docking analysis, quercetin was

found to interact with the Asp129 inside the active site of arginase, which is involved in metal bridge formation with Mn^{2+} in order to disrupt the polyamine biosynthesis for cell proliferation and ROS detoxification by trypanothione [77].

1-7-2 Synthetic Flavonoids

Although the natural flavonoids were active in killing *Leishmania* parasites, their potency was not good as the standard antileishmanial drugs. Structural modification of natural products to improve the pharmacological activity and druggability has been reported [78, 79].

Chow's research group had designed and synthesized flavonoids as modulators to reverse the drug resistance caused by overexpression of transporter proteins such as MDR1, BCRP and MRP1 in cancer cells [80-84]. It was demonstrated that synthetic flavonoid dimers successfully reversed the resistance of promastigotes towards sodium stibogluconate and pentamidine *in vitro* [85, 86]. One compound in the dimer library was found to have antileishmanial activity. Such compound was further modified to yield **FD39** (**Figure 1-11**), which was potent against *Leishmania* (IC_{50} against promastigotes and amastigotes = 0.4 μM) with low toxicity (IC_{50} against murine macrophages > 88 μM) *in vitro* [87]. Its efficacy against cutaneous leishmaniasis was demonstrated in BALB/c mice by intralesional injection into the lesion footpad [88]. However, because of its large molecular size (MW = 723) and low water solubility, a low oral bioavailability of **FD39** was expected. Therefore, modification of the compound is needed.

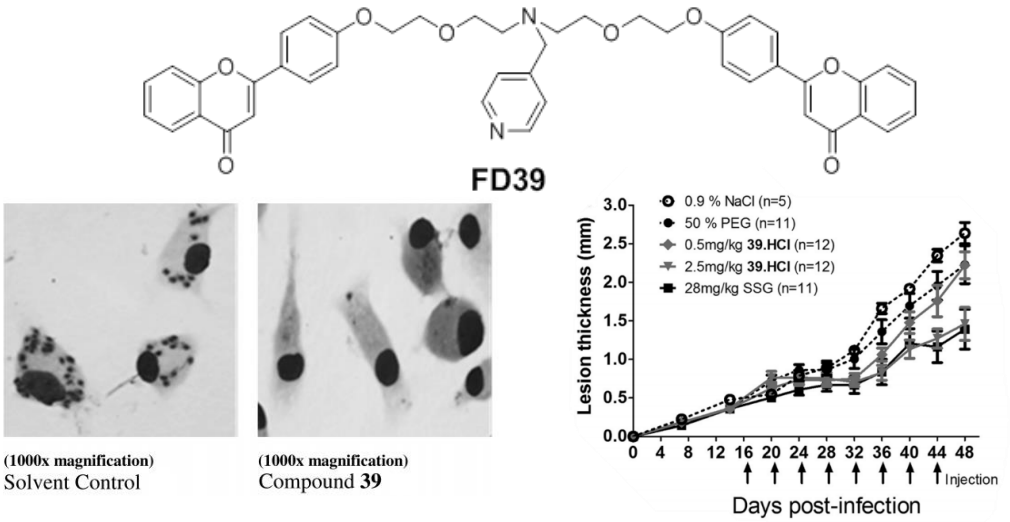


Figure 1-11 Flavonoid dimer, **FD39** was active in treating cutaneous leishmaniasis

Flavonoid dimers were developed by Chow's group and were demonstrated to be effective in reversing SSG resistance in *Leishmania*. One of the candidates, **FD39** was found to be active in killing *Leishmania* parasites. (A) Chemical structure of **FD39**. (B) **FD39** was active in killing SSG-resistant *L. donovani* Ld39 inside the peritoneal elicited murine macrophages *in vitro* at a IC_{50} of 0.4 μM. [87] (C) **FD39** was effective in treating cutaneous leishmaniasis in an *in vivo* BALB/c mouse model. When administered intradermally into the lesion at 2.5 mg/kg, **FD39** could resolve the lesion by 32% compared to the solvent control [89].

1-8 Objectives of the Project

As the use of current antileishmanial drugs was limited by their respective drawbacks, alternative chemotherapeutics are needed. Previously, as synthetic flavonoid **FD39** was potent against *Leishmania* both *in vitro* and *in vivo* with low oral bioavailability, therefore this project is proposed to:

1. to discover potent flavonoid compounds against *Leishmania* by *in vitro* method.
2. to select active, metabolically-stable and orally-available candidate flavonoid monomer compounds.
3. to fully characterize the selected candidate(s) in terms of pharmacokinetics and *in vivo* toxicity.
4. to evaluate the *in vivo* efficacy of the selected candidate(s) against cutaneous and visceral leishmaniasis.

2 In Vitro Screening of Flavonoid Monomers as Antileishmanial Drugs

2-1 Introduction

As mentioned in the Introduction chapter (See **Section 1-7**), the first generation compound **FD39** suffered from low solubility and low oral bioavailability despite having high potency against *Leishmania*. As the flavone moiety is poorly soluble in water, it was proposed to modify the dimeric structure of **FD39** to monomeric structure to improve the drug solubility. Besides, the linkers to connect the flavone moiety and the pyridine and the nitrogen position of the pyridine were also modified to seek better potency. Hence, a library of flavonoid monomers, **FM01** – **FM25 (Figure 2-1**) was synthesized in-house by Dr. Kin-Fai CHAN and Dr. Xue-Zhen ZHU, and tested for their antileishmanial activity.

Using promastigotes in drug testing is simple, fast and reproducible [90]. However, there is a vast difference between promastigotes and amastigotes [91]. While the intracellular amastigotes are prepared by infecting macrophages with promastigotes at 37°C to allow transformation into amastigotes which is complex and time-consuming, such model is widely-used and can provide a better predictive value as it reflects the conditions encountered by a drug such as drug penetration from the media into the macrophages. In this project, both promastigote and intracellular amastigote models were employed to determine the activity of flavonoid monomers. The viability of *Leishmania* promastigotes is measured by CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega).

NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells can reduce the MTS tetrazolium compound in the assay into a colored formazan product in the presence of an electron coupling reagent PMS. The amount of formazan product measured by spectrophotometer can be quantified by absorbance measurement at OD_{490} that is directly proportional to the number of living cells in the culture. The lower is the OD_{490} compared to untreated control, the higher is the antileishmanial activity of the flavonoid monomers. The viability of *Leishmania* amastigotes after incubation with flavonoid monomers is measured by enumerating the number of amastigotes inside infected macrophages under the microscope after Giemsa staining. The lower is the parasite burden compared to untreated control, the higher is the antileishmanial activity of the flavonoid monomers.

In this study, cutaneous *Leishmania*, namely *L. amazonensis* LV78 and *L. major* FV1, and visceral *Leishmania* including wild-type *L. donovani* HU3 and SSG-resistant clinical isolate *L. donovani* Ld39 were employed for *in vitro* drug screening. To determine the cytotoxicity of flavonoid monomers, mouse fibroblast L929, murine macrophages RAW264.7 and peritoneal elicited macrophages (PEM) were used.

After determining the potency and cytotoxicity, some potent flavonoid monomers were selected for further optimization by structural modification to synthesize a second generation flavonoid monomer library by Mr. Zhen LIU (**Figure 2-2**). Potent flavonoid monomer candidates from the second generation flavonoid monomer library were further tested for their killing activity against *L. donovani* strain AG83 that was resistant to amphotericin B, miltefosine, pentamidine and SSG.

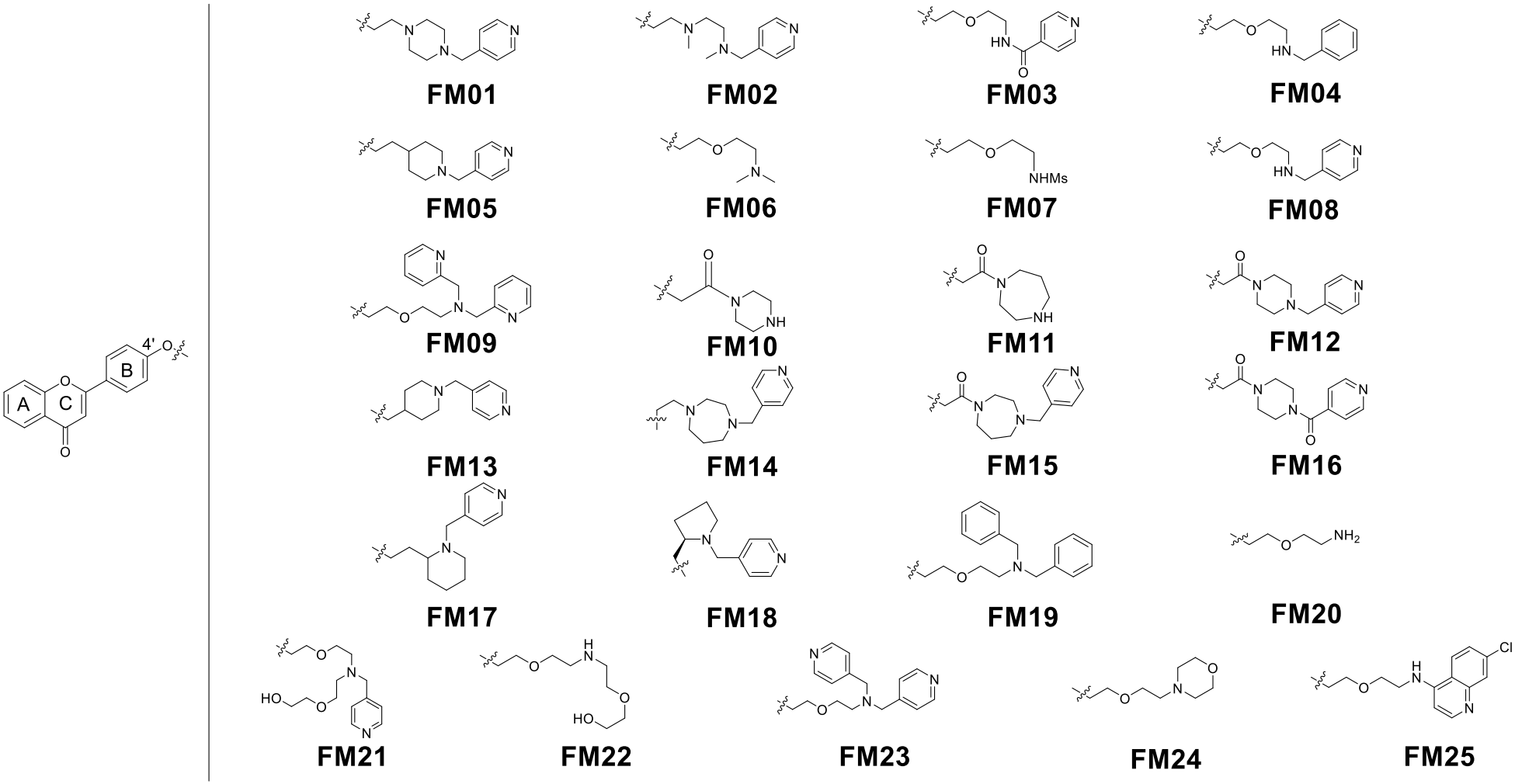


Figure 2-1 Chemical structure of the first generation flavonoid monomers **FM01** – **FM25**

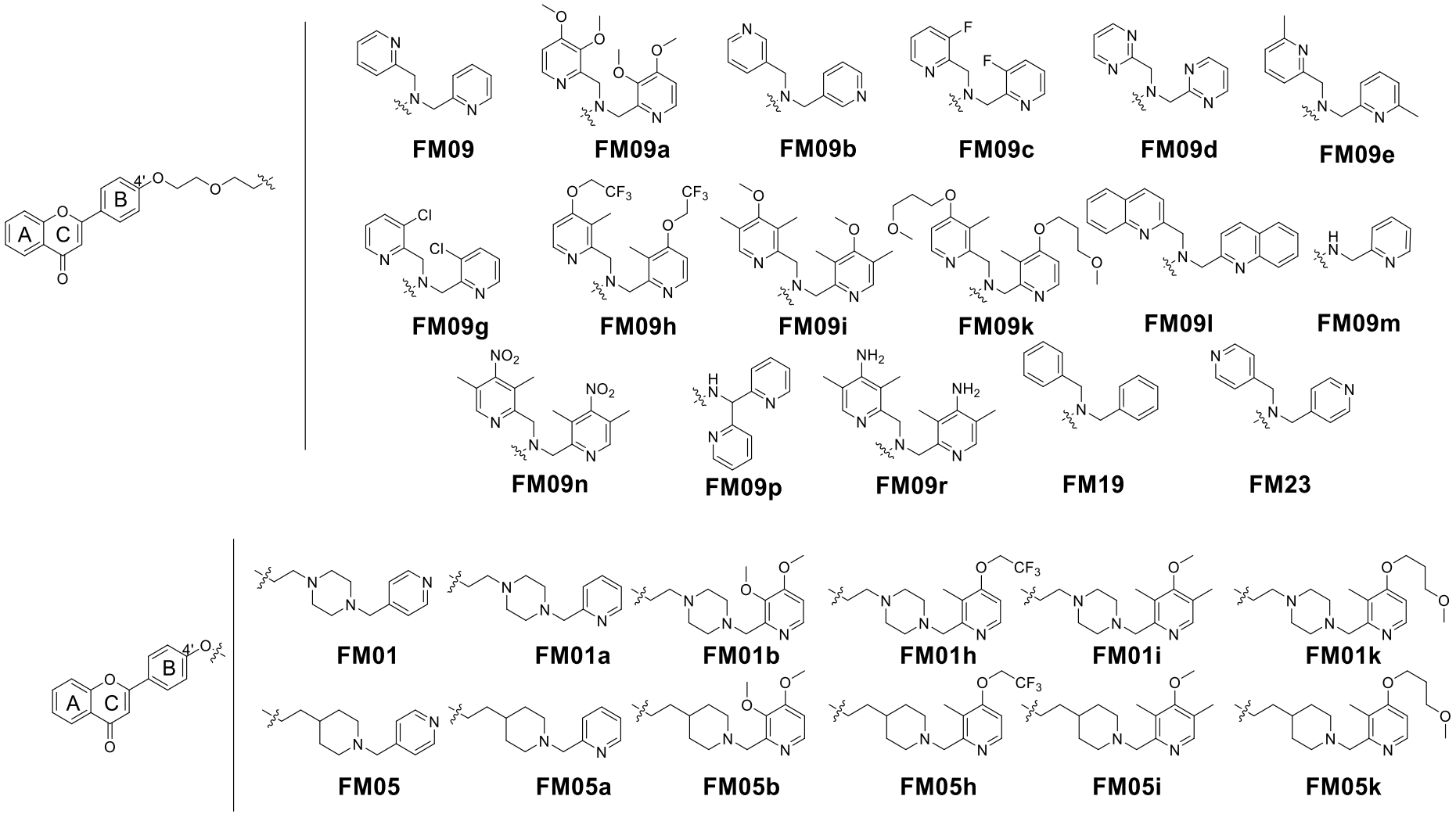


Figure 2-2 Chemical structure of the second generation flavonoid monomers.

2-2 Materials and Methods

2-2-1 Chemicals and Reagents

Schneider Drosophila's medium for culturing promastigotes was purchased from US Biologicals. Glutamine, penicillin, streptomycin and dimethyl sulfoxide (DMSO) were purchased from Sigma. Dulbecco's modified Eagle Medium (DMEM) and gentamicin were purchased from Life Technologies. Fetal Bovine Serum (FBS) was purchased from Hyclone. CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay for determining cell viability of promastigotes and mammalian cells was purchased from Promega.

2-2-2 Cell Lines and Conditions

Promastigotes of two cutaneous *Leishmania*, *L. amazonensis* LV78 and *L. major* Friedlin V1 (FV1), and three visceral *Leishmania*, *L. donovani* HU3, *L. donovani* AG83 and *L. donovani* Ld39, were used to test the potency of flavonoid monomers. LV78 and FV1 was a generous gift from Dr. K. P. Chang from Rosalind Franklin University, AG83 and Ld39 were kindly provided by Dr. Neeleo Singh from the Central Drug Research Institute (CDRI) while HU3 was kindly provided by Dr. Vanessa Yardley from The London School of Hygiene and Tropical Medicine (LSHTM). Ld39 is a clinical isolate of SSG-resistant *L. donovani* and its IC_{50} against SSG was 7090 μM. Another *L. donovani* strain AG83 was drug-selected for 1 month with 0.4 μM amphotericin B, 40 μM miltefosine or 50 μM pentamidine to produce resistant strains, namely AG83-AmBR0.4, AG83-MilR40 and AG83-PentR50.

Promastigotes were cultured in Schneider's Drosophila Medium (US Biologicals) maintained at pH 6.9 and supplemented with 4 mM glutamine (Sigma), 25 μg/mL gentamicin solution (Life Technologies) and 10% (v/v) heat-inactivated fetal bovine serum (Hyclone) at 27°C.

The mouse fibroblast L929, murine macrophages RAW264.7 and primary peritoneal elicited mouse macrophages (PEM) were used to test the cytotoxicity of flavonoid monomers. L929 was purchased from ATCC while RAW264.7 was kindly provided by Dr. Daniel Lee from The Hong Kong Polytechnic University. PEM was elicited in mouse peritoneum by 2% starch suspension and harvested by peritoneal lavage using DMEM. These mammalian cell lines were cultured in DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma) and 10% heat-inactivated fetal bovine serum (Hyclone) at 37°C.

2-2-3 Drug Preparation

Fresh powders of flavonoid monomers were dissolved at 100 mM in 100% DMSO (Sigma) as a stock and stored at -20°C. When testing, flavonoid monomers were further diluted to 10 mM for drug activity assays.

2-2-4 *In Vitro* Promastigote Assay

1.5 x 10^6 cells/mL of *Leishmania* promastigote were seeded into a 96-well flat bottom clear microplate at 100 μL per well. Flavonoid monomers diluted serially starting from

50 μM at 3-fold dilution were added to each well to a final volume of 100 μL in triplicate. The plate was incubated at 27°C for 72 hours. Anti-promastigote activity of flavonoid monomers was determined by the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Fresh solution of 2 mg/mL MTS and 0.92 mg/mL PMS were prepared in a ratio of 20:1 (MTS: PMS). After 72 hour incubation, 10 μL MTS: PMS mixture was added to each well of microplate. The plate was then incubated at 27°C for 4 hours followed by the OD measurement at 490 nm by the automatic microplate reader M680 (Bio-Rad). 0.5% DMSO was used as a negative control.

$$\text{Percentage survival (\%)} = \frac{\text{OD490 of the cell culture with flavonoid monomers}}{\text{OD490 of the cell culture with 0.5\% DMSO control}} \times 100\%$$

The IC_{50} values was determined by the software GraphPad Prism 5.

2-2-5 *In Vitro* Amastigote Assay

The anti-amastigote assay was determined using peritoneal elicited macrophages (PEM) for parasite infection. To prepare PEM cells, 1 mL of 2 % starch suspension (Sigma) was injected into the peritoneum of BALB/c mice (4 to 6 weeks old). Three days later, peritoneal macrophages were harvested by peritoneal lavage using DMEM [92]. Cells were counted and resuspended at a density of 2 x 10^5 cells/mL in supplemented DMEM. 2 x 10^5 cells/mL were seeded into a 24-well culture plate containing a round coverslip (12 mm in diameter) at 500 μL in each well. Macrophages were incubated at 37°C with 5% CO_2 overnight to allow attachment to the coverslip. Then adhered macrophages were washed with warm unsupplemented DMEM to

remove any non-adherent cells. Then stationary phase promastigotes were added to the adherent macrophages at a ratio of 10:1 for *L. amazonensis* LV78 and 40:1 for *L. donovani* HU3 for infection overnight at 37°C. Non-internalized parasites were removed and washed by unsupplemented DMEM twice. Flavonoid monomers diluted serially starting from 10 μM at 3-fold dilution were added to the intracellular amastigote culture to a final volume of 500 μL, then incubated at 37°C with 5% CO_2 for 72 hours. Then the coverslips were picked up from the culture plate, fixed with absolute methanol (ACS), mounted on microscopic slides and stained with 10% Giemsa. The microscopic slides were observed under microscope at 1000X magnification with oil immersion. The number of amastigotes per 100 macrophages was enumerated. At least three areas were selected for cell counting. A 0.1% DMSO was used as a negative control. The percentage survival and IC_{50} were determined according to **Section 2-2-4**.

2-2-6 *In Vitro* Cytotoxicity Assay

1 x 10^5 cells/mL of L929 or RAW264.7, or 2 x 10^5 PEM cells/mL were seeded into a 96-well flat bottom clear microplate at 100 μL per well. Flavonoid monomers serially diluted starting from 100 μM at 3-fold were added to each well to a final volume of 100 μL in triplicate. The plate was incubated at 37°C with 5% CO_2 for 72 hours. The cytotoxicity of flavonoid monomers was determined by CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) mentioned above. The plate was then incubated at 37°C for 1 hour followed by the OD measurement. Cell viability was

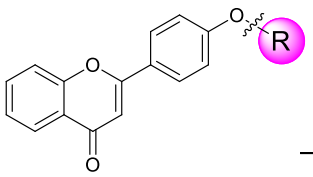
measured in terms of absorbance at 490 nm by automatic microplate reader M680 (Bio-Rad). 1% DMSO was used as a negative control. The percentage survival and IC_{50} were determined according to **Section 2-2-4**.

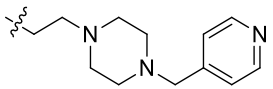
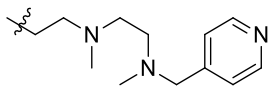
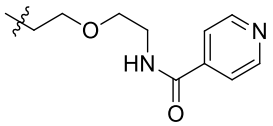
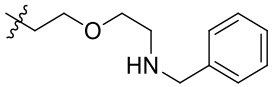
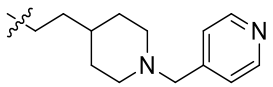
2-3 Results

2-3-1 Activity of the First Generation Flavonoid Monomers FM01 – FM25

The first generation of synthetic flavonoid monomers comprised of a common flavone moiety with different substituents at 4'-position of the B ring via an O-linkage. Among the first generation flavonoid monomers **FM01** to **FM25**, many of them were potent and had an IC_{50} of less than 10 μM against promastigotes and amastigotes (**FM01**, **FM04**, **FM05**, **FM09**, **FM13**, **FM14**, **FM17**, **FM18** and **FM23**) (**Table 2-1**). In general, they were also non-toxic towards L929 fibroblasts (IC_{50} = 13.4 to 95.0 μM) and macrophage cell line RAW264.7 (IC_{50} = 15.1 to > 100 μM) (**Table 2-1**) except for **FM23** (IC_{50} towards L929 = 4.7 μM; RAW264.7 = 12.4 μM). Other flavonoid monomers showed moderate anti-promastigote and anti-amastigote activities (**FM02**, **FM06**, **FM08**, **FM15** and **FM21**) whereas the rest were inactive with IC_{50} greater than 50 μM towards promastigotes and 10 μM towards amastigotes (**FM03**, **FM07**, **FM10**, **FM11**, **FM12**, **FM16**, **FM19**, **FM20**, **FM22**, **FM24** and **FM25**). The most potent one was **FM09,** exhibiting anti-promastigote activity towards cutaneous *Leishmania* (IC_{50} = 1.3 and 0.8 μM for LV78 and FV1 respectively) and visceral *Leishmania* (IC_{50} = 0.9 and 0.5 μM for HU3 and Ld39 respectively). It also displayed the highest anti-amastigote activity towards cutaneous and visceral *Leishmania* (IC_{50} = 0.4 μM for LV78 and HU3) (**Table 2-1**). **FM09** exhibited low toxicity towards L929 mouse fibroblast or RAW264.7 macrophage cell line (IC_{50} = 16.5 – 16.8 μM) (**Table 2-1**).

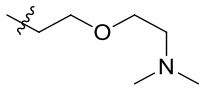
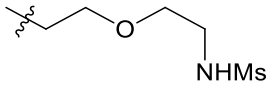
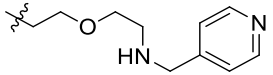
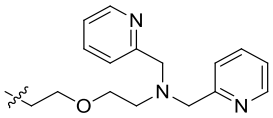
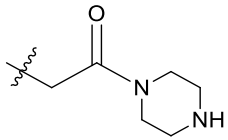
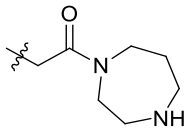
Table 2-1 Anti-promastigote and anti-amastigote activity of synthetic flavonoid monomers



		IC_{50} (μM)							
		Promastigotes				Amastigotes		Mammalian cell lines	
Cpds	R	LV78	FV1	HU3	Ld39	LV78	HU3	L929	RAW264.7
FM01		6.0 ± 4.7	5.6 ± 2.7	1.2 ± 1.0	0.7 ± 0.1	7.4 ± 1.9	8.8 ± 1.9	39.7 ± 23.1	64.4 ± 18.8
FM02		4.6 ± 1.7	5.4 ± 2.5	1.8 ± 0.9	2.1 ± 1.1	4.7 ± 1.7	>10	23.1 ± 9.6	26.1 ± 5.1
FM03		>50	>50	>50	>50	>10	9.7 ± 0.6	85.3 ± 27.2	>100
FM04		2.8 ± 2.5	3.8 ± 1.9	2.4 ± 1.5	2.6 ± 0.8	3.7 ± 2.9	7.4 ± 3.7	14.3 ± 10.3	15.1 ± 9.6
FM05		4.0 ± 2.8	3.9 ± 2.2	0.4 ± 0.3	0.3 ± 0.1	5.5 ± 3.3	7.4 ± 3.3	70.1 ± 30.9	37.3 ± 13.7

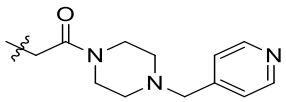
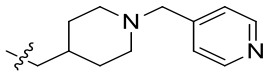
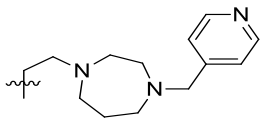
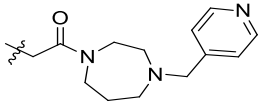
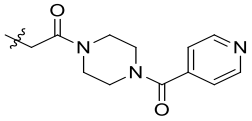
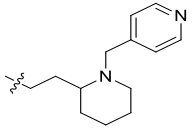
(continued on next page)

Table 2-1 (continued)

		IC_{50} (μM)							
		Promastigotes				Amastigotes		Mammalian cell lines	
Cpds	R	LV78	FV1	HU3	Ld39	LV78	HU3	L929	RAW264.7
FM06		2.0 ± 1.0	3.6 ± 1.9	2.2 ± 1.5	1.1 ± 0.2	6.3 ± 3.7	>10	16.7 ± 7.3	16.8 ± 5.7
FM07		>50	>50	34.7 ± 11.0	21.4 ± 4.4	>10	>10	35.7 ± 28.7	92.2 ± 12.1
FM08		8.3 ± 5.0	9.5 ± 3.1	1.4 ± 0.9	2.7 ± 1.5	6.7 ± 2.5	>10	22.1 ± 9.8	49.5 ± 14.8
FM09		1.3 ± 1.1	0.8 ± 0.4	0.9 ± 1.3	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	16.5 ± 8.9	16.8 ± 5.3
FM10		31.4 ± 7.3	>50	37.6 ± 7.8	49.5 ± 15.9	6.0 ± 0.3	>10	30.0 ± 5.8	34.9 ± 4.9
FM11		>50	>50	>50	>50	7.1 ± 2.2	>10	24.3 ± 5.7	23.5 ± 7.6

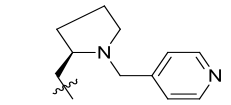
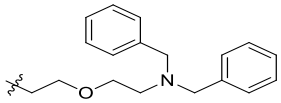
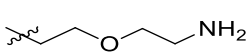
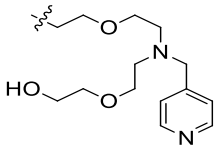
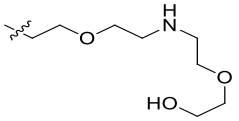
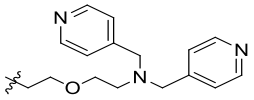
(continued on next page)

Table 2-1 (continued)

		IC_{50} (μM)							
		Promastigotes				Amastigotes		Mammalian cell lines	
Cpds	R	LV78	FV1	HU3	Ld39	LV78	HU3	L929	RAW264.7
FM12		>50	>50	25.4 ± 10.2	15.0 ± 4.5	>10	>10	73.7 ± 25.1	98.4 ± 4.2
FM13		3.5 ± 3.0	2.7 ± 1.8	1.1 ± 1.7	0.7 ± 0.5	5.4 ± 3.9	5.9 ± 4.3	95.0 ± 15.8	>100
FM14		1.4 ± 1.0	1.6 ± 1.6	0.3 ± 0.2	1.4 ± 1.6	4.6 ± 4.3	8.2 ± 2.6	13.9 ± 8.9	21.6 ± 4.9
FM15		4.8 ± 3.9	6.4 ± 4.7	1.5 ± 0.9	1.8 ± 1.5	9.7 ± 0.6	>10	17.2 ± 9.3	47.0 ± 7.3
FM16		>50	>50	35.9 ± 20.2	>50	>10	>10	87.5 ± 23.1	91.7 ± 20.4
FM17		0.9 ± 0.8	2.1 ± 1.2	0.4 ± 0.3	1.5 ± 0.9	4.7 ± 3.4	8.9 ± 2.0	85.9 ± 29.5	85.1 ± 30.4

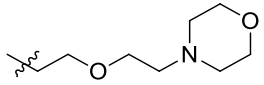
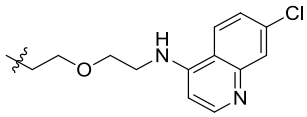
(continued on next page)

Table 2-1 (continued)

		IC_{50} (μM)							
		Promastigotes				Amastigotes		Mammalian cell lines	
Cpds	R	LV78	FV1	HU3	Ld39	LV78	HU3	L929	RAW264.7
FM18		5.5 ± 2.3	4.9 ± 0.9	2 ± 0.9	2.7 ± 1.5	5.4 ± 4.0	9.5 ± 0.7	43.2 ± 13.0	55.0 ± 10.7
FM19		13.2 ± 6.7	21.6 ± 8.5	9.8 ± 5.5	>50	1.3 ± 0.8	>10	82.0 ± 25.9	>100
FM20		14.1 ± 3.7	43.0 ± 16.5	36.4 ± 4.4	26.1 ± 12.6	>10	>10	41.8 ± 11.6	20.8 ± 6.4
FM21		8.7 ± 5.1	14.8 ± 11.5	21.5 ± 5.8	11.5 ± 9.7	4.2 ± 0.4	5.9	13.4 ± 7.0	26.1 ± 3.6
FM22		17.1 ± 3.5	>50	>50	40.2 ± 24.2	>10	>10	18.6 ± 7.9	10.1 ± 2.0
FM23		2.0 ± 1.6	2.7 ± 1.3	7.3 ± 3.1	2.8 ± 2.5	6.5 ± 1.4	7.2 ± 4.0	4.7 ± 1.0	12.4 ± 1.5

(continued on next page)

Table 2-1 (continued)

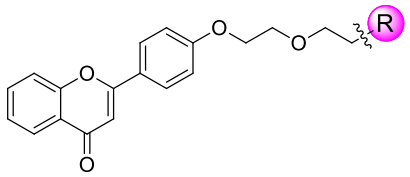
		IC_{50} (μM)							
		Promastigotes				Amastigotes		Mammalian cell lines	
Cpds	R	LV78	FV1	HU3	Ld39	LV78	HU3	L929	RAW264.7
FM24		>50	>50	>50	25.8	>10	>10	40.6 ± 30.3	61.7 ± 37.2
FM25		19.4 ± 16.5	34.4 ± 15.9	39.2 ± 18.7	21.1 ± 20.4	>10	>10	27.6 ± 35.1	28.7 ± 9.8

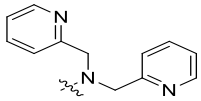
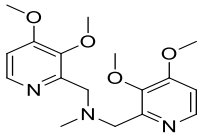
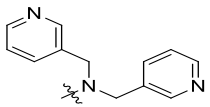
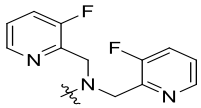
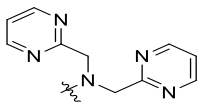
Twenty-five novel synthetic flavonoid monomers (**FM01** to **FM25**) were tested for their *in vitro* anti-promastigote and anti-amastigote activities against *L. amazonensis* (LV78), *L. major* (FV1) and *L. donovani* (HU3 and Ld39). Ld39 is a SSG-resistant cell line. Their cytotoxicity towards mouse fibroblast L929 and mouse macrophage cell line RAW264.7 was also determined as an indicator of general toxicity. All compounds were dissolved in DMSO and the highest amount of DMSO used was 1 % at which no toxicity towards promastigotes, infected macrophages or mammalian cells was observed. IC_{50} values were presented as mean ± SD. N = 1 – 8 independent experiments.

2-3-2 Activity of the Second Generation Flavonoid Monomers Derived from FM01, FM05 and FM09

FM09 was selected to be further modified to seek higher potency and metabolic stability (described in **Chapter 3**) by changing and adding the functional groups on the pyridine rings (**Figure 2-2**). **Table 2-2** summarized the lead optimization of **FM09**. Among the 14 **FM09** derivatives (**FM09a** to **FM09r**), some compounds e.g. **FM09a**, **FM09h**, **FM09i** and **FM09k** could maintain or even slightly improve their potency (IC_{50} = 0.1 – 1.1 μM against promastigotes and IC_{50} = 0.2 – 0.3 μM against amastigotes) but with slight increase in *in vitro* cytotoxicity towards mammalian cell lines (IC_{50} = 2.3 – 12.9 μM). Some **FM09** derivatives had reduced potency against promastigotes and amastigotes, including **FM09b**, **FM09c**, **FM09e**, **FM09g**, **FM09l, FM09m, FM09n, FM09p and FM09r** (IC_{50} = 2.8 to > 50 μM against promastigotes and IC_{50} = 0.8 to > 10 μM against amastigotes). One peculiar observation was that **FM09r** was inactive against promastigotes but active in amastigotes with less cytotoxicity towards mammalian cells (IC_{50} > 36.7 μM against promastigotes and IC_{50} = 4.2 μM against LV78 amastigotes and = 1.2 μM against HU3 amastigotes).

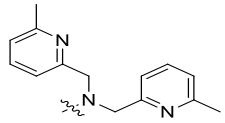
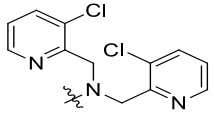
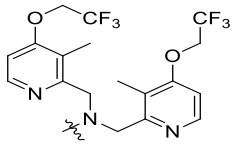
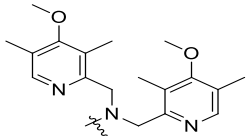
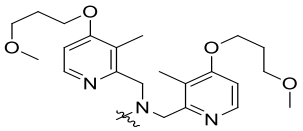
Table 2-2 Anti-promastigote and anti-amastigote activity of **FM09** derivatives



		IC_{50} (µM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM09		1.3 ± 1.1	0.9 ± 1.3	0.4 ± 0.2	0.4 ± 0.2	16.5 ± 8.9	16.8 ± 5.3	26.9
FM09a		0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	12.9 ± 3.4	8.5 ± 2.8	10.4 ± 0.6
FM09b		8.1 ± 4.9	5.7 ± 3.8	>10	>10	75.1 ± 19	90.5 ± 0.5	ND
FM09c		3.2 ± 1.3	5.1 ± 2.5	1.7 ± 0.7	3.0 ± 1.6	39.0 ± 8.6	39.2 ± 11.9	35.4 ± 1.1
FM09d		35.3 ± 5.9	42.4 ± 10.8	>10	>10	>100	>100	>100

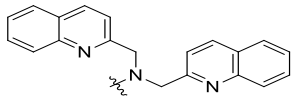
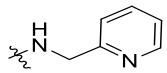
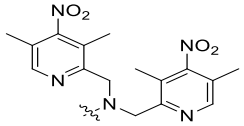
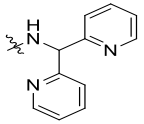
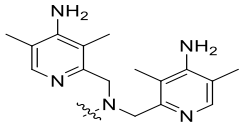
(continued on next page)

Table 2-2 (continued)

		IC_{50} (μM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM09e		7.4 ± 4.3	4.9 ± 1.9	2.4 ± 0.6	3.8 ± 1.2	18.8 ± 1.3	22 ± 5.9	ND
FM09g		28.1 ± 12.8	18.1 ± 11.9	>10	>10	33.7 ± 15.0	34.3 ± 18.9	ND
FM09h		0.5 ± 0.4	1.1 ± 0.5	0.3 ± 0.1	0.3 ± 0.1	3.6 ± 0.9	3.8 ± 1.7	6.4
FM09i		0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	2.3 ± 1.4	3.9 ± 1.1	4.5
FM09k		0.2 ± 0.1	1.1 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	4.7 ± 1.2	4.5 ± 2.8	5.3

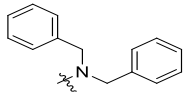
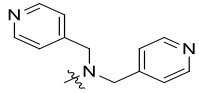
(continued on next page)

Table 2-2 (continued)

		IC_{50} (μM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM09l		4.4 ± 2.8	4.7 ± 1.1	3.0 ± 0.4	4.2 ± 3.4	>100	76.4 ± 20.5	67.1
FM09m		17.6 ± 9.1	25.3 ± 8.8	4.5 ± 1.4	9.4 ± 1.1	23.9 ± 1.6	24.7 ± 3.6	28.4
FM09n		4.1 ± 1.9	8.2 ± 1.3	ND	ND	38.5 ± 7.8	51.9 ± 42.4	ND
FM09p		3.7 ± 1.3	2.8 ± 0.5	0.8 ± 0.5	6.0 ± 1.2	2.1 ± 0.0	6.5	12.5
FM09r		38.3	>50	4.2	1.2	73.5 ± 21.7	36.7 ± 8.9	ND

(continued on next page)

Table 2-2 (continued)

		IC_{50} (μM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM19		13.2 ± 6.7	9.8 ± 5.5	1.3 ± 0.8	>10	82.0 ± 25.9	>100	ND
FM23		2.0 ± 1.6	7.3 ± 3.1	6.5 ± 2.4	7.2 ± 4.0	4.7 ± 1.0	12.4 ± 1.5	29.4 ± 2.5

Derivatives of **FM09** were tested for their anti-promastigote and anti-amastigote activities against LV78 and HU3. Their cytotoxicity towards mouse fibroblast L929, macrophage cell line RAW264.7 and mouse PEM was also measured. All compounds were dissolved in DMSO and the highest amount of DMSO used was 1 % at which no toxicity towards promastigotes, infected macrophages or mammalian cells was observed. The IC_{50} values were presented as mean ± SD. N = 1 – 4 independent experiments. ND = not determined.

Because of the improvement in potency in **FM09a**, **FM09h**, **FM09i** and **FM09k**, similar modifications were applied to **FM01**, producing **FM01a**, **FM01b**, **FM01h**, **FM01i** and **FM01k**, and to **FM05**, producing **FM05a**, **FM05b**, **FM05h**, **FM05i** and **FM05k (Figure 2-2**). Among them, all derivatives except **FM01a**, **FM01h, FM01k** and **FM05a**, showed significant improvement in potency (IC_{50} against promastigotes = < 0.2 – 6.2 µM; IC_{50} against amastigotes = 0.1 – 4.2 µM). Among them, the most potent one was **FM05b** with an IC_{50} of less than 1 µM (IC_{50} against promastigotes = 0.6 – 0.9 µM; IC_{50} against amastigotes = 0.3 µM for LV78 and 0.7 µM for HU3). Cytotoxicity towards mammalian cell lines ranges from 5.1 to 10.6 µM (**Table 2-3**). **Figure 2-3** showed that **FM05b** and **FM09h** at 1.1 µM could kill 90% *L. donovani* HU3 amastigotes inside the peritoneal-elicited macrophages.

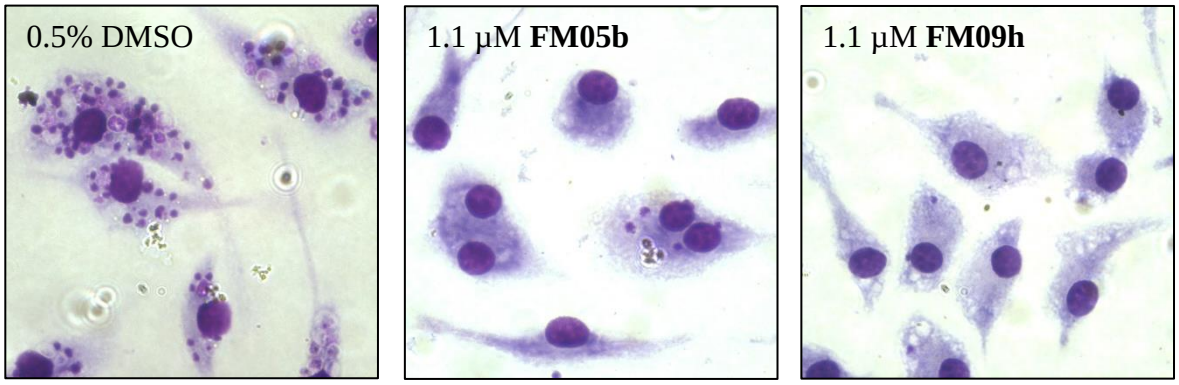
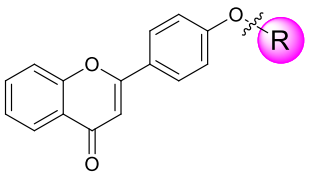
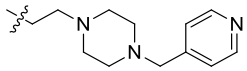
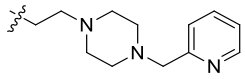
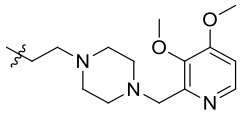
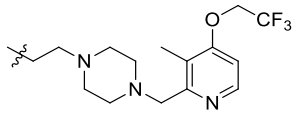


Figure 2-3 FM05b and **FM09h** at 1.1 µM could kill *L. donovani* HU3 amastigotes inside the peritoneal-elicited macrophages

Peritoneal-elicited macrophages infected with *L. donovani* HU3 were treated with 0.1% DMSO (left), 1.1 µM **FM05b** (centre) and 1.1 µM **FM09h** (right). **FM05b** and **FM09h** at such concentration could eliminate 90% amastigotes inside the macrophages. Magnification at 1,000X.

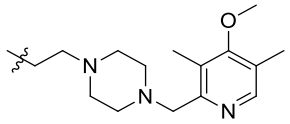
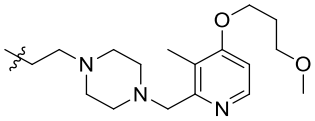
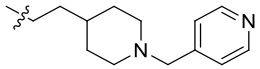
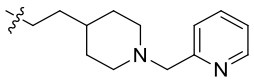
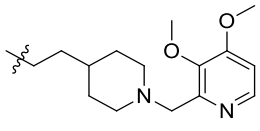
Table 2-3 Anti-promastigote and anti-amastigote activity of **FM01**and **FM05** derivatives



		IC_{50} (μM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM01		6.0 ± 4.7	1.2 ± 1.0	7.4 ± 1.9	8.8 ± 1.9	39.7 ± 23	64.4 ± 18.8	ND
FM01a		11.7 ± 3.3	9.9 ± 6.3	7.5 ± 1.2	>10	60.3 ± 3.3	82.0 ± 3.3	ND
FM01b		4.0 ± 2.4	6.2 ± 3.6	2.4 ± 1.2	4.2 ± 2.3	95.8 ± 6.0	53.6 ± 9.2	ND
FM01h		5.1 ± 2.5	3.4 ± 5.5	2.0	9.8 ± 0.4	32.3 ± 18.0	20.6 ± 8.7	ND

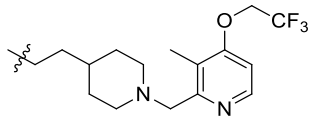
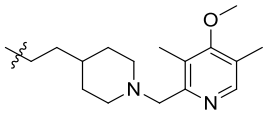
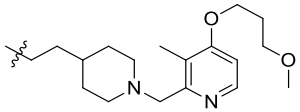
(continued on next page)

Table 2-3 (continued)

		IC_{50} (μM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM01i		0.4 ± 0.1	<0.2	2.4	2.3 ± 0.6	27.5 ± 12.2	18.2 ± 6.2	ND
FM01k		5.8 ± 4.4	<0.2	ND	>10	35.2 ± 11.5	21.6 ± 12.0	ND
FM05		4.0 ± 2.8	0.4 ± 0.3	5.5 ± 3.3	7.4 ± 3.3	70.1 ± 30.9	37.3 ± 13.7	36.5
FM05a		3.0 ± 2.6	7.4 ± 5.7	3.6	6.2 ± 3.4	58.9 ± 40.4	>50	17.6
FM05b		0.6 ± 0.2	0.9 ± 0.3	0.3 ± 0.2	0.7 ± 0.7	10.6 ± 2.0	7.7 ± 2.1	5.1

(continued on next page)

Table 2-3 (continued)

		IC_{50} (µM)						
		Promastigotes		Amastigotes		Mammalian cells		
Cpds	R	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM
FM05h		3.6 ± 2.0	<0.2	0.5	2.0 ± 1.4	24.8 ± 21.9	21.8 ± 21.1	ND
FM05i		2.6 ± 3.3	<0.2	0.1	2.5 ± 1.2	15.2 ± 2.3	12.3 ± 6.2	ND
FM05k		3.1 ± 1.7	<0.2	ND	2.5 ± 1.6	50.6 ± 37.6	15.4 ± 9.6	ND

Derivatives of **FM01** and **FM05** were tested for their anti-promastigote and anti-amastigote activities against LV78 and HU3. Their cytotoxicity towards mouse fibroblast L929, macrophage cell line RAW264.7 and mouse PEM was also measured. All compounds were dissolved in DMSO and the highest amount DMSO used was 1 % at which no toxicity towards promastigotes, infected macrophages or mammalian cells was observed. The IC_{50} values were presented as mean ± SD. N = 1 – 4 independent experiments. ND = not determined.

2-3-3 Comparison Between Potent Flavonoid Monomers, Natural Flavonoids and Antileishmanial Drugs Used Clinically

The synthetic flavonoid monomers (**FM05b**, **FM09**, **FM09a**, **FM09h**, **FM09i** and **FM09k**) selected from the library were then compared with natural flavonoids including luteolin and quercetin. It was found that their anti-promastigote activities were more potent than luteolin by 28 – 475 fold (IC_{50} = 30.8 – 47.5 µM) and quercetin by 32.8 – 427 fold (IC_{50} = 42.7 to > 50 µM) while their anti-amastigote activities were more potent than luteolin and quercetin by more than 43 fold (**Table 2-4**). These potent flavonoid monomers were also compared with the current antileishmanial drugs, amphotericin B, miltefosine, pentamidine and sodium stibogluconate. It was found that their potency was comparable to that of amphotericin B (IC_{50} towards promastigote and amastigotes were 0.4 µM and 0.1 – 0.6 µM respectively) and was higher than that of miltefosine by 10 – 240 fold against promastigotes (miltefosine IC_{50} = 10.7 – 24.0 µM) and 4 – 29 fold against amastigotes (miltefosine IC_{50} = 2.7 – 8.7 µM), pentamidine by 1.2 – 31 fold against promastigotes (pentamidine IC_{50} = 0.6 – 6.2 µM) and 13 – 47 fold against amastigotes (pentamidine IC_{50} = 9.3 µM) and at least 150 fold higher than sodium stibogluconate (IC_{50} towards promastigote and amastigotes > 2000 µM and 61.4 µM respectively) (**Table 2-4**). It should be noted that sodium stibogluconate is an pro-drug that requires reduction from Sb(V) to Sb(III) to become active. Therefore, SSG is only active against amastigotes.

To compare their therapeutic effects, selective index that is determined by the ratio of IC_{50} against amastigotes to IC_{50} against mammalian cells was employed. The selective

index of flavonoid monomers was higher than that of luteolin by 10 – 36 fold (selective index = 1.4), quercetin by 4 – 15 fold (selective index = 3.3), miltefosine by 2 – 6 fold (selective index = 8.3) and pentamidine by 3 – 11 fold (selective index = 4.7) but lower than amphotericin B (selective index = 128.9) and sodium stibogluconate (selective index > 179).

Table 2-4 Comparison between potent flavonoid monomers, natural flavonoids and antileishmanial drugs used clinically

	IC_{50} (µM)							Selective Index
	Promastigotes		Amastigotes		Mammalian cells			
Cpds	LV78	HU3	LV78	HU3	L929	RAW264.7	PEM	
FM09	1.3 ± 1.1	0.9 ± 1.3	0.4 ± 0.1	0.4 ± 0.2	16.5 ± 8.9	16.8 ± 5.3	26.9	50.2
FM09a	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	12.9 ± 3.4	8.5 ± 2.8	10.4 ± 0.6	35.3
FM09h	0.5 ± 0.4	1.1 ± 0.5	0.3 ± 0.1	0.3 ± 0.1	3.6 ± 0.9	3.8 ± 1.7	6.4	15.3
FM09i	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	2.3 ± 1.4	3.9 ± 1.1	4.5	14.3
FM09k	0.2 ± 0.1	1.1 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	4.7 ± 1.2	4.5 ± 2.8	5.3	16.1
FM05b	0.6 ± 0.2	0.9 ± 0.3	0.3 ± 0.2	0.7 ± 0.7	10.6 ± 2.0	7.7 ± 2.1	5.1	15.6
Luteolin	47.5 ± 2.3	30.8 ± 8.1	ND	>30	51.3 ± 22.1	30.2 ± 15.9	ND	1.4
Quercetin	42.7 ± 5.0	>50	ND	>30	>100	>100	ND	3.3
Amphotericin B	0.4 ± 0.5	0.4 ± 0.5	0.6 ± 0.7	0.1	66.4 ± 37.5	42 ± 39.4	27.4 ± 18.2	128.9
Miltefosine	24.0 ± 13	10.7 ± 11	8.7 ± 2.9	2.7 ± 0.8	81.5 ± 20.0	9.9 ± 2.9	50.2 ± 7.3	8.3
Pentamidine	0.6 ± 0.1	6.2 ± 1.8	ND	9.3 ± 0.7	82.9 ± 12	5.0 ± 1.8	ND	4.7
SSG	>2000	>2000	61.4	ND	ND	>11000	ND	>179

Potent flavonoid monomers were compared with natural flavonoids and clinically used antileishmanial drugs, in terms of their activities against promastigotes, amastigotes and cytotoxicity. All compounds were dissolved in DMSO and the highest amount of DMSO used was 1 % at which no toxicity towards promastigotes, infected macrophages or mammalian cells was observed. Selective index was calculated as IC_{50} against mammalian cell lines L929, RAW264.7 and PEM/ IC_{50} against LV78 and HU3 amastigotes. The IC_{50} values were presented as mean ± SD. N = 1 – 4 independent experiments. ND = not determined.

2-3-4 Anti-Promastigote Activity of Flavonoid Monomers Against Drug-Resistant Parasites

Two of the modified and potent flavonoid monomers, **FM05b** and **FM09h** were tested for anti-promastigote activity against parasites resistant to antileishmanial drugs, e.g. amphotericin B, miltefosine, pentamidine and SSG. The four resistant strains were resistant to the respective drug by around 3-fold. It was found that both **FM05b** and **FM09h** was active against all resistant strains that the IC_{50} remained at 1μM. (**Table 2-5**).

Table 2-5 Anti-promastigote activity of flavonoid monomers against drug-resistant parasites

	IC_{50} (μM)				
	Promastigotes				
Cpds	AG83	AG83-AmBR0.4	AG83-MilR40	AG83-PentR50	Ld39
FM09h	0.8 ± 0.5	0.9 ± 0.5	0.7 ± 0.6	1.0 ± 0.6	1.4
FM05b	0.5 ± 0.4	0.6 ± 0.0	1.1 ± 0.7	1.1 ± 0.6	1.2
Amphotericin B	0.1 ± 0.0	0.4 ± 0.1	/	/	/
Miltefosine	11.8 ± 8.8	/	65.4 ± 36.5	/	/
Pentamidine	8.8 ± 4.2	/	/	24.9 ± 0.8	/
SSG	2216 ± 166	/	/	/	7090 ± 747

Potent flavonoid monomers, **FM05b** and **FM09h** were tested for their anti-promastigote activity against drug-resistant parasites. Wild-type *L. donovani* strain, AG83 was selected in Schneider Drosophila's Medium containing 0.4 μM amphotericin B, 40 μM miltefosine and 50 μM pentamidine to produce AG83-AmBR0.4, AG83-MilR40 and AG83-PentR50, respectively. Ld39 was a clinical isolate of *L. donovani* resistant to SSG. All compounds were dissolved in DMSO and the highest amount of DMSO used was 1 % at which no toxicity towards promastigotes was observed. The IC_{50} values were presented as mean ± SD. N = 1 – 4 independent experiments.

2-4 Discussion

Significant improvement in potency found in synthetic flavonoid monomers

In this study, a group of flavonoid monomers were designed and synthesized for characterization. It was found that they have promising anti-promastigote and anti-amastigote activities. The synthetic flavonoid monomers (**FM05b**, **FM09**, **FM09a**, **FM09h**, **FM09i** and **FM09k**) were more active in killing promastigotes and amastigotes, compared with the natural flavonoids, luteolin and quercetin (**Table 2-4**). Besides that, the potency of the compounds could be improved by structural modification. Especially in the **FM01** and **FM05** series, the activity was increased significantly in the second generation compound library. **FM05b** (IC_{50} against promastigotes = 0.6 – 0.9 µM; IC_{50} against amastigotes = 0.3 – 0.7 µM) exhibited the greatest improvements with a 10-fold increase in potency, resulting in an IC_{50} similar to that of **FM09** (IC_{50} = 0.4 µM against amastigote of LV78 and HU3) (**Table 2-4**). Compared with current antileishmanial drugs, the selective indices of **FM05b**, **FM09**, **FM09a**, **FM09h**, **FM09i** and **FM09k** were higher than that of miltefosine by 2 – 6 fold (selective index = 8.3) and pentamidine by 3 – 11 fold (selective index = 4.7) but lower than amphotericin B (selective index = 128.9) and sodium stibogluconate (selective index > 179). These flavonoid monomers were more potent than most of the current antileishmanial drugs such as miltefosine, pentamidine and sodium stibogluconate and comparable with amphotericin B (**Table 2-4**). Although cytotoxicity towards mammalian cell lines, L929, RAW264.7 and PEM were observed *in vitro*, but their IC_{50} was greater than their corresponding IC_{50}s against promastigotes and amastigotes

by 10 fold. The *in vivo* toxicity of flavonoid monomers in animals will be confirmed in subsequent chapter.

Structure-activity relationship of flavonoid monomers

Based on the two generations of synthesized flavonoid monomer libraries, a structure-activity relationship could be drawn as follows:

(1) Pyridine ring improved the antileishmanial activity of flavonoid monomers. In general, flavonoid monomers containing pyridine rings were more active in killing promastigotes and amastigotes. For the 25 flavonoid monomers of first generation library, 9 of them exhibited active and promising activity with IC_{50} less than 10 μM against promastigotes and amastigotes (**Table 2-1**) and 8 of them were found to contain pyridine ring(s) including **FM01**, **FM05**, **FM09**, **FM13**, **FM14 FM17**, **FM18** and **FM23**. Flavonoid monomers linked with morpholino ring which is investigated in antiviral drug development (**FM24**), or heme binding motif of chloroquine (a well-known antimalarial drug) (**FM25**), were not potent against *Leishmania* parasites (IC_{50} > 10 μM in promastigotes and amastigotes) (**Table 2-1**). In the second generation library, **FM09l** that was derived by the replacement of pyridine rings in **FM09** with quinoline, a natural product has been studied in antileishmanial drug development, had decreased activity (IC_{50} = 4.4 – 4.7 μM against promastigotes and IC_{50} = 3.0 – 4.2 μM against amastigotes) (**Table 2-2**). Besides, in comparison of **FM11** with **FM15**, the presence of pyridine ring contributed to the drug activity although such improvement was not seen in **FM10** and **FM12** (**Table 2-1**). For the non-pyridine flavonoid monomers, only **FM04** (IC_{50} = 2.4 – 3.8 μM against promastigotes and IC_{50} = 3.7 –

7.4 μM against amastigotes) that the flavone was linked with a phenyl ring was active in both promastigotes and amastigotes. **FM06**, a tertiary amine was only active against promastigotes (IC_{50} = 1.1 – 3.6 μM against promastigotes and IC_{50} > 6.3 μM against amastigotes) (**Table 2-1**). **FM19** that the flavonoid monomer was linked by two phenyl rings only showed activities against LV78 amastigotes (IC_{50} = 1.3 μM against LV78 amastigotes) but was inactive against promastigotes (IC_{50} = 9.8 to > 50 μM against promastigotes) (**Table 2-2**).

(2) Two pyridine rings contributed to higher activity. Comparing **FM20**, **FM21**, **FM22** and **FM23**, **FM23** that had two pyridine rings was the most potent flavonoid monomer against promastigotes and amastigotes (IC_{50} = 2.0 – 7.3 μM against promastigotes and IC_{50} = 6.5 – 7.2 μM against amastigotes). In contrast, **FM20** and **FM22**, with no pyridine rings, decreased their potency against promastigotes and amastigotes (IC_{50} = 14.1 to > 50 μM against promastigotes and IC_{50} > 10 μM against amastigotes). **FM21** with one less pyridine ring diminished the potency only in cutaneous *Leishmania* promastigotes (IC_{50} = 8.7 – 21.5 μM against promastigotes and IC_{50} = 4.2 – 5.9 μM against amastigotes) (**Table 2-1**). **FM09m**, (IC_{50} = 17.6 – 25.3 μM against promastigotes and IC_{50} = 4.5 – 9.4 μM against amastigotes) which had only one pyridine ring showed poorer activity as compared with the **FM09** that contained two pyridine rings (**Table 2-2**). These suggested that two instead of one pyridine rings was crucial for antileishmanial activity.

(3) Ortho-pyridine flavonoid monomers showed higher antileishmanial activities. The position of nitrogen atom in pyridine ring also affects the antileishmanial potency.

In the **FM09** series, **FM09** (ortho-, IC_{50} = 0.9 – 1.3 μM against promastigotes and IC_{50} = 0.4 μM against amastigotes) had a better potency than **FM23** (para-, IC_{50} = 2.0 – 7.3 μM against promastigotes and IC_{50} = 6.5 – 7.3 μM against amastigotes) and **FM09b** (meta-, IC_{50} = 5.7 – 8.1 μM against promastigotes and IC_{50} > 10 μM against amastigotes) (**Table 2-2**). Therefore, the relative importance of the nitrogen in the pyridine rings in antileishmanial activity can be ranked as ortho- > para- > meta-. This order of activity was also reported in aminothiazoles in antileishmanial drug development [93]. Intriguingly, **FM09d** with pyrimidine rings that two nitrogen atoms are on both ortho positions on the aromatic rings totally lost the activity (IC_{50} = 35.3 – 42.4 μM against promastigotes and IC_{50} > 10 μM against amastigotes) (**Table 2-2**). In contrast to dual pyridine ring flavonoid monomer **FM09**, such variations were not significant in single pyridine ring flavonoid monomers such as **FM09m** (ortho-) and **FM08** (para-), **FM01** (para-) and **FM01a** (ortho-), and **FM05** (para-) and **FM05a** (ortho-) (**Table 2-1**, **Table 2-2** and **Table 2-3**). The structure activity relationship in term of nitrogen position in pyridine rings may only be applied and optimized in certain structures.

(4) Amide groups in the linker of flavonoid monomers abolished the activity while amine groups did not. Three pairs of compounds, **FM01** and **FM12**, **FM08** and **FM03**, and finally **FM14** and **FM15**. The amine-linking **FM01** (IC_{50} = 0.7 – 6.0 μM against promastigotes and IC_{50} = 7.4 – 8.8 μM against amastigotes), **FM08** (IC_{50} = 1.4 – 9.5 μM against promastigotes and IC_{50} = 6.7 to > 10 μM against amastigotes) and **FM14** (IC_{50} = 0.3 – 1.6 μM against promastigotes and IC_{50} = 4.6 – 8.2 μM against amastigotes) contributed to a higher antileishmanial activity than their counterparts

FM12 (IC_{50} = 15.0 to > 50 μM against promastigotes and IC_{50} > 10 μM against amastigotes), **FM03** (IC_{50} > 50 μM against promastigotes and IC_{50} > 10 μM against amastigotes) and **FM15** (IC_{50} = 1.5 – 6.4 μM against promastigotes and IC_{50} = 9.7 to > 10 μM against amastigotes) respectively, linking with amide groups instead (**Table 2-1**).

(5) Modification of functional groups on pyridine rings affected the activity. Substitution in pyridine rings with electron donating groups rather than electron withdrawing groups can slightly improve the potency. Electron donating groups such as methoxy (**FM09a** and **FM09i**), trifluoroethoxy (**FM09h**) and methoxypropoxy (**FM09k**) groups could maintain or slightly enhance the potency against amastigotes (IC_{50} = 0.2 – 0.3 μM against amastigotes versus 0.4 μM in **FM09**). On the other hand, electron withdrawing groups such as fluoro- (**FM09c**), chloro- (**FM09g**) and nitro- (**FM09n**) abolished the killing activity in both promastigotes and amastigotes (IC_{50} = 3.2 – 28.1 μM against promastigotes and IC_{50} = 1.7 to > 10 μM against amastigotes) (**Table 2-2**). Improvements in potency after insertion of electron donating groups were also observed in some of the derivatives in **FM01** and **FM05** series. For **FM01** series, improvements were also found in **FM01b** (methoxy-substituted, IC_{50} = 2.4 – 4.2 μM against amastigotes) and **FM01i** (methoxy and methyl-substituted, IC_{50} = 2.3 – 2.4 μM against amastigotes). Their potency was 2 – 4 fold higher than **FM01a** (IC_{50} > 7.5 μM against amastigotes) For **FM05** series, improvements in potency was found in **FM05b** (methoxy-substituted, IC_{50} = 0.3 – 0.7 μM against amastigotes), **FM05h** (trifluoroethoxy-substituted, IC_{50} = 0.5 – 2.0 μM against amastigotes), **FM05i**

(methoxy and methyl-substituted, IC_{50} = 0.1 – 2.5 μM against amastigotes) and **FM05k** (methoxypropoxy-substituted, IC_{50} = 2.5 μM against amastigotes) that their potency was 3 to 36 fold higher than that of **FM05a** (IC_{50} = 3.6 – 6.2 μM against amastigotes) (**Table 2-3**).

Advantages of flavonoid monomers as an antileishmanial candidate

The flavonoid monomers were very active in killing both promastigotes and amastigotes with an IC_{50} of around 1 μM, and second only to amphotericin B. In addition, different species of *Leishmania* might have different similarity towards antileishmanial drugs like miltefosine and SSG [26]. In contrast, **FM05b** and **FM09h** showed a consistently high potency towards different species of *Leishmania*. Also, **FM05b** and **FM09h** were potent against drug-resistant *Leishmania*, implying that flavonoid monomers could be used in treating drug-resistant leishmaniasis.

2-5 Conclusion

Synthetic flavonoid monomer library was generated for *in vitro* screening of antileishmanial activity. **FM05b**, **FM09**, **FM09h**, **FM09i** and **FM09k** had potent antileishmanial activities towards both promastigotes and amastigotes at the sub-micromolar range. Structure-activity relationship of pyridine rings in flavonoid monomers was summarized although it did not apply to all flavonoid compounds.

1. Pyridine ring was active against both *Leishmania* promastigotes and amastigotes.
2. Dual pyridine rings contributed to higher activity against promastigotes and amastigotes, better than single ring, shown in **FM09**.
3. The position of nitrogen in pyridine was crucial in activity of **FM09** in an order of ortho- > para- > meta-.
4. Presence of amide groups abolished the antileishmanial activity.
5. Substitution of electron withdrawing groups to pyridine rings abolished the activity while electron donating groups slightly increase the activity.

3 Metabolic Stability and Pharmacokinetic Study of Flavonoid Monomers

3-1 Introduction

Metabolism and pharmacokinetic study play an important role in drug discovery and development, allowing optimization of bioavailability and duration of action of new drugs. Whether a drug will be metabolized and how long a drug is remained in the body is crucial in drug discovery and development [94].

Many *in vitro* models may be used to assess the metabolic stability of drug candidates, such as recombinant enzymes, liver microsomes, fresh and cryopreserved hepatocytes and tissue slices. Because of the ease in preparation and long shelf life of liver microsomes obtained by ultracentrifugation, it has recently become a popular model for metabolism study [95]. Besides, the use of primates that are physiologically closer to humans can generate useful information about metabolic stability of drug candidates too [96, 97]. Liquid chromatography and mass spectrometry can be used for quantification with high accuracy and sensitivity [98, 99]. In industrial scale, 96-well- or 384-well plate based screening and rational AVID-based HTS (automation, validation, integration and database management) have also been developed to support drug discovery [96, 100]. After metabolic stability was determined, characterization of drug candidates in terms of pharmacokinetics will be conducted. To reduce the number

of mice and rats required in pharmacokinetic study, a rapid pharmacokinetic screening may be used, by using pooled plasma and reducing the collection period [101].

To improve metabolic stability to decrease intrinsic clearance and increase bioavailability, one of the most commonly-used strategies is structural modification by adjusting lipophilicity, functional group substitution, changing conformation and blockage or modification of metabolically-labile groups. Literature reviews have summarized many successful examples of how structural modification could lead to improvements in metabolic stability, pharmacokinetic profile and oral bioavailability [94, 102, 103].

In this chapter, the pharmacokinetic profile of the lead compound, **FM09** selected from the first generation library, was determined. However, rapid clearance of **FM09** from plasma was found. Metabolic instability of **FM09** was demonstrated in *in vitro* liver microsome assay and could lead to rapid clearance. To improve the pharmacokinetic profile, a metabolic stability screening was conducted to identify stable candidates. Metabolic stability of flavonoid monomers was first studied using a single-time point pharmacokinetic study. Finally, two flavonoid monomers, **FM05b** and **FM09h** were selected for further characterization due to their improved pharmacokinetic properties.

3-1 Materials and Methods

3-1-1 Chemicals and Reagents

Human 150-Donor Pooled Liver Microsomes, Male Pooled SD Rat Liver Microsome and nicotinamide adenine dinucleotide phosphate (NADPH) used in *in vitro* metabolic stability experiment were purchased from Research Institute for Liver Diseases, RILD (Shanghai, China). Organic solvents such as diethyl ether, acetonitrile, methanol and ethanol were at high-performance liquid chromatography (HPLC) grade and purchased from Anaqua. Cremophor EL and heparin, used in pharmacokinetic study were purchased from Sigma. The Waters Acquity UPLC BEH C8 (1.7 μm, 2.1 x 50 mm) column, sample insert, vials and caps used in running UPLC-MS were purchased from Tegent Technology (Hong Kong).

3-1-2 Pharmacokinetic Study of FM09 in Plasma

FM09 was administered intravenously (10 mg/kg) or orally (50 mg/kg) to overnight-starved BALB/c mice (N = 3). At different time points (5, 15, 30, 60, 120, 240 and 420 min), blood was collected via cardiac puncture and placed in a heparinized Eppendorf tube. Blood was centrifuged at 14,000 rpm for 5 minutes to obtain the plasma. 40 μL plasma samples were added with 160 μL acetonitrile for protein precipitation followed by centrifugation at 14,000 rpm for 10 minutes to pellet precipitated proteins. Supernatant was collected and filtered through a 0.22 μm nylon filter for LC-MS/MS analysis.

3-1-3 *In Vitro* Metabolic Stability Experiment

Flavonoid monomers (10 μM) dissolved in methanol (less than 0.2% in the reaction) [104] were incubated in the 0.1 mg human or rat liver microsomes in a final volume of 200 μL in a microcentrifuge tube. The reaction mixture was incubated with or without CYP450 cofactor, NADPH (final concentration 2 mM) at 37°C for 30 minutes. After incubation, 200 μL acetonitrile was added to stop the reaction. 1.2 mL diethyl ether was added to extract the flavonoid monomers and any formed metabolites. Then the tube was heated to evaporate diethyl ether. The flavonoid monomers were reconstituted in 100 μL acetonitrile and filtered through a 0.22 μm nylon filter for liquid chromatography-mass spectrometry. The equation of how the metabolic stability of a compound is expressed as follows:

$$\text{Metabolic Stability (\%)} = \frac{\text{Peak Area of compound incubated with NADPH in LM}}{\text{Peak Area of compound incubated without NADPH in LM}} \times 100\%$$

3-1-4 Preliminary Pharmacokinetic Screening of Flavonoid Monomers

Flavonoid monomers at 10 mg/kg for i.v. and 50 mg/kg for p.o. were administered to starved BALB/c mice (N = 3). After 30 minutes, blood was collected via cardiac puncture and placed in a heparinized microcentrifuge tube. Blood was centrifuged at 14,000 rpm for 5 minutes to obtain the plasma. Proteins were precipitated for 15 minutes by adding 160 μL acetonitrile to 40 μL plasma samples. Any precipitated proteins were centrifuged at 14,000 rpm for 10 minutes and supernatant was filtered through a 0.22 μm nylon filter for LC-MS/MS.

3-1-5 Analytical Method for Metabolism Study and Preliminary Pharmacokinetic Study by UPLC/qTOF-MS or UPLC/QQQ-MS

Agilent 6540 and Agilent 6460 Ultra Performance Liquid Chromatography (UPLC) were used in sample separation in *in vitro* metabolic stability experiment and preliminary pharmacokinetic study, respectively. The samples were dissolved in acetonitrile and kept in the autosampler at 4°C. 10 μL of the sample was injected into UPLC for separation and analysis. Waters Acquity UPLC BEH C8 (1.7 μm, 2.1 x 50 mm) column was used in stationary phase, while 50 mM ammonium formate (0.1% formic acid, v/v) and acetonitrile (0.1% formic acid, v/v) were used in the mobile phase. The flow rate was kept at 0.2 mL/min. During separation, a gradient elution protocol was used as follows: equilibration: 0 – 1 min 95% ammonium formate; elution gradient: 1 – 10 min 5 → 100% acetonitrile; regeneration: 10 – 12 min 100% acetonitrile then 12 – 14 min 100 → 5% acetonitrile and re-equilibration:14 – 15 min 95% ammonium formate (**Table 3-1**). The eluent was then delivered to Electrospray Ionization (ESI+) Quadrupole–TOF mass spectrometer or Electrospray Ionization (ESI+) Triple Quadrupole mass spectrometer to detect the analytes.

The Electrospray Ionization (ESI+) Quadrupole–TOF mass spectrometer was operated in positive electrospray ionization (ESI+) mode with supply of 300°C gas temperature and sheath gas temperature, 8 L/min drying gas, and 11 L/min sheath gas flow and 3.5 kV capillary voltage. In full scan setting LC/qTOF-MS, the mass range was set at m/z 100 – 1,000. The collision energy used for metabolite identification by LC/qTOF-MSMS was 20 eV.

For the Electrospray Ionization (ESI+) Triple Quadrupole mass spectrometer, it was operated in positive electrospray ionization (ESI+) mode with supply of 300°C gas temperature and sheath gas temperature, 8 L/min drying gas, and 11 L/min sheath gas flow and 3.5 kV capillary voltage. The ion pairs for various flavonoid monomers were monitored at optimized collision voltage (**Table 3-2)**.

Table 3-1 Liquid chromatography profile for the LC/qTOF-MS and LC/QQQ-MS

Time (min)	Flow Rate (mL/min)	Ammonium formate (%)	Acteonitrile (%)
0	0.2	95	5
1	0.2	95	5
10	0.2	0	100
12	0.2	0	100
14	0.2	95	5
15	0.2	95	5

The reaction mixtures in metabolic stability study and the plasma samples collected from mice in PK study were extracted and subjected to LC/qTOF-MS and LC/QQQ-MS. The analytes were separated by gradient elution of 5 mM ammonium formate (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid).

Table 3-2 Ion pairs selected for ESI(+)-QQQ-MS

Flavonoid Monomers	Parent Ion (m/z)		Daughter Ion (m/z)	Collision Energy (eV)
FM09	508	>	415	22
FM09a	628	>	475	30
FM09c	544	>	447	30
FM09h	732	>	527	38
FM09i	624	>	473	34
FM09k	712	>	517	38
FM01b	502	>	152	34
FM01i	500	>	262	30
FM05b	501	>	152	30
FM05h	553	>	315	34
FM05i	499	>	150	42
FM05k	543	>	233	46

The flavonoid monomers extracted and eluted from the UPLC are subjected to QQQ-MS. Flavonoid monomers are detected according to the parent and daughter ion pair given above.

3-2 Results

3-2-1 Pharmacokinetic study of FM09 in Plasma and Its Metabolite Identification

FM09 was selected for pharmacokinetic study due to its high potency against both promastigotes and amastigotes (**Table 2-4**). A pharmacokinetic study of **FM09** in BALB/c mice was conducted by oral administration at 50 mg/kg or intravenous injection at 10 mg/kg. It was found that the **FM09** level in plasma decreased quickly from 2,000 to 100 ng/mL within 30 minutes when administered intravenously. Besides, a low level of **FM09** (100 - 300 ng/mL) in plasma was observed when it was administered orally (**Figure 3-1**).

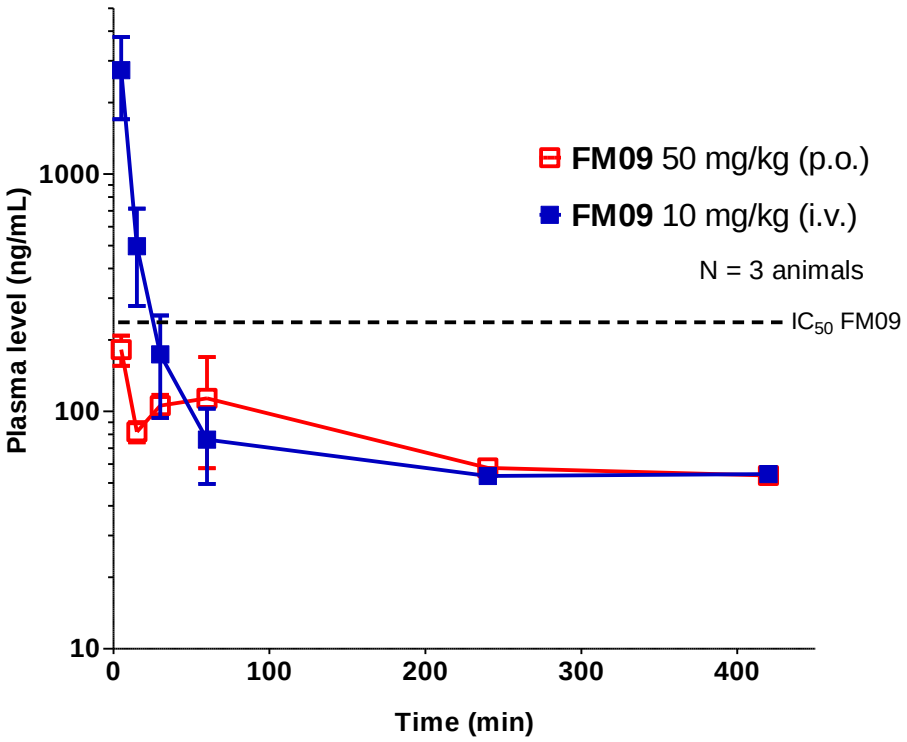


Figure 3-1 The semi-logarithmic plot of **FM09** concentration in plasma versus time after oral administration and intravenous injection in mice.

Flavonoid monomer **FM09** was administered to BALB/c mice either orally (50 mg/kg, p.o.) or intravenously (10 mg/kg, i.v.). Animals were sacrificed at different time points (5, 15, 30, 60, 120, 240 and 420 min). Plasma levels of **FM09** were quantified by LC/MSMS. IC_{50} of corresponding flavonoids were indicated. *In vitro* IC_{50} of **FM09** (200 ng/mL or 0.4 μM) was indicated as a dashed line. The values were presented as mean ± SD. N = 3 animals.

To explain the rapid clearance of **FM09**, an *in vitro* metabolic stability study was performed. In the metabolic stability experiment, human or microsomes enriched with cytochrome P450 enzymes obtained by ultracentrifugation were used. In the presence of cofactor NADPH, these CYP enzymes catalyze the breakdown of xenobiotics as well as the drugs tested. A reduction in compound level after incubation with liver microsomes with NADPH indicated metabolic instability of the tested compound.

After 30-minute incubation with either human or rat liver microsomes in the presence of NADPH, **FM09** disappeared quickly with simultaneous appearance of metabolites, **M1** (m/z 417) and **M2** (m/z 327) (**Figure 3-2** & **Figure 3-3**). The identity of **M1** and **M2** was predicted by qTOF-MS/MS fragmentation and the mass to charge ratio of the generated metabolites. Then the predicted structures, namely **FM09m** and **FM327**, were synthesized and subjected to MS/MS fragmentation. The metabolites shared the same mass to charge ratio and fragmentation patterns with the predicted structures. **M1** was found to have m/z of 417 which was the same as **FM09m** (**Figure 3-4**) whereas **M2** has the m/z of 327 which was the same as **FM327** (**Figure 3-5**). Taken together, it was suggested that **FM09** was metabolized via N-dealkylation at the amine linkage in liver microsome system (**Figure 3-6**). These metabolites were also detected in the plasma after oral administration of **FM09**. They were rapidly generated within 5 minutes after oral administration, possibly from metabolism of **FM09** (**Figure 3-7**). Such metabolic instability may explain the quick clearance of **FM09** in plasma when it was administered to BALB/c mice by either intravenous or oral route (**Figure 3-1**).

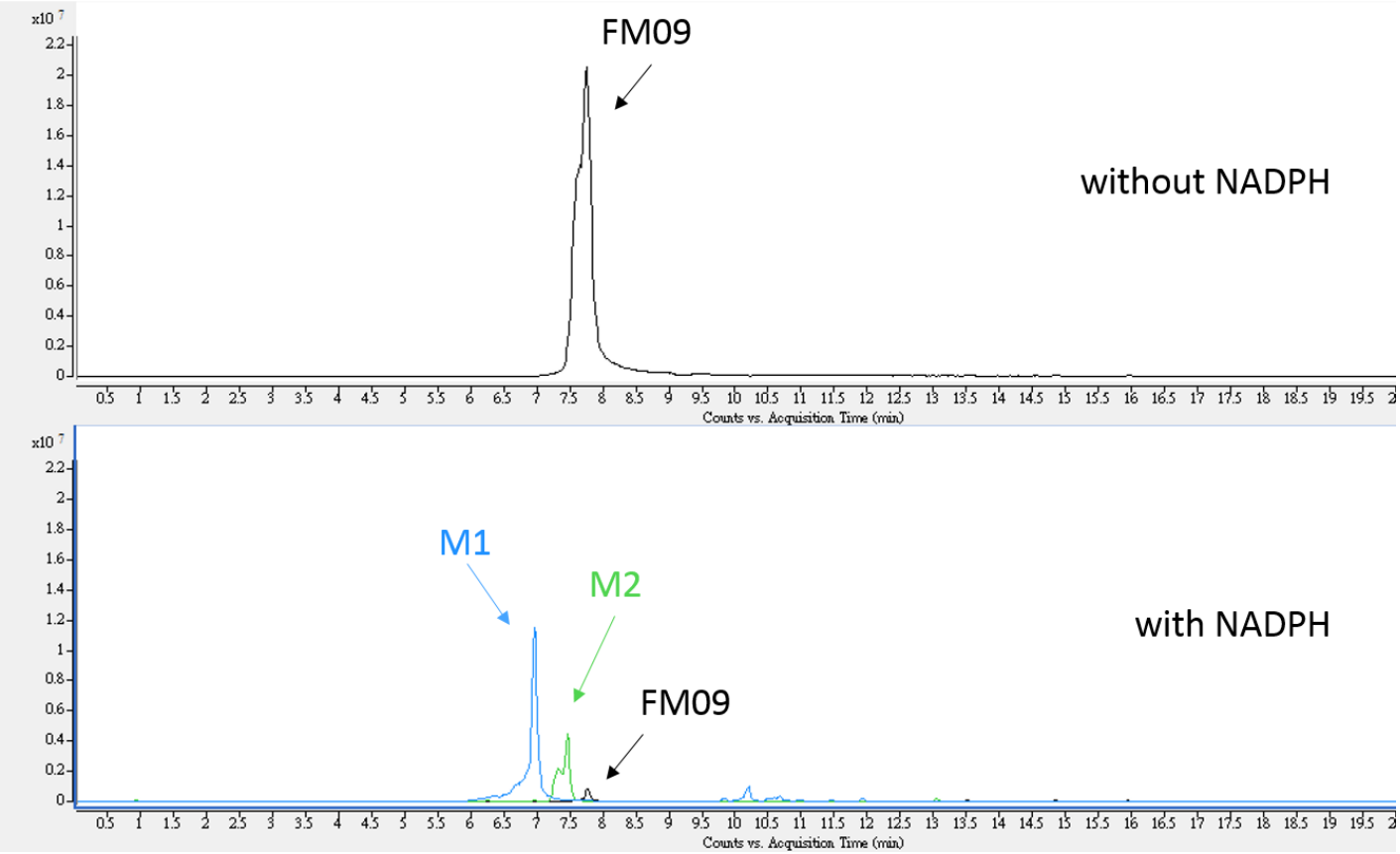


Figure 3-2 UPLC/qTOF-MS extracted ion chromatograms of **FM09** after incubation with human liver microsomes (HLM)

FM09 was incubated with HLM in the absence (upper panel) or presence (lower panel) of NADPH at 37°C for 30 minutes. **FM09** and any metabolites generated was extracted from the reaction mixture for UPLC/qTOF-MS analysis. Two metabolites (**M1** and **M2**) were found. **FM09** (m/z = 508), **M1** (m/z = 417) and **M2** (m/z = 327).

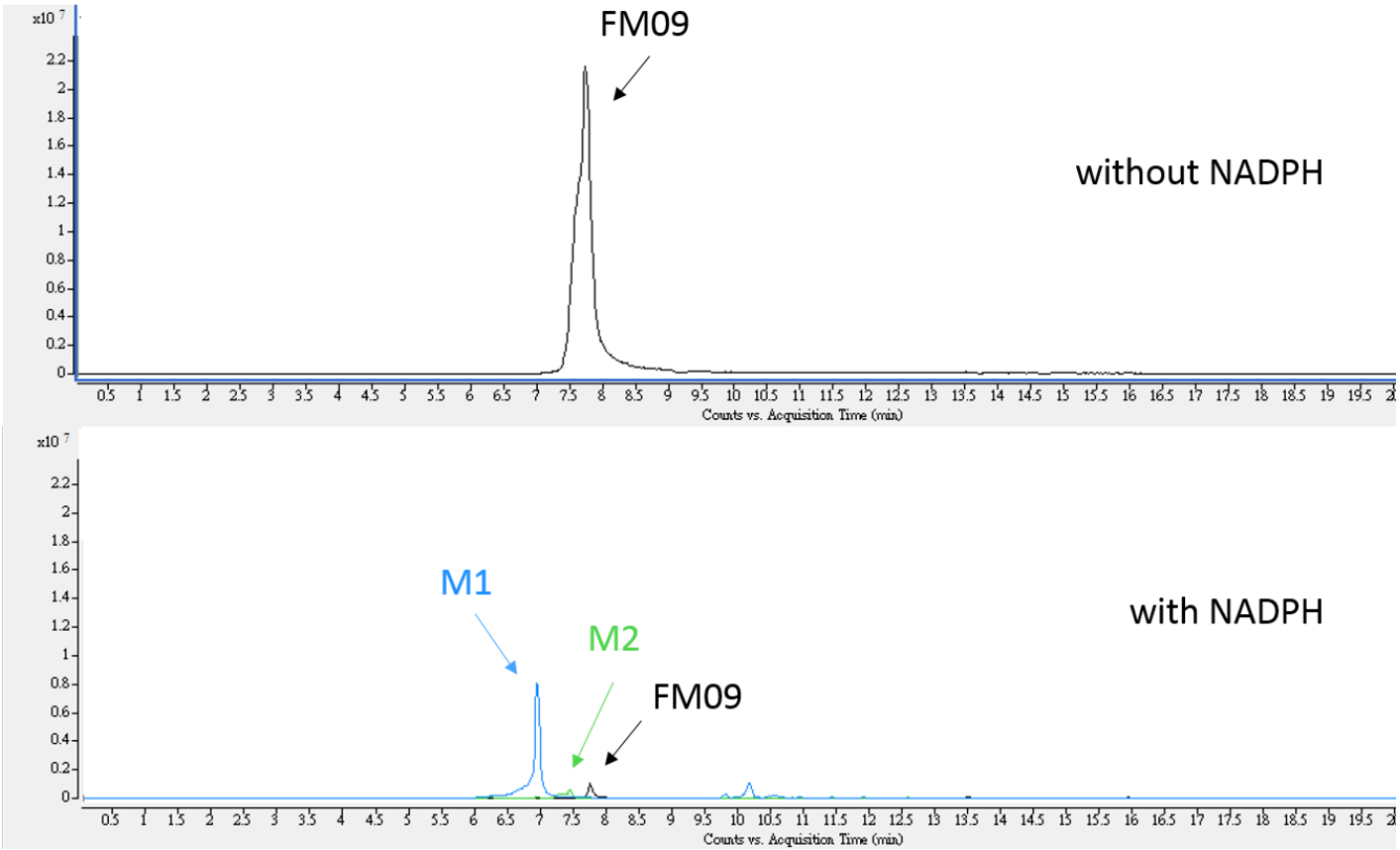


Figure 3-3 UPLC/qTOF-MS extracted ion chromatograms of **FM09** after incubation with rat liver microsomes (RLM)

FM09 was incubated with RLM in the absence (upper panel) or presence (lower panel) of NADPH at 37°C for 30 minutes. **FM09** and any metabolites generated was extracted from the reaction mixture for UPLC/qTOF-MS analysis. Two metabolites (**M1** and **M2**) were found. **FM09** (m/z = 508), **M1** (m/z = 417) and **M2** (m/z = 327).

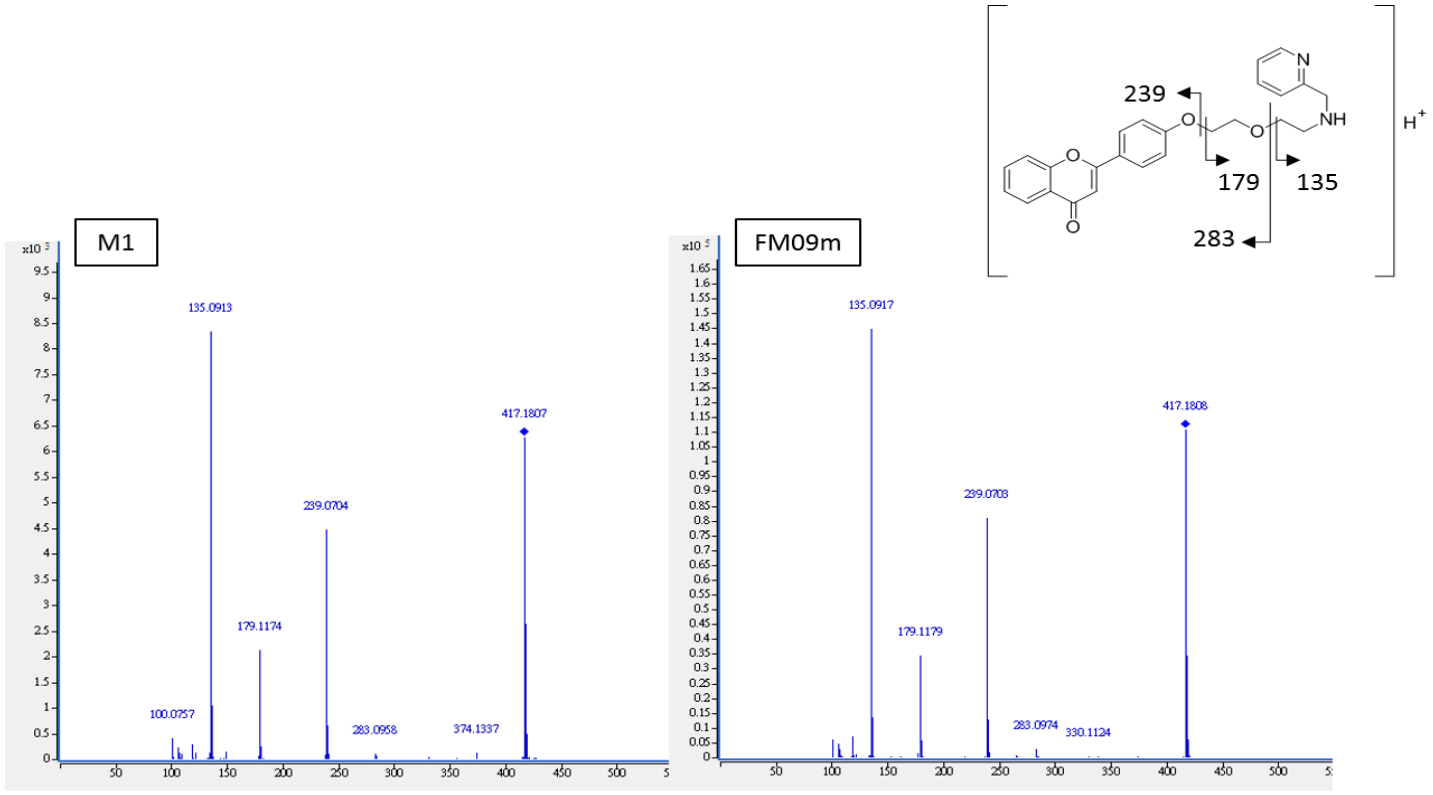


Figure 3-4 Product ion spectra comparison of *in vitro* metabolites **M1** and synthetic compound **FM09m**

qTOF-MSMS of **M1** was conducted to confirm its identity. The MS/MS fragmentation was conducted at collision energy of 20 eV. (Left panel) Product ion spectrum of **M1**. (Right panel) Product ion spectrum of synthesized compound **FM09m**. The same fragmentation pattern of **M1** and **FM09m** confirmed they have the same structure.

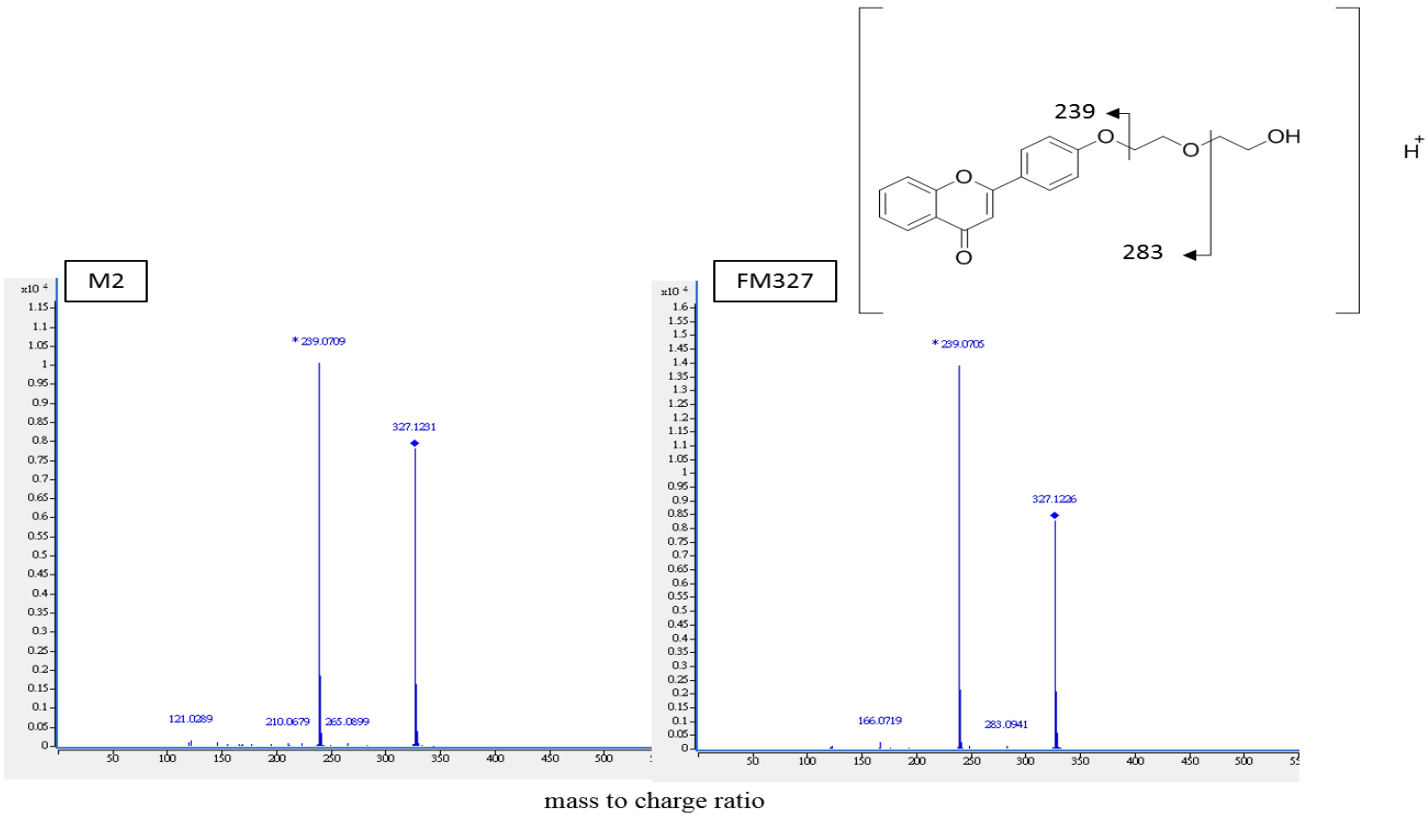


Figure 3-5 Product ion spectra comparison of *in vitro* metabolites **M2** and synthetic compound **FM327**

qTOF-MSMS of **M2** was conducted to confirm its identity. The MS/MS fragmentation was conducted at collision energy of 20 eV. (Left panel) Product ion spectrum of **M2**. (Right panel) Product ion spectrum of synthesized compound **FM327**. The same fragmentation pattern of **M2** and **FM327** confirmed they have the same structure.

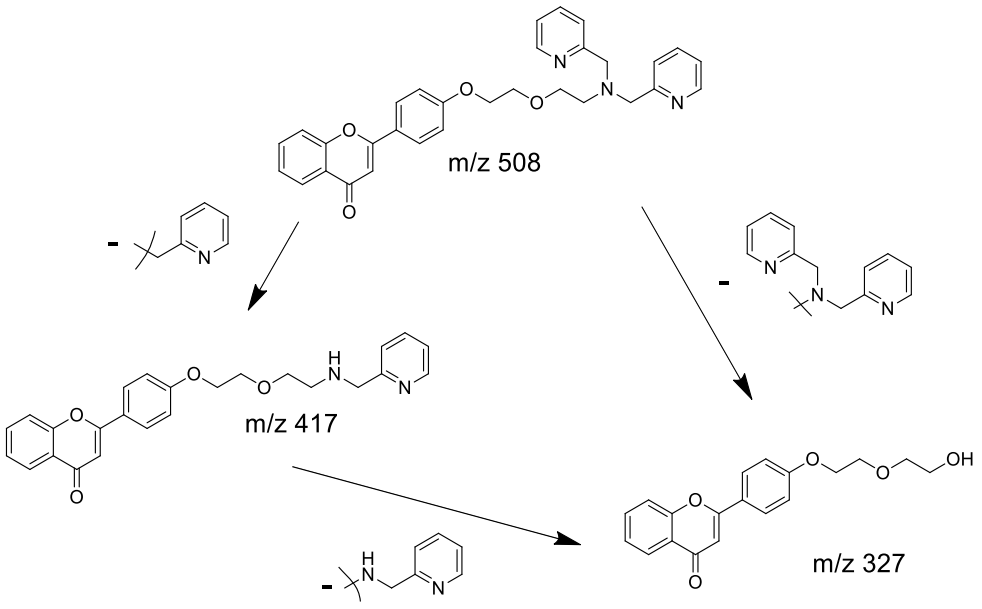


Figure 3-6 Proposed metabolic pathway of **FM09** by liver microsomes

FM09 (m/z 508) may be metabolized into **FM09m** (m/z 417) and **FM327** (m/z 327) via N-dealkylation at the amine linkage by cytochrome P450.

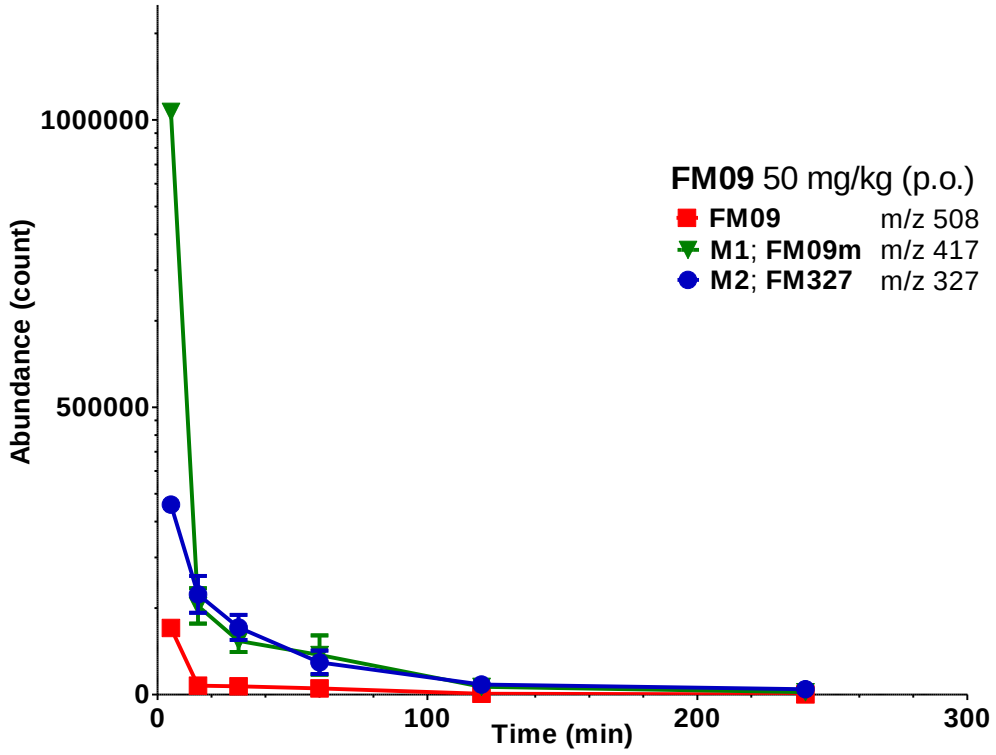


Figure 3-7 Monitoring of **FM09** and its metabolites in plasma after oral administration of **FM09**

FM09 was administered to BALB/c mice (N = 3) orally (50 mg/kg, p.o.). Animals were sacrificed at different time points (5, 15, 30, 60, 120 and 240 min). Plasma levels of **FM09**, **FM09m** and **FM327** were determined by LC/MSMS and expressed in relative abundance. The values were presented as mean ± SD. N = 3 animals.

3-2-2 Lead Optimization to Improve Metabolic Stability of Flavonoid Monomers

Whether structural modification of flavonoid monomers could protect them from degradation, thereby improving their metabolic stability was investigated. Therefore, flavonoid monomers were incubated with CYP-rich liver microsomes to assess their metabolic stability. For the **FM09** series, some derivatives showed improvements in metabolic stability. **FM09h** (methyl and trifluorethoxy substituted **FM09**), that was active against both promastigotes and amastigotes and **FM09r** (amino and methyl substituted **FM09**), that was active only in amastigotes, had improved metabolic stability in both HLM and RLM over the parent compound **FM09** (HLM = 2.3%, RLM = 18.1%) after 30-minute incubation (**FM09h** in HLM = 39.4%, in RLM = 65.7%; **FM09r** in HLM = 60.5%, in RLM = 70.4%) (**Table 3-3**). **FM09d** was another metabolically-stable derivative (HLM = 84.6%, RLM = 83.3%), but it was non-potent. Other potent or weak **FM09** derivatives (**FM09a**, **FM09b**, **FM09c**, **FM09e**, **FM09i**, **FM09k**, **FM09l**, **FM09n** and **FM09p)** were metabolically-unstable with less than 30% metabolic stability in both HLM and RLM (HLM = 4.4 – 17.5%, RLM = 4.9 – 19.4%) (**Table 3-3**).

For the **FM01** and **FM05** derivatives (**FM01**, **FM01a**, **FM01b**, **FM01h**, **FM01i**, **FM01k**, **FM05**, **FM05a**, **FM05b**, **FM05h**, **FM05i** and **FM05k**), all of them could maintain high metabolic stability in both HLM and RLM (HLM from 65.9% to 95.8%; RLM from 63.7% to 98.8%) (**Table 3-3**), indicating that piperazine-linked **FM01** series and piperidine-linked **FM05** series were metabolically-stable. Besides, the functional groups substituted in **FM01** and **FM05** could resist the CYP-mediated degradation. The most potent derivative **FM05b**, with two methoxy

groups added to the pyridine rings, had a high stability in both HLM and RLM (89.0% and 83.1% in HLM and RLM respectively).

Table 3-3 Metabolic stability of **FM01**, **FM05** and **FM09** derivatives

	Percentage of compounds remaining after 30 minutes incubation with	
Flavonoid monomers	Human Liver Microsome	Rat Liver Microsome
FM09	2.3 ± 2.4	18.1 ± 18.6
FM09a	4.6 ± 4.2	4.9 ± 3.7
FM09b	8.7 ± 4.3	7.2 ± 3.9
FM09c	17.5 ± 12.9	17.7 ± 12.7
FM09d	84.6 ± 17.8	83.3 ± 11.1
FM09e	6.0 ± 3.7	7.2 ± 2.4
FM09h	39.4 ± 24.2	65.7 ± 29.2
FM09i	5.3 ± 2.3	14.8 ± 10.0
FM09k	4.4 ± 3.4	10.6 ± 9.3
FM09l	7.8 ± 3.8	16.8 ± 8.4
FM09n	16.5 ± 16.0	13.1 ± 5.6
FM09p	7.4 ± 4.7	19.4 ± 14.7
FM09r	60.5 ± 15.1	70.4 ± 8.1
FM01	80.8 ± 16.9	90.2 ± 13.3
FM01a	81.7 ± 6.3	91.3 ± 12.0
FM01b	87.1 ± 13.3	96.5 ± 6.0
FM01h	93.0 ± 6.1	91.2 ± 7.7
FM01i	78.3 ± 18.8	82.3 ± 25.8
FM01k	80.3 ± 7.0	79.7 ± 5.2
FM05	65.9 ± 26.0	75.2 ± 21.5
FM05a	88.1 ± 13.6	63.7 ± 27.8
FM05b	89.0 ± 16.2	83.1 ± 14.1
FM05h	95.8 ± 4.7	90.8 ± 15.6
FM05i	94.7 ± 5.7	82.5 ± 3.2
FM05k	91.9 ± 3.8	98.8 ± 2.0

Derivatives of flavonoid monomers **FM01**, **FM05 and FM09** were incubated with either Human Liver Microsome (HLM) or Rat Liver Microsome (RLM) for 30 minutes. Metabolic stability was defined as the percentage of compounds remaining after 30 minutes of incubation. The values were presented as mean ± SD. N = 2 to 6 independent experiments.

3-2-3 Preliminary Pharmacokinetic Screening of Flavonoid Monomers

It was questioned whether the metabolically-stable flavonoid monomers determined in the previous section can be administered orally. Therefore, a single time-point pharmacokinetic study in BALB/c mice was performed to estimate their oral bioavailability. Among all **FM09** derivatives tested, **FM09h**, was the most metabolically-stable derivative of **FM09**, yielding the highest plasma level of 5,227 and 10,747 ng/mL 30 minutes post oral administration at 50 mg/kg and intravenous injection at 10 mg/kg, respectively. This resulted in a 10% oral bioavailability (**Table 3-4**). **FM09k** gave the second highest plasma level among all **FM09** derivatives with plasma levels of 1,665 and 8,735 ng/mL after oral and intravenous administration, respectively, yielding a 4% oral bioavailability (**Table 3-3**). Other unstable derivatives, such as **FM09a**, **FM09c** and **FM09i,** yielded low plasma levels (less than 1,200 ng/mL for both p.o. and i.v.). Compared with **FM09**, **FM09h** with substituted trifluoroethoxy groups and methy groups was found at a greatest increase in abundance in plasma after administration via both the oral and intravenous routes by 49- and 61-fold, respectively. For **FM01** and **FM05** derivatives, in general, all derivatives gave a high plasma level except **FM05i**. **FM05b** generated a level of 4,757 and 1,849 ng/mL 30 minutes post oral administration at 50 mg/kg and intravenous injection at 10 mg/kg respectively, yielding the highest oral bioavailability of 60% (**Table 3-3**). **FM05h** gave the second highest plasma level among all **FM05** derivatives with plasma level of 3,413 ng/mL after oral administration (**Table 3-3**). Other derivatives such as **FM01b**, **FM01i** and **FM05k** gave a plasma level higher than 1,200 ng/mL.

Table 3-4 Single time-point pharmacokinetic study of **FM01**, **FM05** and **FM09** derivatives

Flavonoid monomers	Plasma level (ng/mL)		F_{30}
	50 mg/kg (p.o.)	10 mg/kg (i.v.)	(%)
FM09	106 ± 12	176 ± 80	12
FM09a	130 ± 28	1 196 ± 455	2
FM09c	88 ± 6	278 ± 13	3
FM09h	5 227 ± 1614	10 747 ± 3768	10
FM09i	215 ± 28	1 073 ± 165	4
FM09k	1 665 ± 528	8 735 ± 1154	4
FM01b	1 478 ± 858	ND	ND
FM01i	3 291 ± 175	ND	ND
FM05b	4 757 ± 773	1 849 ± 713	60
FM05h	3 413 ± 968	ND	ND
FM05i	431 ± 76	ND	ND
FM05k	1 377 ± 93	ND	ND

Flavonoid derivatives of **FM09**, **FM01** and **FM05** were administered to BALB/c mice (N = 3) either orally (50 mg/kg p.o.) or intravenously (10 mg/kg i.v.). Blood samples were collected after 30 minutes. After extraction, plasma level of flavonoid monomers was quantified by LC/MS-MS analysis. Oral bioavailability (F) was calculated using the formula: [(quantity of flavonoid monomer detected when it is given p.o. / p.o. dosage) / (quantity of flavonoid monomer detected when it is given i.v. / i.v. dosage)] x 100%. The values were presented as mean ± SD. N = 3 animals. ND = not determined.

3-3 Discussion

The amine linkage connecting pyridine rings to the flavonoid moiety in **FM09** was thought to be the labile group that caused metabolic instability to **FM09**. It was found that substitution of trifluoroethoxy and methyl groups to pyridine rings, generating **FM09h** can improve its metabolic stability (metabolic stability improved from 2% to 39% in HLM; from 19% to 66% in RLM) (**Table 3-3**). It has been reported that the use of trifluoroethoxy groups may improve the metabolic stability of lipid peroxidation inhibitor [105] and phosphodiesterase (PDE) 5 inhibitor [106]. In other flavonoid monomers, substitution with amino groups (**FM09r**) or replacement of pyridine rings with pyrimidine rings (**FM09d**) was found to greatly improve the metabolic stability compared with **FM09h** (**Table 3-3**). Unfortunately, **FM09d** and **FM09r** were less active against *Leishmania* (**Table 2-2**). Their activities may be improved by further structural modification. Especially in **FM09r**, the amine group can be modified with different functional groups to improve its activity; this may be investigated in the future.

On the other hand, the **FM01** and **FM05** derivatives can maintain a high metabolic stability. The rigid six-membered rings (piperazine for **FM01** series and piperidine for **FM05** series) joining the flavone moiety and pyridine rings together might protect the molecules from CYP degradation. **FM13**, **FM14**, **FM15**, **FM16**, **FM17** and **FM18** in the first generation flavonoid library also shared similar structure with wide range of activities in promastigotes and amastigotes that may also be metabolically-stable. Modification can be done to improve their potencies.

In the single time point pharmacokinetic study, the metabolic stability data positively correlated with the quantity of flavonoid monomers found in plasma after

drug administration. Only metabolically-stable candidates could be found in plasma at a relatively high level, such as **FM01b**, **FM01i**, **FM05b**, **FM05h**, **FM05k** and **FM09h**. Compared with all **FM09** derivatives, **FM09h** with substituted trifluoroethoxy groups and methy groups into **FM09**, was found at a greatest increase in abundance in plasma after administration via both the intravenous and oral routes, implying the success of lead optimization in order to improve metabolic stability.

3-4 Conclusion

Metabolically-stable flavonoid monomers can be identified by metabolic stability assay. Among the potent flavonoid monomers, **FM05b** and **FM09h** were selected for further characterization because of their higher metabolic stability and levels detected in plasma 30 minutes post oral administration.

4 Pharmacokinetics and Toxicity of Novel Antileishmanial Candidates, FM05b and FM09h

4-1 Introduction

After the screening based on antileishmanial activity, metabolic stability and pharmacokinetic profile, **FM05b** and **FM09h** were the best candidates over **FM09** (**Figure 4-1**) because they were potent, metabolically-stable and could be highly detected in plasma when administered orally (**Table 4-1**).

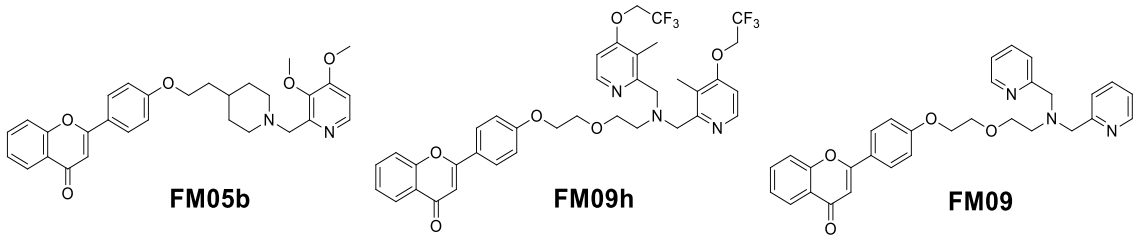


Figure 4-1 Chemical structure of potent flavonoid monomers **FM05b** and **FM09h** compared to **FM09**

Table 4-1 Summary of **FM05b** and **FM09h** in terms of potency, metabolic stability and pharmacokinetics

	Amastigote IC_{50} (µM)		Metabolic Stability (%)		Plasma level at 30th minute (ng/mL)
Cpds	LV78	HU3	HLM	RLM	Oral 50mg/kg
FM09	0.4 ± 0.1	0.4 ± 0.1	1.9 ± 1.0	18.1 ± 1.4	106 ± 12
FM09h	0.3 ± 0.1	0.3 ± 0.0	39.4 ± 12.1	67.1 ± 14.0	5227 ± 714
FM05b	0.3 ± 0.1	0.7 ± 0.4	91.5 ± 7.8	83.1 ± 6.8	5539 ± 904

FM05b and **FM09h** were selected as potential candidates to replace **FM09**. They could maintain an IC_{50} comparable to **FM09** with improved metabolic stability and high abundance in plasma when administered orally.

The next step was to fully characterize the pharmacokinetic and toxicity profile of these two drug candidates. The pharmacokinetic study, including ADMET (absorption, distribution, metabolism and excretion and toxicity) may help to design a safe and effective dosage and dose regimen for subsequent *in vivo* efficacy study [107]. The ADME of a drug is related to the intrinsic properties of its chemical structure such as molecular weight, lipophilicity and water solubility [108].

In this chapter, an extended pharmacokinetic study and *in vivo* toxicity study of **FM05b** and **FM09h** was conducted. In the pharmacokinetic study, how long the flavonoid monomers can be maintained in plasma, liver and spleen above their corresponding effective concentration (IC_{50}) was determined. In the toxicity assessment, healthy BALB/c mice were administered orally with **FM05b** and **FM09h** at 50 or 100 mg/kg once every day for 10 days to see if there was any apparent toxicity to the animals.

4-2 Materials and Methods

4-2-1 Chemicals and Reagents

Cremophor EL and heparin in the pharmacokinetic study were purchased from Sigma. Organic solvents such as acetonitrile were of high-performance liquid chromatography (HPLC) grade and purchased from Anaqua. The Waters Acquity UPLC BEH C8 (1.7 μm, 2.1 x 50 mm) column, sample insert, vials and caps used to run UPLC-MS were purchased from Tegent Technology (Hong Kong).

4-2-2 Animal Housing

BALB/c mice were obtained from the Centralised Animal Facilities (CAF), The Hong Kong Polytechnic University. Animals were kept in cages at 12-hour light-dark cycles with standard diet and water.

4-2-3 Drug Preparation of FM05b and FM09h

FM05b and **FM09h** were dissolved in 5% ethanol, 5% Cremophor EL and 90% saline at 5 mg/mL. To make a 1 mL drug solution, flavonoid monomers were first dissolved in 50 μL ethanol. Then 50 μL Cremophor EL was added to the solution followed by vortex mixing to form a homogenous solution. Finally, 900 μL of saline solution was added to generate the working solution. **FM09h** was stable for over 24 hours without any precipitation. For **FM05b**, it was stable for about 30 minutes before precipitation occurred.

4-2-4 Pharmacokinetic Study of FM05b and FM09h in Plasma, Liver and Spleen

Flavonoid monomers were administered intravenously (10 mg/kg) or orally (50 mg/kg) to overnight-starved BALB/c mice (N = 3). At different time points (5, 15, 30, 60, 120, 240 and 420 min), blood was collected via cardiac puncture and placed in a heparinized Eppendorf tube. Blood was centrifuged at 14,000 rpm for 5 minutes to obtain the plasma. 40 µL plasma samples were added with 160 µL acetonitrile for protein precipitation followed by centrifugation at 14,000 rpm for 10 minutes to pellet precipitated proteins. Supernatant was collected and filtered through a 0.22 µm nylon filter for LC-MS/MS analysis. To quantify the level of flavonoid monomers in organs, livers and spleens were collected, weighed and homogenized in 2 and 3 volumes of 1X PBS respectively. Extraction of flavonoid monomers from 40 µL of homogenate was performed as in the plasma samples. The chromatographic and mass spectrometry condition can be found in the previous chapter. (See **Chapter 3-1-5**)

4-2-5 *In Vivo* Toxicity of Flavonoid Monomers

Flavonoid monomers **FM05b** and **FM09h** were administered orally to BALB/c mice (N = 4) every day at 50 or 100 mg/kg once daily for 10 days. The body weight of the mice was measured every day. Any toxicity symptoms like behavioral changes or shivering were recorded. Weight loss of more than 15% for 3 consecutive days suggested toxicity.

4-3 Results

4-3-1 Pharmacokinetic Profiles of FM09, FM09h and FM05b in Plasma

A detailed pharmacokinetic study lasting 7 hours was performed on **FM09**, **FM09h** and **FM05b**. After oral administration (50 mg/kg) or intravenous administration (10 mg/kg), the plasma drug concentration-time curves were obtained (**Figure 4-2**) and their corresponding pharmacokinetic parameters were summarized in **Table 4-2**.

For **FM09**, a low level of **FM09** and quick clearance in plasma was observed in both p.o. and i.v. routes. The maximal concentration (C_{max}) when administered i.v. was 2,742 ng/mL (after 5 minutes) while the C_{max} in p.o. route was 183 ng/mL at T_{max} of 5 minutes. The distribution half-lives ($T_{1/2\alpha}$) for i.v. and p.o. administration were short at 5 and 14 minutes, respectively while the elimination half-lives ($T_{1/2\beta}$) for i.v. and p.o. administration were 354 and 185 minutes, respectively. The mean residence time (MRT) calculated for **FM09** in i.v. and p.o. were 339 and 328 minutes, respectively. The area under curve ($AUC_{(0\text{-}7h)}$) for i.v. and p.o. routes were 63,673 and 31,949 ng/mL, respectively, yielding an oral bioavailability ($F_{0\text{-}7h}$) of 7% (**Table 4-2**).

For **FM09h**, both i.v. and p.o. routes could achieve a high C_{max} in plasma. For i.v. injection, the C_{max} was 13,881 ng/mL (after 5 minutes) while, the C_{max} for p.o. administration appeared 15 minutes post administration at 6,844 ng/mL. The $T_{1/2\alpha}$ for i.v. and p.o. administration was 42 and 14 minutes, respectively while the $T_{1/2\beta}$ for i.v. and p.o. administration were 135 and 368 minutes, respectively. The MRT for **FM09h** was similar for both administration routes at around 150 minutes. The

$AUC_{(0\text{-}7h)}$ for i.v. and p.o. routes were 1,647,892 and 388,462 ng-min/mL, respectively, yielding an oral bioavailability ($F_{0\text{-}7h}$) of 5% (**Table 4-2**).

Similarly, **FM05b** was administered to BALB/c mice using the same dose regimens and administration routes. For i.v. injection, the C_{max} was 6,678 ng/mL (after 5 minutes) while the C_{max} in p.o. administration was 4,757 ng/mL at T_{max} of 30 minutes. The $T_{1/2\alpha}$ for i.v. and p.o. administration were 4 and 35 minutes, respectively while the $T_{1/2\beta}$ for i.v. and p.o. administration were 98 and 261 minutes, respectively. **FM05b** exhibited a different MRT of 93 and 284 minutes for i.v. and p.o. routes, respectively. The $AUC_{(0\text{-}7h)}$ for i.v. and p.o. routes were 465,643 and 750,406 ng-min/mL, respectively, yielding an oral bioavailability ($F_{0\text{-}7h}$) of 32% (**Table 4-2**).

Compared to **FM09**, both **FM05b** and **FM09h** could exhibit a substantially higher plasma level and be maintained for a longer period in plasma when administered by both i.v. and p.o. routes. The short $T_{1/2\alpha}$ for the two compounds in i.v. and p.o. routes suggested a rapid distribution into different parts of body. Oral **FM05b** and **FM09h** level could maintain above its *in vitro* IC_{50} for over at least 7 and 3 hours respectively when they were administered orally at 50 mg/kg.

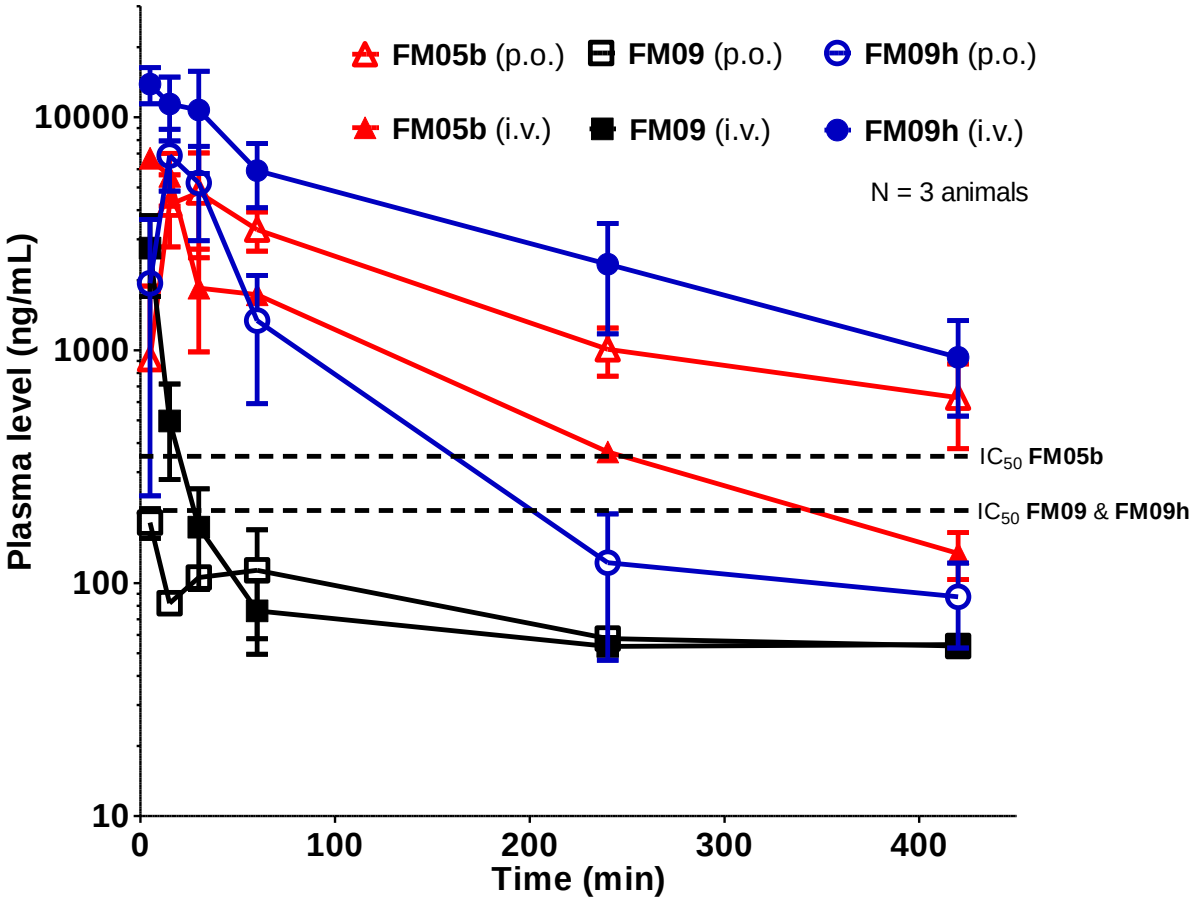


Figure 4-2 The semi-logarithmic plot of **FM05b** and **FM09h** concentration in plasma versus time after oral administration and intravenous injection in mice.

Flavonoid monomers **FM05b** and **FM09h** were administered to BALB/c mice either orally (50 mg/kg, p.o.) or intravenously (10 mg/kg, i.v.). Animals were sacrificed at different time points (5, 15, 30, 60, 120, 240 and 420 min). Plasma levels of these flavonoid monomers were quantified by LC/MSMS. IC_{50} of corresponding flavonoids was indicated. *In vitro* IC_{50} of **FM05b** (350 ng/mL or 0.7 μM), **FM09** (200 ng/mL or 0.4 μM) and **FM09h** (220 ng/mL or 0.3 μM) were indicated as dashed lines. The values were presented as mean ± SD. N = 3 animals.

Table 4-2 Pharmacokinetic parameters of **FM09**, **FM05b** and **FM09h** after intravenous (10 mg/kg) and oral (50 mg/kg) administration in BALB/c mice

		Plasma		**Plasma**		**Plasma**	
		p.o.	**i.v.**	**p.o.**	**i.v.**	**p.o.**	**i.v.**
Parameter	Unit	**FM09**		**FM09h**		**FM05b**	
Dose	mg/kg	50	10	50	10	50	10
AUC (0-7h)	ng-min/mL	31 949	63 673	388 462	1 647 892	750 406	465 643
T1/2α	min	14	5	14	42	35	4
T1/2β	min	185	354	368	135	261	98
MRT (area)	min	328	339	152	161	284	93
Cmax (obs)	ng/mL	183	2 742	6 844	13 881	4 757	6 678
Tmax (obs)	min	5		15		30	
CL (obs)	mL/min	1 565	157	129	6	67	21
Oral Bioavailability (F)	%	7		5		32	

AUC: area under curve, $T_{1/2\alpha}$: distribution half-life, $T_{1/2\beta}$: elimination half-life, MRT: mean residence time, C_{max}: maximal concentration, T_{max}: time at which C_{max} is attained, CL: clearance rate

Flavonoid monomers **FM05b** and **FM09h** were administered to BALB/c mice either orally (50 mg/kg p.o.) or intravenously (10 mg/kg i.v.). Plasma levels of these flavonoid monomers were quantified by LC/MS-MS at different time points for up to 7 hours. Pharmacokinetic parameters were calculated by the pharmacokinetic software-Summit® PK solution.

4-3-2 Accumulation of FM05b and FM09h in the Liver and Spleen After Oral Administration

As visceral leishmaniasis-causing parasites infect particularly the liver, spleen and bone marrow, it is important to see if flavonoid monomers can accumulate in these organs. Therefore, the pharmacokinetic study (24 hours) of **FM05b** and **FM09h** in the liver and spleen by oral administration was also performed and the result was shown in **Figure 4-3**. The corresponding pharmacokinetic parameters of **FM05b** were summarized in **Table 4-3**. Accumulation of **FM05b** in the liver and spleen was found to be higher than that in plasma. The C_{max} in the liver and spleen appeared after 30 minutes and were 56,949 and 28,652 ng/g, respectively. The $AUC_{(0\text{-}24h)}$ of **FM05b** in the liver and spleen were 13,061,108 and 11,304,240 ng-min/g, respectively. **FM05b** displayed $T_{1/2\alpha}$ from 41 – 128 minutes and $T_{1/2\beta}$ from 461 – 698 minutes in plasma, liver and spleen. The MRTs were similar at around 486 – 631 minutes in plasma, liver and spleen. This result suggested that **FM05b** could accumulate in the liver and spleen after oral administration. Its level at 24h post oral administration in the liver (2,273 ng/g or 4.5 μM) or spleen (1,608 ng/g or 3.2 μM) remained higher than its *in vitro* IC_{50} of 0.7 μM (**Figure 4-3**). Accumulation of **FM09h** in the liver was also determined. **FM09h** did not accumulate in liver at a high level but was cleared more quickly compared with **FM05b**. Its liver level dropped below its *in vitro* IC_{50} of 0.7 μM at about 200 minutes post oral administration (**Figure 4-3**).

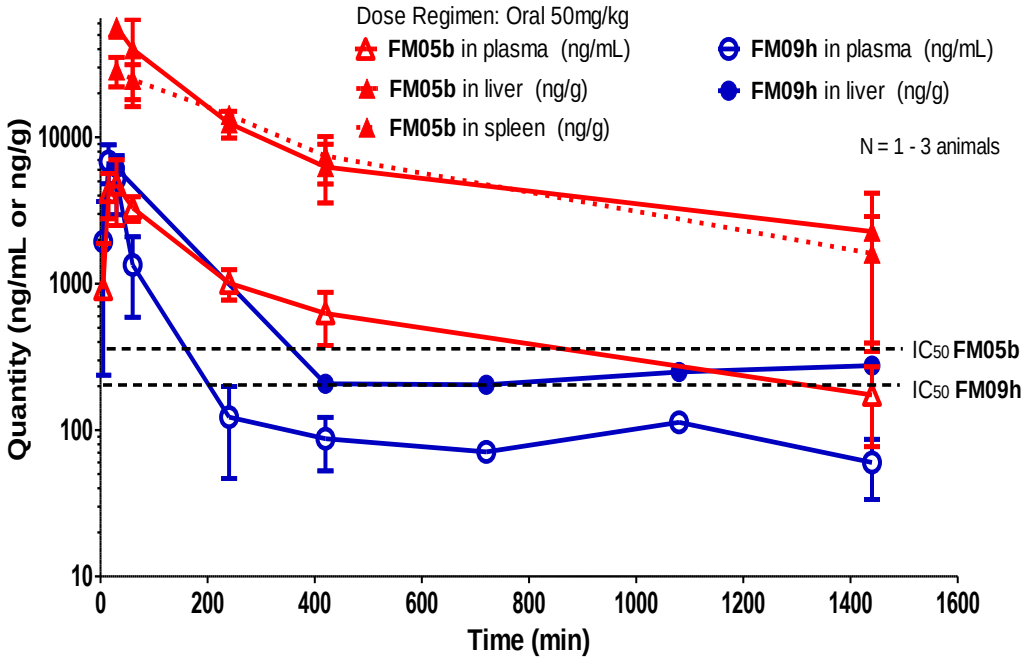


Figure 4-3 The semi-logarithmic plot of **FM05b and FM09h** concentration in plasma, liver and spleen versus time after oral administration at 50 mg/kg in mice.

FM05b or **FM09h** was administered orally to BALB/c mice at 50 mg/kg. At the indicated time points (30, 60, 120, 240, 420 and 1,440 min), mice were sacrificed and the level of **FM05b** or **FM09h** in the liver and spleen was determined using LC-MS/MS. IC_{50} of **FM05b** (350 ng/mL or 0.7 μM) and **FM09h** (220 ng/mL or 0.3 μM) were indicated as dash line. The values were presented as mean ± SD. N = 1 - 3 animals

Table 4-3 Pharmacokinetic parameters of **FM05b** in plasma, liver and spleen after oral administration (50 mg/kg) in BALB/c mice

Parameter	Unit	**Plasma p.o. FM05b**	**Liver p.o. FM05b**	**Spleen p.o. FM05b**
Dose	mg/kg	50	50	50
AUC (0-24h)	ng-min/mL or ng-min/g	1 158 236	13 061 108	11 304 240
T1/2α	min	41	66	128
T1/2β	min	497	698	461
MRT (area)	min	486	631	494
Cmax (obs)	ng/mL or ng/g	4 757	56 949	28 652
Tmax (obs)	min	30	30	30

AUC: area under curve, $T_{1/2\alpha}$: distribution half-life, $T_{1/2\beta}$: elimination half-life, MRT: mean residence time, C_{max}: maximal concentration, T_{max}: time at which C_{max} is attained, CL: clearance rate. ng-min/mL described the quantity of compounds in plasma while ng-min/g described the quantity of compounds in liver and spleen.

Flavonoid monomer **FM05b** or **FM09h** was administered orally to BALB/c mice at 50 mg/kg. Levels of these flavonoid monomers in the plasma, liver and spleen were quantified by LC/MS-MS at different time points for up to 24 hours. Pharmacokinetic parameters were calculated by the pharmacokinetic software-Summit® PK solution.

4-3-3 *In Vivo* Toxicity of FM05b and FM09h

Healthy BALB/c mice were administered orally with either **FM05b** or **FM09h** at 50 or 100 mg/kg for 10 days. Their body weight and other signs of toxicity were recorded daily. It was found that oral administration of **FM05b** or **FM09h** at 50 or 100 mg/kg for 10 consecutive days resulted in no animal death. The body weight of the mice varied very slightly between -4 and +2 % during the treatment period (**Figure 4-4**). It was concluded that **FM05b** and **FM09h** did not possess any apparent toxicity to the animals.

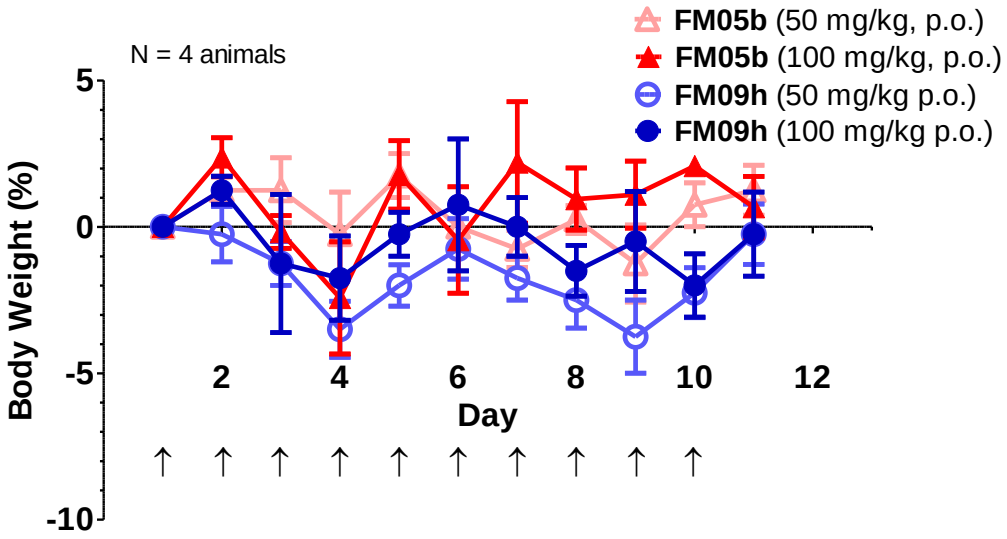


Figure 4-4 *In vivo* toxicity of **FM05b** and **FM09h** in BALB/c mice

BALB/c mice were administered orally with **FM05b** or **FM09h** 50 mg/kg once every day for 10 days. Their body weight was monitored every day. The values were presented as mean ± SD. N = 4 animals.

4-4 Discussion

Results from this chapter demonstrated that structural modification of **FM09** to **FM09h** could lead to improvement not only in *in vitro* metabolic stability but also in the pharmacokinetic profile. Substitution at pyridine ring of **FM09** with trifluoroethoxy and methyl groups, producing **FM09h**, could significantly improve its pharmacokinetic profile. For i.v. injection, the $AUC_{(0\text{-}7h)}$ was increased by 26-fold (from 63,673 to 1,647,892 ng-min/mL). This result further confirmed that **FM09h** was metabolically more stable than **FM09** (**Table 3-3**). Similarly, the $AUC_{(0\text{-}7h)}$ in p.o. route was increased by 12-fold (from 31,949 to 388,462 ng-min/mL) (**Table 4-2**). It should be noted that the increased $AUC_{(0\text{-}7h)}$ in p.o. route may be contributed not only by improvement in metabolic stability but also by elevation in oral absorption. Although decrease in value of $T_{1/2\beta}$ calculated was not observed in **FM09h** administered by either i.v. or p.o. routes, a significant decrease in clearance rate (CL) for i.v. from 157 to 6 mL/min and for p.o. from 1,565 to 129 mL/min demonstrated a lower drug metabolism. Although the **FM09h** level in both plasma and the liver were dropped below IC_{50} after 3 hours post administration, the insertion of trifluoroethoxy groups into pyridine rings, though not very efficient, could delay the elimination/excretion of the flavonoid monomer from the body for the drug to exhibit its function.

On the other hand, **FM05b** also demonstrated a positive correlation between metabolic stability, longer half-life and increased oral bioavailability (**Table 3-3**, **Table 4-2** and **Figure 4-2**). The rigid piperidine ring of **FM05b** contributed to high *in vitro* metabolic stability and high plasma level of **FM05b** *in vivo*. Compared with **FM09h**, **FM05b** administered orally had a greater drug exposure and reduced metabolism. The level of **FM05b** can be maintained in the liver and spleen above

its IC_{50} level for more than 24 hours. Such observation could not be found in **FM09h**. This further confirmed the high metabolic stability of **FM05b.** It was also found that **FM05b** accumulated in the liver more efficiently than **FM09h** when administered orally. Such a prolonged liver and spleen accumulation of **FM05b** above IC_{50} might be advantageous for treating visceral leishmaniasis.

The toxicity assessment showed that neither **FM05b** nor **FM09h** at 50 and 100 mg/kg caused any severe body weight loss in the mice. When designing the dose regimen to determine the *in vivo* efficacy, increasing **FM05b** and **FM09h** to 100 mg/kg could be an option if 50 mg/kg was not effective.

4-5 Conclusion

The pharmacokinetic profile of **FM09** was significantly improved by structural modification, producing **FM09h**, with elevated C_{max} and AUC seen in both i.v. and p.o. routes. **FM05b** displayed a good pharmacokinetic profile not only in plasma but also in the liver and spleen, which are target organs of VL. **FM05b** could be maintained for over 24 hours in the liver and spleen.

5 *In Vivo* Efficacy of Flavonoid Monomers Against Leishmaniasis

5-1 Introduction

Only a few antileishmanial drugs are available namely pentavalent antimony as well as sodium stibogluconate and meglumine antimoniate, paromomycin, pentamidine, amphotericin B and miltefosine. Their drawbacks have been reviewed in the Introduction chapter of this thesis (see **Section 1-6**). Among them, only miltefosine can be administered orally while the others require parenteral administration such as intramuscular, intradermal or intravenous injection. This imposes limitation to poor countries which lack hygienic places for injection or hospitalization. Besides, there are increasing relapse cases after treatment with miltefosine [109, 110]. There is an urgent need for more novel oral therapeutics for the treatment of leishmaniasis.

In **Chapter 2**, *in vitro* screening has identified several active flavonoid monomers with IC_{50} at around 1 μM against both promastigotes and intracellular amastigotes of *L. amazonensis* and *L. donovani*. In **Chapter 3**, the metabolic stability and preliminary pharmacokinetic study have further narrowed down suitable candidates to **FM05b** and **FM09h** that were metabolically-stable after 30-minute of liver microsome incubation (**FM05b** in HLM = 89.0%, in RLM = 83.1%, **FM09h** in HLM = 39.4%, in RLM = 65.7%). In **Chapter 4**, **FM09h** demonstrated improved pharmacokinetics and its plasma level could maintain above its IC_{50} for more than 3 hours. Besides, **FM05b** could even maintain its plasma level above IC_{50} for more

than 7 hours and in spleen and liver for more than 24 hours. Moreover, repeated oral administration of **FM05b** and **FM09h** at 50 or 100 mg/kg did not show any apparent toxicity, including animal death or significant weight loss in mice. The above data suggested that **FM05b** and **FM09h** were safe to use orally.

In this chapter, the *in vivo* efficacy of selected flavonoid monomers **FM05b** and **FM09h** was evaluated in cutaneous leishmaniaisis (*L. amazonensis*) and visceral leishmaniasis (*L. donovani*) BALB/c mice model. These two animal models were chosen for *in vivo* efficacy experiments because of their extensive uses in drug testing [74, 88, 111-115]. In cutaneous leishmaniasis, two administration routes were studied including intralesional injection and oral administration. Intralesional injection was performed by administering **FM05b** and **FM09h** 2.5 or 10 mg/kg once every 4 days for a total of 8 injections (q4d x 8). For oral administration, the dose regimen of oral administration was decided based on the pharmacokinetic data and *in vivo* toxicity data gathered in **Chapter 4**: The plasma level of **FM05b** and **FM09h** could maintain above their IC_{50} for more than 7 and 3 hours. **FM05b** or **FM09h** 50 and 100 mg/kg were administered orally once daily for a total of 12 administrations. In visceral leishmaniasis, only **FM05b** was tested because **FM05b** can accumulate in the target organs of infection as well as liver and spleen. **FM05b** 50 mg/kg was administered orally to infected mice once daily for 14 days. As miltefosine works effectively in treating both cutaneous and visceral leishmaniasis, the pharmacokinetics of miltefosine in BALB/c mice was also studied to compare its biodistribution with **FM05b**.

5-2 Materials and Methods

5-2-1 Animal Housing

BALB/c mice in efficacy experiments were obtained from the Laboratory Animal Services Centre (LASEC), The Chinese University of Hong Kong. Animals were kept in IVC cages with temperature and humidity controlled and 12-hour light-dark cycles with standard diet and water. The animal experiment protocol was approved by the Animal Subjects Ethics Sub-Committee of The Chinese University of Hong Kong in 16/206/MIS-5-B.

5-2-2 Drug Preparation

FM05b and **FM09h** were dissolved in a formulation of 5% ethanol, 5% Cremophor EL and 90% saline. The final concentration of the drug solution was 1, 4 or 5 mg/mL used in intralesional injection 2.5 mg/kg, intralesional injection 10 mg/kg and oral administration 50 and 100 mg/kg, respectively. To make a 1 mL drug solution, flavonoid monomers were first dissolved in 50 μL ethanol. Then the solution was added with 50 μL Cremophor EL followed by vortex mixing to form a homogenous solution. Finally, 900 μL of saline solution was added to generate the working solution. **FM09h** was stable for over 24 hours without any precipitation. For **FM05b**, it was stable for about 30 minutes before precipitation.

5-2-3 *In Vivo* Efficacy of Cutaneous Leishmaniasis Mouse Model

5-2-3-1 By Intralesional Administration

4-week-old female BALB/c mice that were maintained under specific pathogen-free conditions were used to evaluate the *in vivo* efficacy of novel flavonoid monomers on cutaneous leishmaniasis. 1 x 10^7 stationary phase *L. amazonensis* promastigotes (resuspended in 50 µL antibiotic-free DMEM) was inoculated intradermally in the left hind footpad of mice using a 30-gauge needle. The treatment was initiated when the lesion thickness reached approximately 0.5 mm after infection. The mice were first randomized into separate groups (experimental groups, positive control, solvent control and untreated control). Then 50 µL flavonoid monomers (**FM05b** and **FM09h** 2.5 or 10 mg/kg), positive control (SSG 28 mg/kg dissolved in saline), solvent control (5% ethanol, 5% Cremophor EL and 90% saline) or untreated control (saline) was injected intradermally into the lesion once every 4 days (a total of total 8 injections on day 25, 29, 33, 37, 41, 45, 49 and 53). Anesthesia of mice using ketamine/xylazine before injection was needed. After the last injection, the animals were monitored for 4 more days before sacrificed on day 57. The size of the lesion was measured every 4 days before drug administration using a digital caliper. The lesion size was determined by subtraction of the thickness of the left lesion-bearing footpad from that of the right uninfected footpad.

5-2-3-2 By Oral Administration

The same infection scheme was implemented as mentioned above. The treatment was initiated 21 days after infection when the lesion thickness reached approximately 0.5 mm. The mice were randomized into separate groups (experimental groups, positive control and solvent control). Flavonoid monomers (**FM05b** and **FM09h** 50 or 100 mg/kg), positive control (miltefosine 13 mg/kg dissolved in saline [114]), or solvent control (5% ethanol, 5% Cremophor EL and 90% saline) was administered orally by gavage once daily (a total of 12 administrations from day 21 to day 33). After the last administration, the animals were monitored for 2 more days before sacrificed on day 35. The lesion size was measured every 4 days as described in **Section 5-2-3-1**. Anesthesia of mice using ketamine/xylazine before the measurement.

5-2-4 *In Vivo* Efficacy of Visceral Leishmaniasis Mouse Model

5-week-old female BALB/c mice that were maintained under specific pathogen-free condition were used to evaluate the *in vivo* efficacy of novel flavonoid monomers on visceral leishmaniasis. 5 x 10^7 stationary phase *L. donovani* HU3 promastigotes (resuspended in 100 μL antibiotic-free DMEM) was intravenously injected via the tail vein using a 27-gauge needle. The mice were randomized into separate groups (experimental group, positive control and solvent control). After 21 days, **FM05b** 50 mg/kg, positive control (miltefosine 20 mg/kg dissolved in saline) or solvent control (5% ethanol, 5% Cremophor EL and 90% saline) was administered orally by oral gavage once daily (a total of 14 administrations from

day 22 to day 35). After the last administration, the animals were sacrificed on day 36. Liver and spleen were excised from the animals, and their weight were measured. To evaluate the parasite burden in tissue after different treatments, a traditional method that determines Leishman-Donovan Units (LDU) can be used. First, impression smears of the liver and spleen were taken. The smears were fixed in methanol and stained with 10% Giemsa. The number of amastigotes per 1,000 host cell nuclei was enumerated to calculate the Leishman-Donovan units (LDU) using the formula: LDU = number of amastigotes per host cell nucleus x organ weight in mg. Then the reduction in parasitemia was calculated relative to the LDU of the control and expressed as percentage inhibition.

5-2-5 Pharmacokinetic Study of Oral Miltefosine in Plasma, Liver and Spleen

Miltefosine was administered orally at 20 mg/kg to overnight-starved BALB/c mice (N = 3). At different time points (5, 15, 30, 60, 120, 240, 420, 720, 1,080 and 1,440 min), blood was collected via cardiac puncture and placed in a heparinized Eppendorf tube. Blood was centrifuged at 14,000 rpm for 5 minutes to obtain the plasma. 40 µL plasma samples were added with 160 µL acetonitrile for protein precipitation followed by centrifugation at 14,000 rpm for 10 minutes to pellet precipitated proteins. Supernatant was collected and filtered through a 0.22 µm nylon filter for LC-MS/MS analysis. To quantify the level of miltefosine in the liver and spleen, the organs were collected, weighed and homogenized in 2 and 3 volumes of 1X PBS respectively. Extraction of miltefosine from 40 µL of homogenate was performed as in the plasma samples (See **Section 3-1-5**). The

chromatographic and mass spectrometry condition can be found in **Section 3-1-5**. The ion pair of miltefosine for MS detection was 408 to 125 at 30 eV collision voltage.

5-3 Results

5-3-1 *In Vivo* Efficacy of FM05b and FM09h Against Cutaneous Leishmaniasis

5-3-1-1 Antileishmanial Efficacy by Intralesional Injection

FM05b and **FM09h** were tested for their antileishmanial efficacy in a mouse model of cutaneous leishmaniasis infected with *L. amazonensis* LV78 in the footpad of mice. Lesion thickness and weight were used as indicators of disease progression. Intralesional injection of **FM05b** (10 mg/kg, 8 times every 4 days) or **FM09h** (10 mg/kg, 8 times every 4 days) could inhibit lesion growth as efficiently as that of SSG (28 mg/kg, 8 times every 4 days) (**Figure 5-1** & **Figure 5-2**). After 8 injections, **FM05b** and **FM09h** could reduce the lesion thickness by 49 ± 22% (1.43 ± 0.62 mm, $p < 0.001$) and 72 ± 15% (0.78 ± 0.41 mm, $p < 0.001$), respectively, compared with the saline group (2.82 ± 0.58 mm) while SSG could reduce the lesion size by 63 ± 10% (1.04 ± 0.28 mm, $p < 0.001$). **FM05b** and **FM09h** showed a dose-dependent efficacy with a greater inhibition of lesion growth (**Figure 5-1**) and lesion weight (**Figure 5-2**). Throughout the experiment, the body weight of all mice increased steadily and there were no abnormalities in the lesion (**Figure 5-3**), suggesting that **FM05b** and **FM09h** at the indicated dosages did not cause any toxicity to the animals. The footpad lesion after the last treatment clearly showed that there was a significant inhibition of lesion growth after intralesional administration of **FM05b** or **FM09h** at 10 mg/kg (

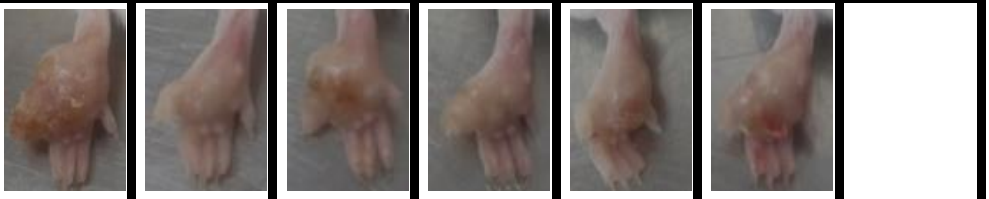
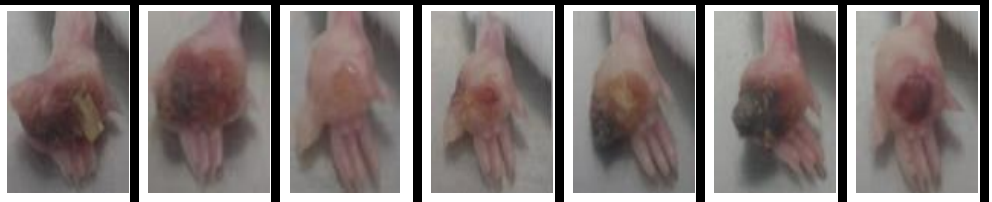
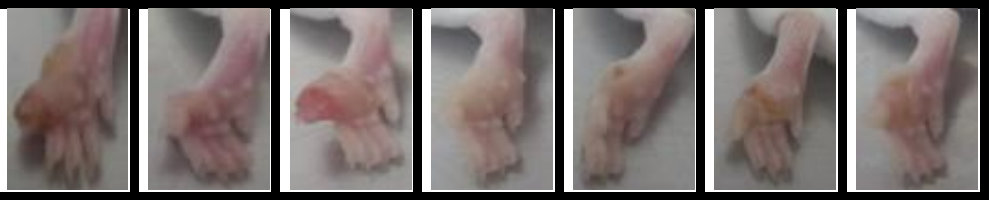
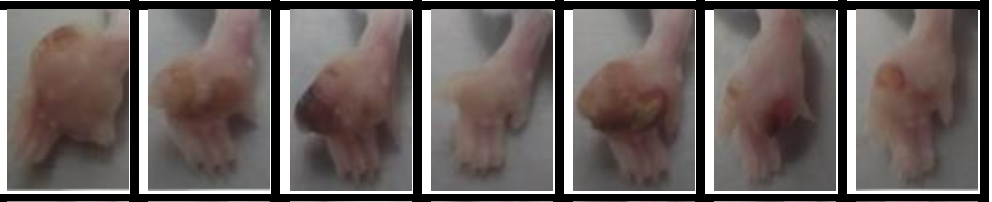
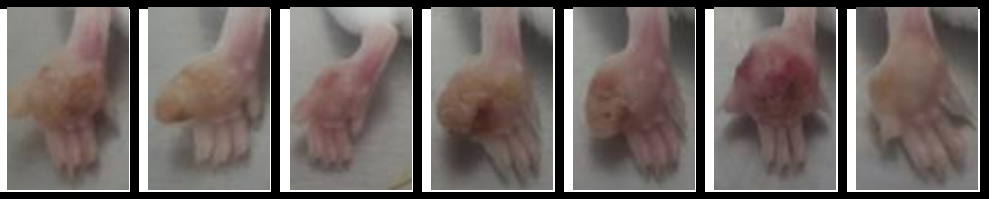
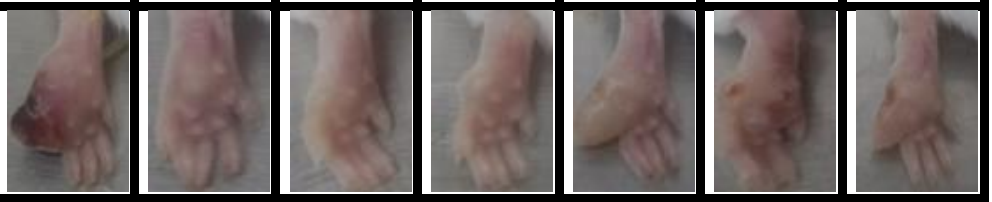
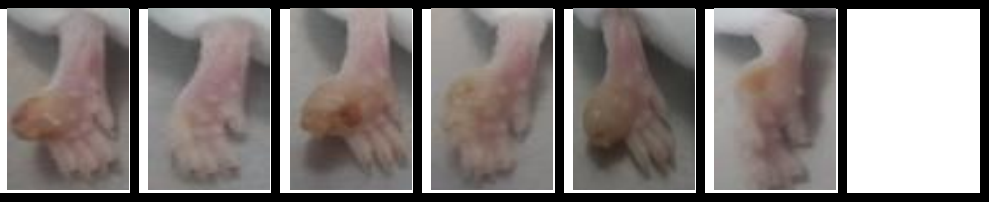
Treatment	Lesion size of the hind footpad of CL-infected mice on day 57
Saline	
Solvent	
SSG 28mg/kg	
FM05b 2.5mg/kg	
FM09h 2.5mg/kg	
FM05b 10mg/kg	
FM09h 10mg/kg	

Figure 5-4).

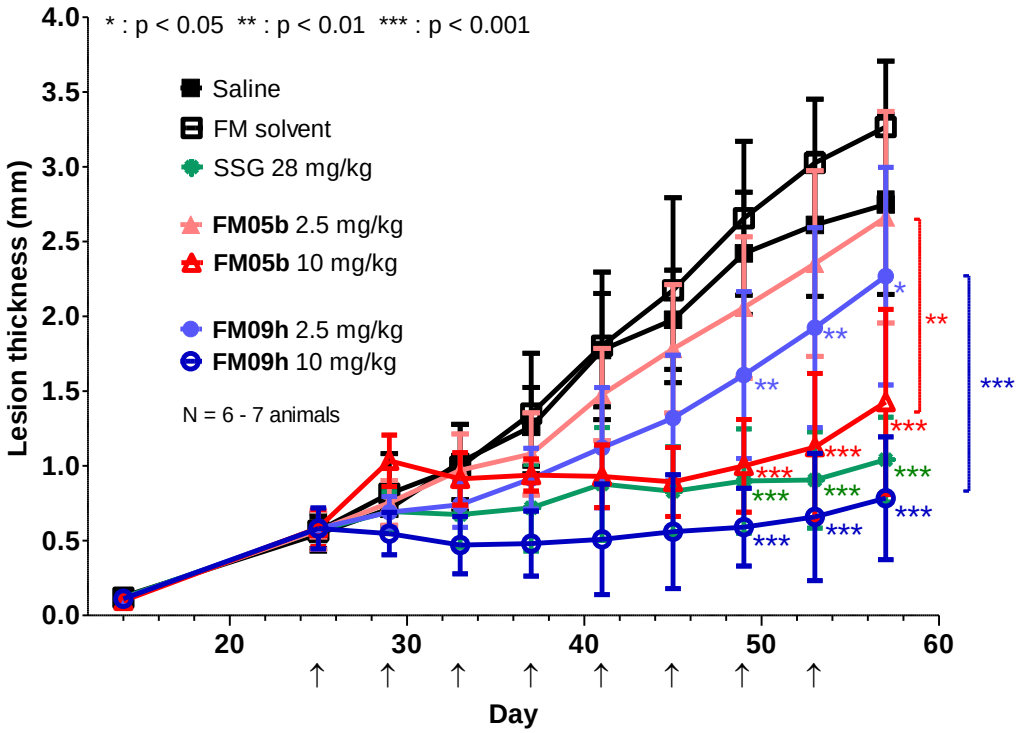


Figure 5-1 *In vivo* antileishmanial efficacy of **FM05b** and **FM09h** in treating cutaneous leishmaniasis by intralesional injection

BALB/c mice were infected with 1 x 10^7 stationary phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. The mice were randomized into different groups including saline control, solvent control, positive control (SSG 28 mg/kg) and treatment groups (**FM05b** or **FM09h** at 2.5 or 10 mg/kg**)**. The lesion thickness was plotted as an indicator of disease progression. Lesion thickness was measured by a digital caliper to measure the footpad thickness. Lesion thickness was expressed as footpad thickness of infected footpad thickness – footpad thickness of uninfected hind footpad. The experimental values were considered significantly different when p-value was < 0.05 as compared to FM solvent control (*: p <0.05; **: p < 0.01; *** = p < 0.001). The arrows (↑) indicated the day of drug administration. The values were presented as mean ± SD. N = 6 – 7 animals.

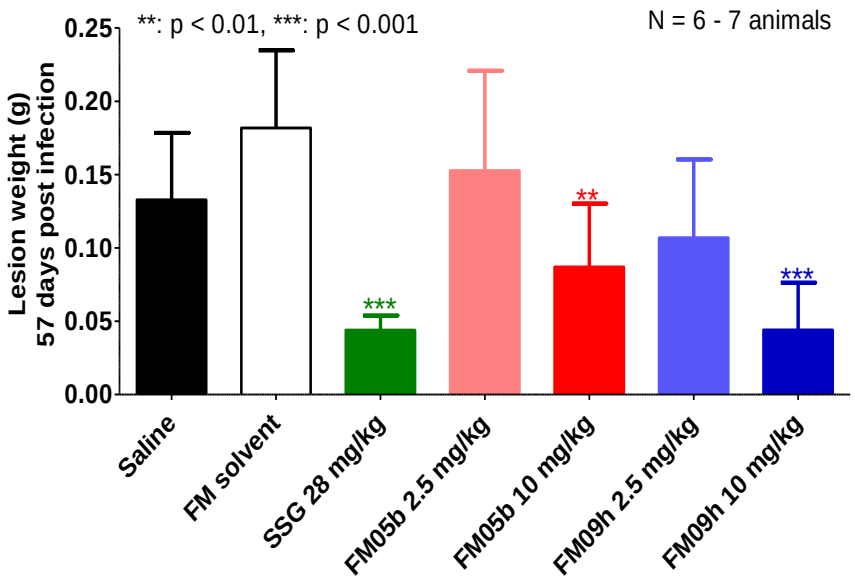


Figure 5-2 Lesion weight of the CL-infected footpad of BALB/c mice treated with **FM05b** and **FM09h** by intralesional injection

BALB/c mice were infected with 1 x 10^7 stationary phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. The mice were randomized into different groups including saline control, solvent control, positive control (SSG 28 mg/kg) and treatment groups (**FM05b** or **FM09h** 2.5 and 10 mg/kg**)**. On day 57, animals were sacrificed, and their lesion weight was measured by subtracting the weight of uninfected footpad from that of infected footpad and expressed in gram. The experimental values were considered significantly different when p-value was < 0.05 as compared to FM solvent control (**: $p < 0.01$; ***: $p < 0.001$). The values were presented as mean ± SD. N = 6 – 7 animals.

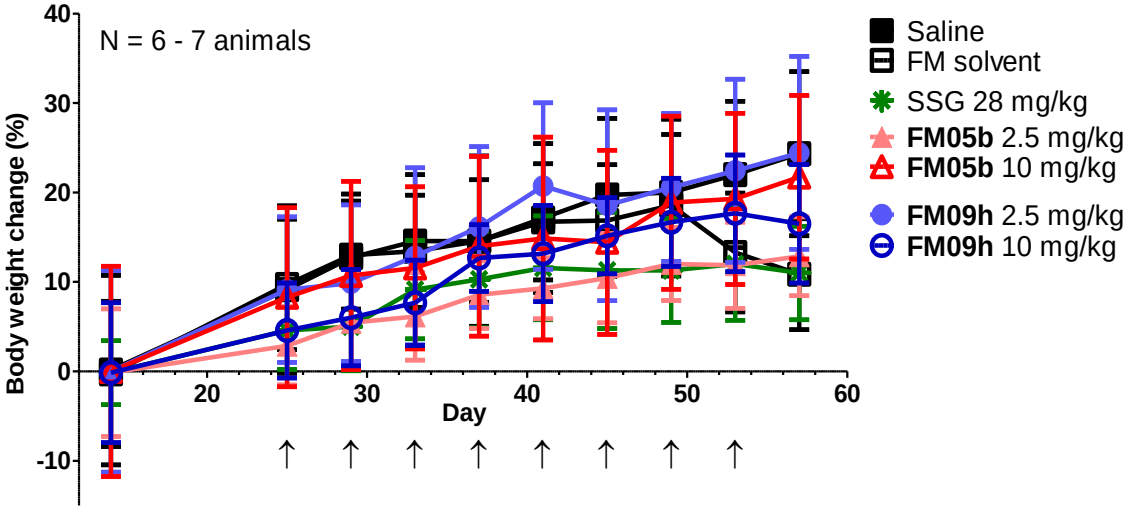


Figure 5-3 Body weight change of CL-infected mice treated with **FM05b** and **FM09h** by intralesional injection

BALB/c mice were infected with 1 x 10^7 stationary phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. The mice were randomized into different groups including saline control, solvent control, positive control (SSG 28 mg/kg) and treatment groups (**FM05b** or **FM09h** 2.5 and 10 mg/kg). The body weight of mice in each group was measured. The values were presented as mean ± SD. Weight loss of more than 15% for 3 consecutive days suggests toxicity. The arrows (↑) indicated the day of drug administration. The values were presented as mean ± SD. N = 6 – 7 animals.

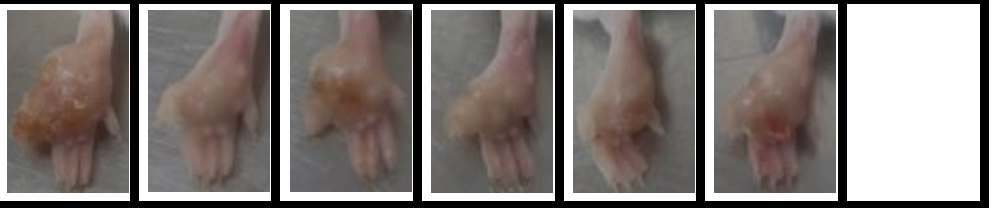
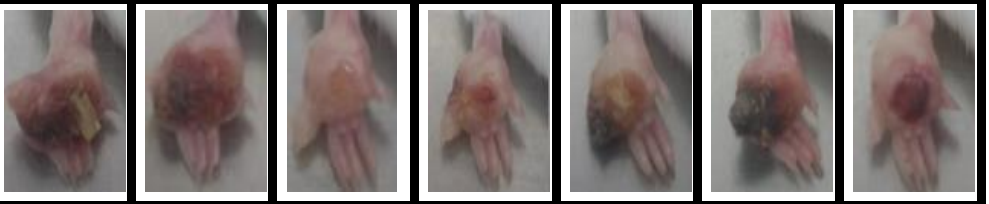
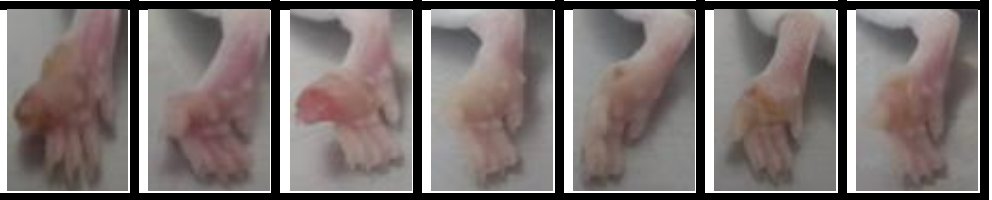
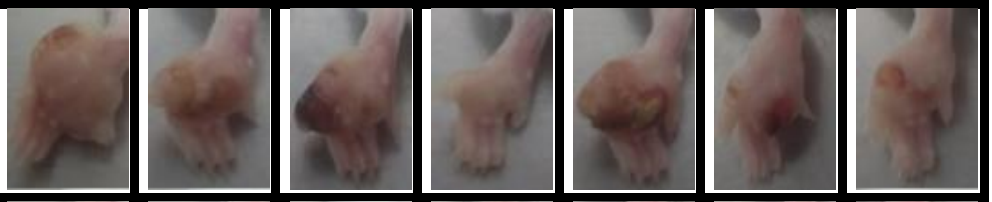
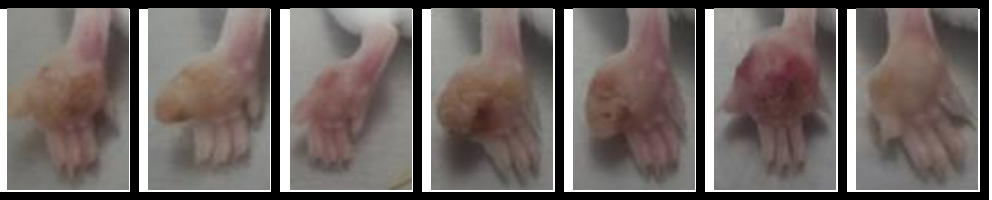
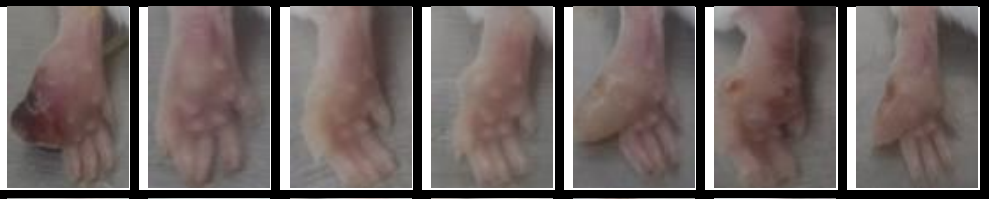
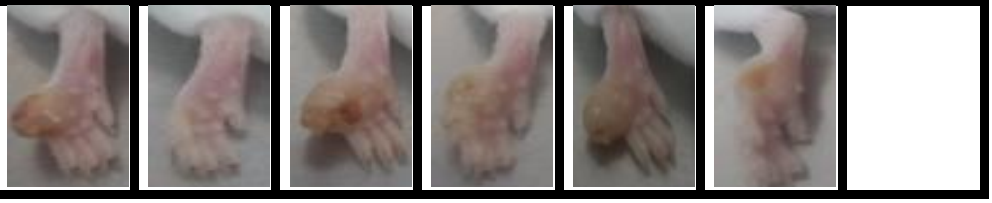
Treatment	Lesion size of the hind footpad of CL-infected mice on day 57
Saline	
Solvent	
SSG 28mg/kg	
FM05b 2.5mg/kg	
FM09h 2.5mg/kg	
FM05b 10mg/kg	
FM09h 10mg/kg	

Figure 5-4 The footpad lesion of BALB/c mice with cutaneous leishmaniasis treated by **FM05b** and **FM09h**

Pictures were taken on the termination day (day 57). In the saline and control group, swellings in the footpads were obvious. When treated with **FM05b** or **FM09h** at 10 mg/kg, cessation of lesion was obvious, and the potency was comparable to the treatment group of SSG 28 mg/kg. **FM05b** and **FM09h** were effective against cutaneous leishmaniasis in a dose-dependent manner. N = 6 – 7 animals.

5-3-1-2 Antileishmanial Efficacy by Oral Administration

After intralesional administration of **FM05b** or **FM09h** was found to be effective in treating cutaneous leishmaniasis, oral administration was also tested. **FM05b** and **FM09h** were administered orally at 50 or 100 mg/kg to CL-infected BALB/c mice for 12 days. This dose regimen was found to be non-toxic to healthy BALB/c mice with no weight loss (**Figure 4-4**). After the treatment, however, **FM05b** and **FM09h** were found to be ineffective at both 50 and 100 mg/kg (**FM05b** 50 mg/kg: 0.92 ± 0.28 mm; **FM09h** 50 mg/kg: 0.83 ± 0.20 mm; **FM05b** 100 mg/kg: 0.75 ± 0.22 mm; **FM09h** 100 mg/kg: 0.92 ± 0.24 mm) (**Figure 5-5** & **Figure 5-6**). The positive control of miltefosine 13 mg/kg (given once daily for 12 days) could efficiently reduce the lesion thickness by 75 ± 3% (0.23 ± 0.03 mm, $p < 0.001$) and lesion weight by 70% (0.0097 g) compared with the solvent control (0.93 ± 0.38 mm, 0.032 g) (**Figure 5-5** & **Figure 5-6**).

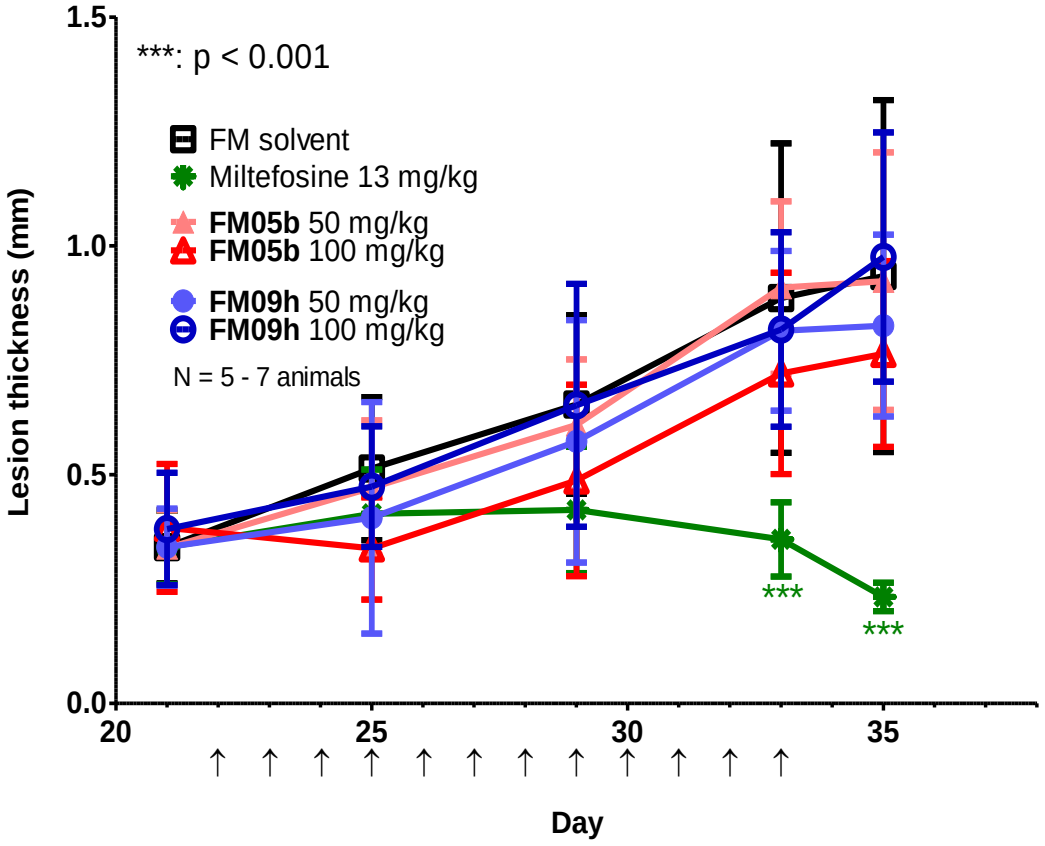


Figure 5-5 *In vivo* antileishmanial efficacy of **FM05b** and **FM09h** in treating cutaneous leishmaniasis by oral administration

BALB/c mice were infected with 1 x 10^7 stationary phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. The mice were randomized into different groups including saline control, positive control (miltefosine 13 mg/kg) and treatment groups (**FM05b** or **FM09h** 50 or 100 mg/kg). The lesion thickness was plotted as an indicator of disease progression. Lesion thickness was measured by a digital caliper to measure the footpad thickness. Lesion thickness was expressed as footpad thickness of infected footpad thickness – footpad thickness of uninfected hind footpad. The experimental values were considered significantly different when p-value was < 0.05 as compared to FM solvent control (***: p < 0.001). The arrows (↑) indicated the day of drug administration. The values were presented as mean ± SD. N = 5 – 7 animals.

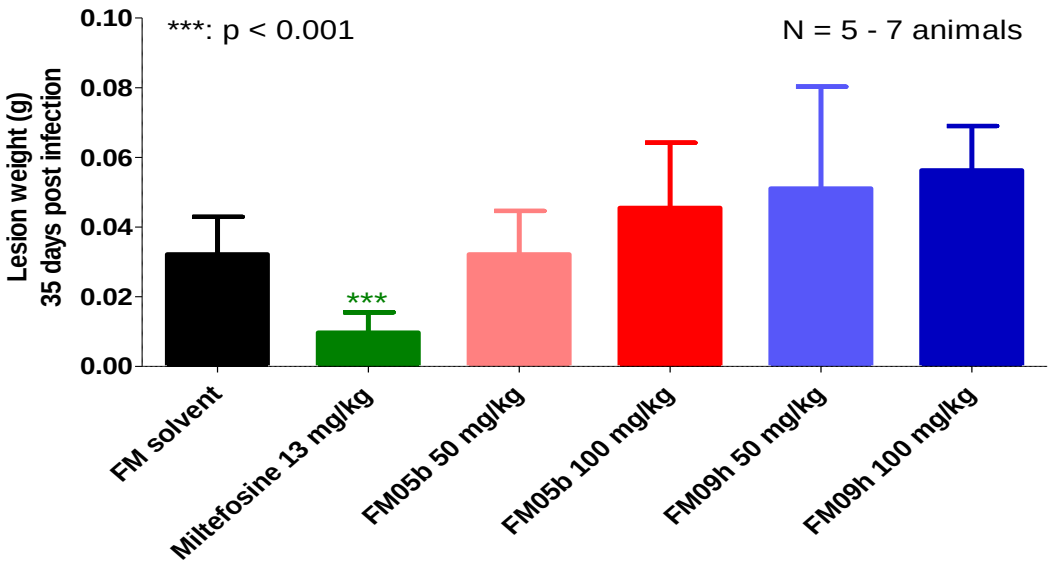


Figure 5-6 Lesion weight of the CL-infected footpad of BALB/c mice treated with **FM05b** and **FM09h** by oral administration

BALB/c mice were infected with 1 x 10^7 stationary phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. The mice were randomized into different groups including saline control, positive control (miltefosine 13 mg/kg) and treatment groups (**FM05b** or **FM09h** 50 or 100 mg/kg). The lesion weight was measured by subtraction of weight of uninfected footpad by that of infected footpad and expressed in gram. The experimental values were considered significantly different when p-value was < 0.05 as compared to solvent control (***: p < 0.001). The values were presented as mean ± SD. N = 5 – 7 animals.

5-3-2 *In Vivo* Efficacy of FM05b against Visceral Leishmaniasis by Oral Administration

In addition to cutaneous leishmaniasis, visceral model was also studied. As the level of **FM05b** in liver was above its IC_{50} 24 hours post oral administration (**Figure 4-3**), a dose regimen of oral administration of **FM05b** 50 mg/kg once daily for 14 days was tested for its efficacy against visceral leishmaniasis. After 14 days of treatment, it was found that **FM05b** was potent in reducing the parasite burden in the liver by 32 ± 18% (LDU = 1,429 ± 376, $p < 0.05$) compared to the solvent control (LDU = 2,102 ± 622) (**Figure 5-7**). The positive control, oral miltefosine (20 mg/kg once daily for 14 days), could reduce the parasite burden by 93 ± 1% (LDU = 149 ± 24, $p < 0.001$) (**Figure 5-7**). The treatment groups of **FM05b** 50 mg/kg and miltefosine 20 mg/kg did not result in any significant weight loss (**Figure 5-9**).

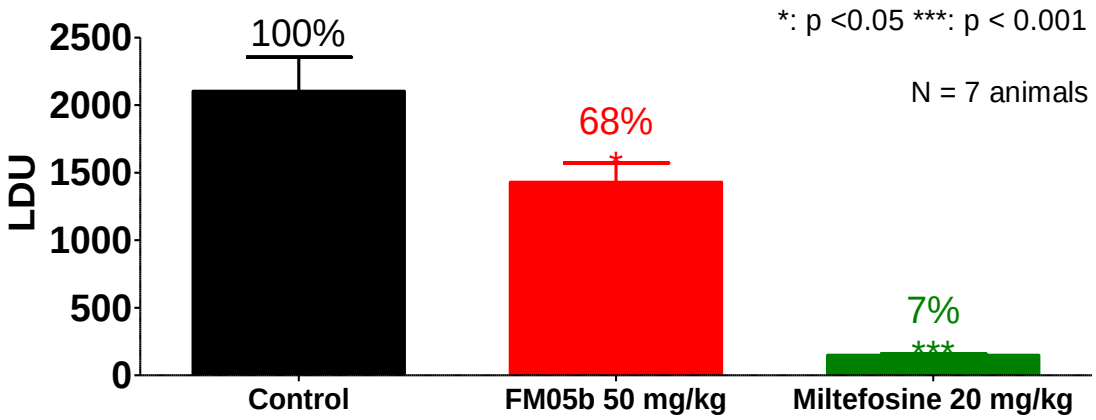


Figure 5-7 Parasite burden in the liver of VL-infected mice treated with **FM05b** by oral administration

BALB/c mice were infected with 5 x 10^7 stationary phase *L. donovani* HU3 promastigotes via intravenous injection. The mice were randomized into different groups including solvent control, positive control (miltefosine 20 mg/kg) and treatment group (**FM05b** 50 mg/kg). The parasite burden in the liver was plotted as an indicator of disease progression. Parasite burden was measured by enumeration of amastigotes on liver smears after treatment. Parasite burden was expressed in Leishman-Donovan Unit (LDU) = number of amastigotes per host cell nuclei x liver weight (mg). The experimental values were considered significantly different when p-value was < 0.05 as compared to control group (*: $p < 0.05$; ***: $p < 0.001$). The values are presented as mean ± SD. N = 7 animals.

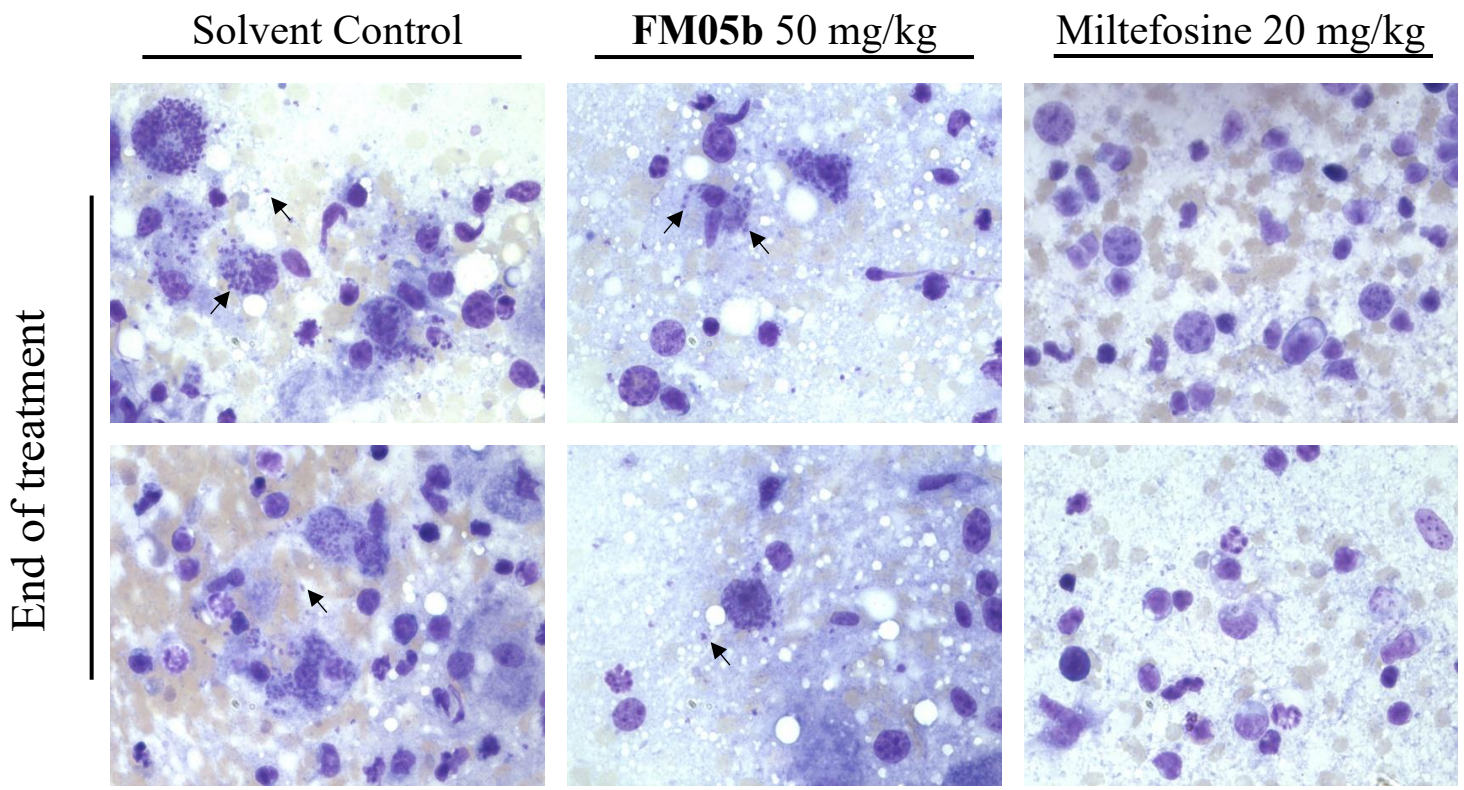


Figure 5-8 The microscopy of liver smears obtained from VL-infected BALB/c mice treated with **FM05b** 50 mg/kg and miltefosine 20 mg/kg

BALB/c mice were infected with 5 x 10^7 stationary phase *L. donovani* HU3 promastigotes via intravenous injection. The mice were administered with **FM05b** 50 mg/kg and miltefosine 20 mg/kg once daily for 14 days. The microscopic slides were prepared by pressing flat cut surface of liver obtained from VL-infected mice onto microscopic slides followed by fixation and Giemsa staining. The arrows indicated the *L. donovani* HU3 amastigotes.

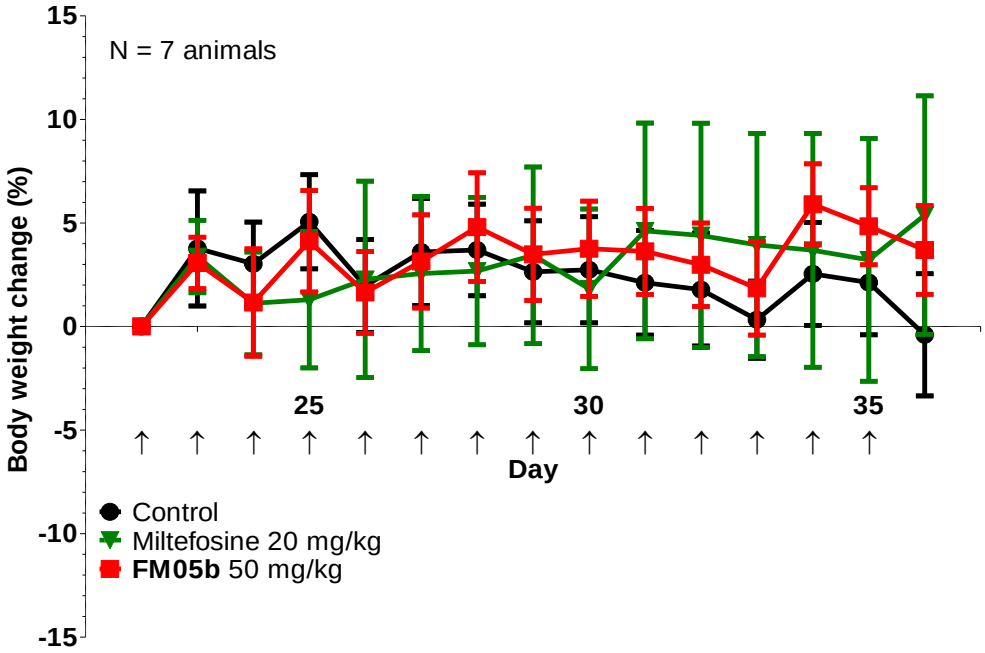


Figure 5-9 Body weight of VL-infected mice treated with **FM05b** by oral administration

BALB/c mice were infected with 5 x 10^7 stationary phase *L. donovani* HU3 promastigotes via intravenous injection. The mice were randomized into different groups including solvent control, positive control miltefosine 20 mg/kg and treatment group **FM05b** 50 mg/kg. The body weight of mice in each group was measured to monitor any toxicity. Weight loss of more than 15% for 3 consecutive days suggests toxicity. The arrows (↑) indicated the day of drug administration. The values were presented as mean ± SD. N = 7 animals.

5-3-3 Pharmacokinetic Study of Oral Miltefosine in Plasma, Liver and Spleen

Oral miltefosine was more effective than **FM05b** in treating both cutaneous leishmaniasis (**FM05b** not effective whereas miltefosine showed 75 ± 3% growth inhibition) and visceral leishmaniasis (**FM05b** showed 32 ± 18% versus miltefosine showed 93 ± 1% growth inhibition). Here, the pharmacokinetic profile of miltefosine was determined and its biodistribution with **FM05b** was compared. The plasma level of miltefosine consistently increased after oral administration and reached C_{max} of 5,663 ng/mL 12 hours post oral administration (**Figure 5-10**). Similarly, the level of miltefosine in liver and spleen also gradually increased and approached plateau level at 18 and 24 hours after oral administration with a C_{max} of 70,592 and 26,046 ng/g, respectively (**Figure 5-10** and **Table 5-1**). Although the C_{max} of **FM05b** and miltefosine in these tissues were comparable, the T_{max} of miltefosine was much longer than that of **FM05b** (T_{max} in plasma, liver and spleen were 15, 30 and 30 minutes, respectively). Unlike **FM05b** that was distributed rapidly to different tissues, miltefosine after oral administration had a prolonged absorption into blood circulation and biodistribution to different tissues including liver and spleen. The $AUC_{(0\text{-}24h)}$ of miltefosine in plasma was 5,587,488 ng-min/mL and the $AUC_{(0\text{-}24h)}$ of miltefosine in liver and spleen were 62,024,692 and 27,351,739 ng-min/g that they are 5-, 5- and 2-fold higher than that of **FM05b** in the tissues, respectively, indicating a long residence of miltefosine inside the body (**Figure 5-10** and **Table 5-1**). As a full distribution phase and elimination phase could not be determined within the 24-hour period, some pharmacokinetic parameter such as $T_{1/2\alpha}$, $T_{1/2\beta}$ and CL were not generated.

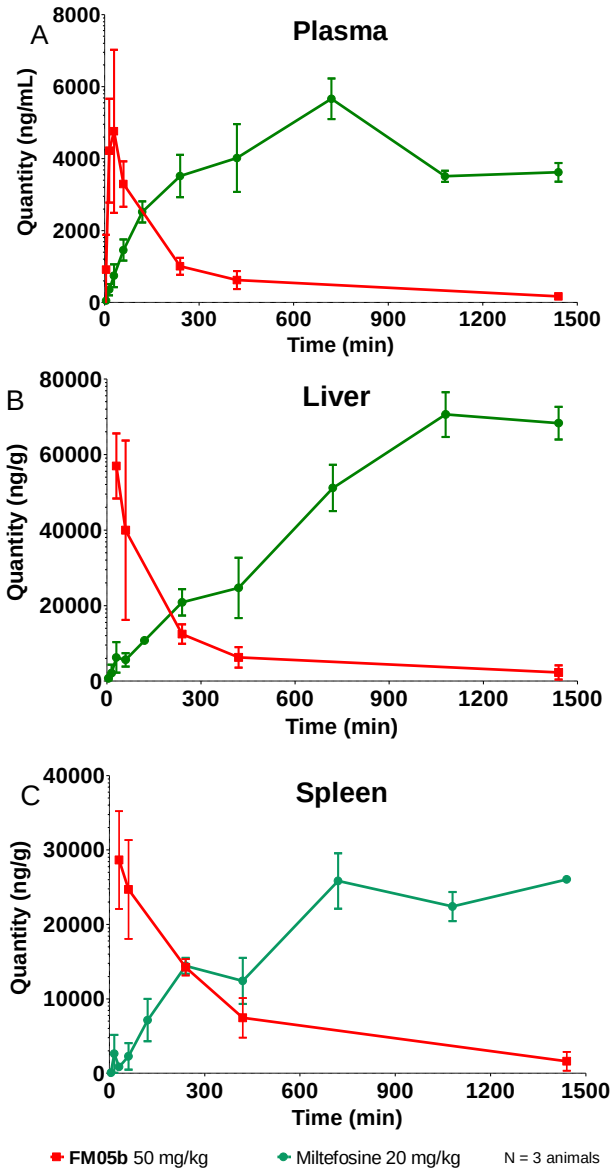


Figure 5-10 The semi-logarithmic plot of miltefosine concentration in plasma, liver and spleen versus time after oral administration at 20 mg/kg in mice

Miltefosine was administered orally to BALB/c mice at 20 mg/kg. At the indicated time points (5, 15, 30, 60, 120, 240, 420, 720, 1,080 and 1,440 min), mice were sacrificed and the level of miltefosine in (A) plasma, (B) liver and (C) spleen was determined using LC-MS/MS. The values were presented as mean ± SD. N = 3 animals.

Parameter	Unit	**FM05b Plasma**	**Miltefosine Plasma**	**FM05b Liver**	**Miltefosine Liver**	**FM05b Spleen**	**Miltefosine Spleen**
Dose	mg/kg	50	20	50	20	50	20
AUC (0-24h)	ng-min/mL or ng-min/g	1 158 236	5 587 488	13 061 108	62 024 692	11 304 240	27 351 739
Cmax (obs)	ng/mL or ng/g	4 757	5 663	56 949	70 592	28 652	26 046
Tmax (obs)	min	15	720	30	1080	30	1 440

AUC: area under curve,C_{max}: maximal concentration, T_{max}: time at which C_{max} is attained, CL: clearance rate. ng-min/mL described the quantity of compounds in plasma while ng-min/g described the quantity of compounds in liver and spleen.

Table 5-1 Pharmacokinetic parameters of **FM05b** and miltefosine in plasma, liver and spleen

Flavonoid monomer **FM05b** 50 mg/kg or miltefosine 20 mg/kg was administered orally to BALB/c mice. Levels of these flavonoid monomers in the plasma, liver and spleen were quantified by LC/MS-MS at different time points for up to 24 hours. Pharmacokinetic parameters were calculated by the pharmacokinetic software-Summit® PK solution

5-4 Discussion

Results from this chapter illustrated that intralesional injection of **FM05b** and **FM09h** was as effective as SSG in reducing lesion size, even when the dosage used was half that of SSG. Therefore, both **FM05b** and **FM09h** were active against *Leishmania* parasites *in vitro* and *in vivo*. When the compounds reached the site of infection, it could effectively cure cutaneous leishmaniasis. Oral administration of flavonoid monomers, however, was ineffective against cutaneous leishmaniasis, even when administering **FM05b** or **FM09h** at 50 mg/kg or 100 mg/kg. To understand why oral administration route failed, the accumulation of **FM05b** and **FM09h** in the footpad was determined 30 and 60 minutes after oral administration. A very little amount of **FM05b** or **FM09h** (< 400 ng/g tissue) was found in the footpad. Low distribution of flavonoid monomers to the localized site of infection (footpad) may be the cause of treatment failure. To address this problem in the future, strategy to improve the drug distribution to the skin and mucosal regions should be focused. Plasma protein binding of drugs is a well-recognized phenomena that controls the free drug concentration in plasma and in compartments in equilibrium, thereby attenuating drug potency *in vivo* [116]. Further studies to increase solubility or membrane permeation of the flavonoid monomers should be done to improve drug distribution so as to improve oral drug efficacy [117]. While improving the drug delivery via oral administration is a feasible approach, topical application of flavonoid monomers onto the lesion site as an alternative route can deliver drugs localized lesion with high cost-effectiveness, fewer adverse effects and low risk of developing complications [118]. Structural changes in the epidermal and dermal layers of the skin of CL-infected mice and how these changes affected the topical use of drugs have been reported. Reduced density of collagen and

epidermal hyperplasia caused by extensive inflammation, ulceration and necrosis have been reported to reduced barrier function. This could promote the penetration of hydrophilic but not hydrophobic drugs [119]. However, currently only paromomycin is available for topical use [120, 121] while the other antileishmanial drugs were hindered by their undesirable physiochemical properties [118]. For example, amphotericin b were shown unable to permeate through the skin because of its high molecular weight and high lipophilicity [119] while miltefosine, due to its amphipathic nature, has a lower thermodynamic activity and low permeation even various formulations were tested [122]. New antileishmanial candidates can offer advantages if they can be administered by topical application. Further studies to measure the permeability of skin to **FM05b** and **FM09h** and to choose suitable formulations are needed to see if they are available for topical use.

In case of visceral leishmaniasis where infection occurs in the bone marrow, liver and spleen, **FM05b** (orally at 50 mg/kg, qd x 14), was effective in reducing the parasite burden in the liver by 32 ± 18% (LDU = 1,429 ± 376, $p < 0.05$) compared to the solvent control (LDU = 2,102 ± 622). On the other hand, miltefosine 20 mg/kg can reduce the parasite burden by 93 ± 1% (LDU = 149 ± 24, $p < 0.001$). The miltefosine pharmacokinetics strongly indicated a more prolonged miltefosine absorption, accumulation and residence in plasma, liver and spleen (**Figure 5-10**) although $T_{1/2\alpha}$, $T_{1/2\beta}$ and CL were not determined. A rat pharmacokinetics of miltefosine published in 2016 determined the $T_{1/2}$ of 102 hours after oral administration at 5 mg/kg, suggesting a long residence and slow clearance [123]. Therefore, repeated dosage together with the superior miltefosine accumulation made it more effective in parasite killing. Although not as active as miltefosine, **FM05b** did not suffer from the unfavorably long residence time of miltefosine

which would otherwise select for drug-resistant parasites. To improve the therapeutic effect, increasing the dosage and frequency of administration may be considered in the future. Besides, a combination with other oral drugs will provide a more convenient and effective way to treat leishmaniasis.

5-5 Conclusion

FM05b and **FM09h** were effective in treating cutaneous leishmaniasis via intralesional injection but not oral administration, suggesting that flavonoid monomers given orally might not reach the site of infection. The use of topical application as an alternative administration route may solve the problem of low distribution to the dermal layers. In visceral leishmaniasis, **FM05b** could suppress the parasite growth by 32 ± 18%. Improving the drug efficacy by increasing the dose regimen may be considered in the future.

6 Concluding Remarks

6-1 Overview of the Project

In the absence of effective vaccine against leishmaniasis, it is widely accepted that the control of leishmaniasis primarily relies on chemotherapy. The therapeutic options available for leishmaniasis are limited (pentavalent antimonials, pentamidine, paromomycin, amphotericin B and miltefosine) and they suffer from shortcomings such as clinical resistance, relapse, high cost and toxicity [17, 26, 124, 125]. Novel therapeutic drugs are still needed.

The use of synthetic flavonoids in killing *Leishmania* promastigote and amastigotes was investigated. Potential antileishmanial candidates were selected from a library of flavonoid monomers based on their *in vitro* antileishmanial activity, toxicity and metabolic stability. Structural modification was carried out to further improve the potency of drug candidates. Finally, two candidate flavonoid monomers (**FM05b** and **FM09h**) were selected for further characterization in terms of *in vivo* pharmacokinetics, toxicity and efficacy. When administered orally at 50 mg/kg, **FM05b** and **FM09h** can achieve 32% and 5% oral bioavailability and maintain the plasma level above their IC_{50} for 7 and 3 hours, respectively (**Figure 4-2** and **Table 4-2**). Besides, **FM05b** can accumulate in the target organs of liver and spleen at a level above its IC_{50} for more than 24 hours. Repeated dosing of **FM05b** or **FM09h** 50 or 100 mg/kg for 10 days resulted in no significant weight loss or animal death. In *in vivo* efficacy experiments, **FM05b** and **FM09h** at 10 mg/kg were effective in treating cutaneous leishmaniasis via intralesional injection, but not oral administration, due to low drug penetration into footpads. For treating VL, orally-

available **FM05b** (50 mg/kg) could inhibit parasite burden in the liver by 32 ± 18%. Though not very efficient, the model suggested the potential of **FM05b** to treat visceral leishmaniasis that worth further investigation in drug development.

6-2 Structural Modification and Metabolic Stability Assessments are Important Steps in Drug Screening

In the first generation flavonoid monomer library, **FM09** stood out from the library because of its high potency and low cytotoxicity *in vitro*. However, pharmacokinetic study showed that **FM09** is a metabolically-unstable compound that is quickly cleared from the body via metabolism, making it an unsuitable drug candidate. Metabolic stability of a compound is one of crucial factors to be considered during drug discovery and development as the liver metabolism will affect drug bioavailability and their effectiveness for the treatment. It should be highly considered in the future for drug screening.

Structural modification may be used to improve physiochemical properties of small molecules. In this project, modification can increase both the potency and metabolic stability of the compounds. It also allows us to better understand the structure-activity relationship (SAR) and structure-metabolism relationship. **FM09h** derived from **FM09** by adding methyl and trifluoroethoxy groups to pyridine rings mainly improved the metabolic stability, resulting in a significant increase in residence time in plasma after both p.o. and i.v. administration (**Table 3-3**, **Figure 4-2** and **Table 4-2**). On the other hand, **FM05b** was derived from **FM05** by changing the nitrogen position from para- to ortho- position and adding two methoxy groups to the pyridine ring, which significantly potentiate the activity by 10-fold.

6-3 Topical Application of FM09h as Alternative methods to Treat Cutaneous Leishmaniasis

Flavonoid monomers **FM05b** and **FM09h** had been demonstrated to be effective in resolving the skin lesion in cutaneous leishmaniasis mouse model via intralesional injection (10 mg/kg, q4d x 8). Despite not effective when administered orally, flavonoid monomers, especially **FM09h** (IC_{50} against *L. amazonensis* LV78 promastigotes and amastigotes = 0.5 and 0.3 µM respectively) may be designed in a topical form. Topical application provides a more convenient and direct way to administer **FM09h** to the infected skin lesions to treat cutaneous leishmaniasis. Besides, **FM09h** did not maintain for a long period in plasma or accumulate inside the tissues. Topical **FM09h** may exhibit its action and be cleared from the body that does not lead to severe toxicity.

6-4 FM05b as a Potential Candidate for Future Investigation in Visceral Leishmaniasis

FM05b represents an important candidate for future antileishmanial drug development. It is highly potent against visceral *Leishmania* (IC_{50} against *L. donovani* HU3 promastigotes and amastigotes = 0.9 and 0.7 µM respectively) *in vitro* (**Table 2-4**). In term of chemical structure, the piperidine ring in **FM05** series (also piperazine ring in **FM01** series) as the linker between the flavonoid moiety and the pyridine ring serves as a rigid and stable structure that can resist liver metabolism. Therefore, **FM05b** is metabolically-stable (in HLM = 89%; in RLM = 83%) after 30-minute incubation with liver microsomes. It can be easily absorbed through oral administration. Upon oral administration at 50 mg/kg, plasma C_{max} can reach 4,757 ng/mL 30 minutes post oral administration with an $AUC_{(0\text{-}7h)}$ of

750,406 ng-min/mL and oral bioavailability (F_{7h}) of 32% (**Table 4-2**). It can also accumulate in the target organs of the liver and spleen, with an $AUC_{(0\text{-}24h)}$ of 13,061,108 and 11,304,240 ng-min/g tissue respectively and remains at a level higher than its *in vitro* IC_{50} by more than 24 hours (**Table 4-3**). When administered orally at 50 mg/kg (qd x 14), it was effective in reducing parasite burden in the liver by 32 ± 18% compared to the control group in VL model (**Figure 5-7**). Although not as active as miltefosine, **FM05b** did not suffer from the unfavorable property of miltefosine such as long residence time, which would otherwise select for drug-resistant parasites. In addition, **FM05b** was effective against miltefosine-resistant *L. donovani in vitro* (**Table 2-5**).

7 References

1. Alvar, J., et al., *Leishmaniasis worldwide and global estimates of its incidence.* PloS one, 2012. **7**(5): p. e35671.
2. Organization, W.H., *Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, Switzerland, 22-26 March 2010.* WHO technical report series, 2010(949).
3. Bern, C., J.H. Maguire, and J. Alvar, *Complexities of assessing the disease burden attributable to leishmaniasis.* PLoS neglected tropical diseases, 2008. **2**(10): p. e313.
4. mondiale de la Santé, O. and W.H. Organization, *Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014.* Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire, 2016. **91**(22): p. 286-296.
5. Farrell, J.P., *Leishmania*. Vol. 4. 2002: Springer Science & Business Media.
6. Akhoundi, M., et al., *A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies.* PLoS neglected tropical diseases, 2016. **10**(3): p. e0004349.
7. Oryan, A., *Plant-derived compounds in treatment of leishmaniasis.* Iranian journal of veterinary research, 2015. **16**(1): p. 1.
8. Dagger, F., et al., *Regulatory volume decrease in Leishmania mexicana: effect of anti-microtubule drugs.* Memórias do Instituto Oswaldo Cruz, 2013. **108**(1): p. 84-90.
9. Gupta, N., N. Goyal, and A.K. Rastogi, *In vitro cultivation and characterization of axenic amastigotes of Leishmania.* Trends in parasitology, 2001. **17**(3): p. 150-153.
10. Carlsen, E.D., et al., *Leishmania amazonensis amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms.* Infection and immunity, 2013. **81**(11): p. 3966-3974.
11. Myler, P.J. and N. Fasel, *Leishmania: after the genome*. 2008: Horizon Scientific Press.
12. Githeko, A.K., et al., *Climate change and vector-borne diseases: a regional analysis.* Bulletin of the World Health Organization, 2000. **78**: p. 1136-1147.
13. Semenza, J.C. and B. Menne, *Climate change and infectious diseases in Europe.* The Lancet infectious diseases, 2009. **9**(6): p. 365-375.
14. Ready, P., *Leishmaniasis emergence in Europe.* Eurosurveillance, 2010. **15**(10): p. 19505.
15. González, C., et al., *Climate change and risk of leishmaniasis in North America: predictions from ecological niche models of vector and reservoir species.* PLoS neglected tropical diseases, 2010. **4**(1): p. e585.
16. Stebut, E., *Leishmaniasis.* JDDG: Journal der Deutschen Dermatologischen Gesellschaft, 2015. **13**(3): p. 191-201.
17. Santos, D.O., et al., *Leishmaniasis treatment—a challenge that remains: a review.* Parasitology research, 2008. **103**(1): p. 1-10.
18. Reithinger, R., et al., *Cutaneous leishmaniasis.* The Lancet infectious diseases, 2007. **7**(9): p. 581-596.
19. Satoskar, A. and R. Durvasula, *Pathogenesis of Leishmaniasis*. 2016: Springer.
20. Murray, H.W., et al., *Advances in leishmaniasis.* The Lancet, 2005. **366**(9496): p. 1561-1577.

21. Purohit, H.M., et al., *Diffuse cutaneous leishmaniasis–A rare cutaneous presentation in an HIV-positive patient.* Indian journal of sexually transmitted diseases, 2012. **33**(1): p. 62.
22. Chappuis, F., et al., *Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?* Nature reviews microbiology, 2007. **5**(11supp): p. S7.
23. Zijlstra, E., et al., *Post-kala-azar dermal leishmaniasis.* The Lancet infectious diseases, 2003. **3**(2): p. 87-98.
24. Jain, K. and N. Jain, *Vaccines for visceral leishmaniasis: A review.* Journal of immunological methods, 2015. **422**: p. 1-12.
25. Miranda-Verastegui, C., et al., *First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiquimod in combination with pentavalent antimony.* PLoS neglected tropical diseases, 2009. **3**(7): p. e491.
26. Croft, S.L., S. Sundar, and A.H. Fairlamb, *Drug resistance in leishmaniasis.* Clinical microbiology reviews, 2006. **19**(1): p. 111-126.
27. Sneader, W., *Drug discovery: a history*. 2005: John Wiley & Sons.
28. Denton, H., J.C. McGREGOR, and G.H. Coombs, *Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1*. Biochemical Journal, 2004. **381**(2): p. 405-412.
29. Zhou, Y., et al., *Leishmania major LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam.* Journal of Biological Chemistry, 2004. **279**(36): p. 37445-37451.
30. Herwaldt, B.L. and J.D. Berman, *Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies.* The American journal of tropical medicine and hygiene, 1992. **46**(3): p. 296-306.
31. Frézard, F., C. Demicheli, and R.R. Ribeiro, *Pentavalent antimonials: new perspectives for old drugs.* Molecules, 2009. **14**(7): p. 2317-2336.
32. Sundar, S., et al., *Clinicoepidemiological study of drug resistance in Indian kala-azar.* Bmj, 1994. **308**(6924): p. 307.
33. Gourbal, B., et al., *Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin.* Journal of Biological Chemistry, 2004.
34. Rijal, S., et al., *Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 2003. **97**(3): p. 350-354.
35. Croft, S.L. and G.H. Coombs, *Leishmaniasis–current chemotherapy and recent advances in the search for novel drugs.* Trends in parasitology, 2003. **19**(11): p. 502-508.
36. Kumar, A., S.C. Pandey, and M. Samant, *Slow pace of antileishmanial drug development.* Parasitology Open, 2018. **4**.
37. Singh, S. and R. Sivakumar, *Challenges and new discoveries in the treatment of leishmaniasis.* Journal of infection and chemotherapy, 2004. **10**(6): p. 307-315.
38. Sundar, S. and M. Rai, *Advances in the treatment of leishmaniasis.* Current opinion in infectious diseases, 2002. **15**(6): p. 593-598.
39. Basselin, M., et al., *Resistance to pentamidine in Leishmania mexicana involves exclusion of the drug from the mitochondrion.* Antimicrobial agents and chemotherapy, 2002. **46**(12): p. 3731-3738.
40. Thakur, C., et al., *Amphotericin B deoxycholate treatment of visceral leishmaniasis with newer modes of administration and precautions: a study of*

938 cases. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1999. **93**(3): p. 319-323.
41. Stone, N.R., et al., *Liposomal amphotericin B (AmBisome®): a review of the pharmacokinetics, pharmacodynamics, clinical experience and future directions.* Drugs, 2016. **76**(4): p. 485-500.
42. Stone, N.R.H., et al., *Liposomal Amphotericin B (AmBisome®): A Review of the Pharmacokinetics, Pharmacodynamics, Clinical Experience and Future Directions.* Drugs, 2016. **76**(4): p. 485-500.
43. Saravolatz, L.D., et al., *Liposomal amphotericin B for the treatment of visceral leishmaniasis.* Clinical Infectious Diseases, 2006. **43**(7): p. 917-924.
44. Sundar, S., et al., *Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations.* Clinical Infectious Diseases, 2004. **38**(3): p. 377-383.
45. Sundar, S., et al., *Single-dose liposomal amphotericin B in the treatment of visceral leishmaniasis in India: a multicenter study.* Clinical infectious diseases, 2003. **37**(6): p. 800-804.
46. Sundar, S., et al., *Single-dose liposomal amphotericin B for visceral leishmaniasis in India.* New England Journal of Medicine, 2010. **362**(6): p. 504-512.
47. Purkait, B., et al., *Mechanism of amphotericin B resistance in clinical isolates of Leishmania donovani.* Antimicrobial agents and chemotherapy, 2012. **56**(2): p. 1031-1041.
48. Wasan, K.M., et al., *Highly effective oral amphotericin B formulation against murine visceral leishmaniasis.* Journal of Infectious Diseases, 2009. **200**(3): p. 357-360.
49. Kip, A.E., et al., *Clinical Pharmacokinetics of Systemically Administered Antileishmanial Drugs.* Clinical pharmacokinetics, 2017: p. 1-26.
50. Wiwanitkit, V., *Interest in paromomycin for the treatment of visceral leishmaniasis (kala-azar).* Therapeutics and clinical risk management, 2012. **8**: p. 323.
51. Davidson, R.N., M. den Boer, and K. Ritmeijer, *Paromomycin.* Transactions of The Royal Society of Tropical Medicine and Hygiene, 2009. **103**(7): p. 653-660.
52. Sundar, S., et al., *Injectable paromomycin for visceral leishmaniasis in India.* New England Journal of Medicine, 2007. **356**(25): p. 2571-2581.
53. Ben Salah, A., et al., *Topical paromomycin with or without gentamicin for cutaneous leishmaniasis.* New England Journal of Medicine, 2013. **368**(6): p. 524-532.
54. Jhingran, A., et al., *Paromomycin: uptake and resistance in Leishmania donovani.* Molecular and biochemical parasitology, 2009. **164**(2): p. 111-117.
55. Fong, D., et al., *Paromomycin resistance in Leishmania tropica: lack of correlation with mutation in the small subunit ribosomal RNA gene.* The American journal of tropical medicine and hygiene, 1994. **51**(6): p. 758-766.
56. Kip, A.E., et al., *Clinical pharmacokinetics of systemically administered antileishmanial drugs.* Clinical pharmacokinetics, 2018: p. 1-26.
57. Dorlo, T.P., et al., *Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis.* Journal of Antimicrobial Chemotherapy, 2012. **67**(11): p. 2576-2597.

58. Paris, C., et al., *Miltefosine induces apoptosis-like death in Leishmania donovani promastigotes.* Antimicrobial agents and chemotherapy, 2004. **48**(3): p. 852-859.
59. ul Bari, A., *Miltefosine: a breakthrough in treatment of leishmaniasis.* Journal of Pakistan Association of Dermatologists, 2006. **16**: p. 225-230.
60. Jha, T., et al., *Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis.* New England Journal of Medicine, 1999. **341**(24): p. 1795-1800.
61. Sundar, S., et al., *Oral miltefosine for Indian visceral leishmaniasis.* New England Journal of Medicine, 2002. **347**(22): p. 1739-1746.
62. Sundar, S., et al., *Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use.* Clinical infectious diseases, 2012. **55**(4): p. 543-550.
63. Dorlo, T.P., et al., *Pharmacokinetics of miltefosine in Old World cutaneous leishmaniasis patients.* Antimicrobial agents and chemotherapy, 2008. **52**(8): p. 2855-2860.
64. Sundar, S. and P.L. Olliaro, *Miltefosine in the treatment of leishmaniasis: clinical evidence for informed clinical risk management.* Therapeutics and clinical risk management, 2007. **3**(5): p. 733.
65. Sen, R. and M. Chatterjee, *Plant derived therapeutics for the treatment of Leishmaniasis.* Phytomedicine, 2011. **18**(12): p. 1056-1069.
66. Di Carlo, G., et al., *Flavonoids: old and new aspects of a class of natural therapeutic drugs.* Life sciences, 1999. **65**(4): p. 337-353.
67. Ayaz, M., et al., *Flavonoids as prospective neuroprotectants and their therapeutic propensity in aging associated neurological disorders.* Frontiers in Aging Neuroscience, 2019. **11**.
68. Onozuka, H., et al., *Nobiletin, a citrus flavonoid, improves memory impairment and Aβ pathology in a transgenic mouse model of Alzheimer's disease.* Journal of Pharmacology and Experimental Therapeutics, 2008. **326**(3): p. 739-744.
69. Ginwala, R., et al., *Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin.* Antioxidants, 2019. **8**(2): p. 35.
70. Kikuchi, H., et al., *Chemopreventive and anticancer activity of flavonoids and its possibility for clinical use by combining with conventional chemotherapeutic agents.* American journal of cancer research, 2019. **9**(8): p. 1517.
71. Cushnie, T.T. and A.J. Lamb, *Antimicrobial activity of flavonoids.* International journal of antimicrobial agents, 2005. **26**(5): p. 343-356.
72. Mittra, B., et al., *Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis.* Molecular Medicine, 2000. **6**(6): p. 527-541.
73. Fonseca-Silva, F., et al., *Effect of Apigenin on Leishmania amazonensis is associated with reactive oxygen species production followed by mitochondrial dysfunction.* Journal of natural products, 2015. **78**(4): p. 880-884.
74. Fonseca-Silva, F., et al., *Oral efficacy of apigenin against cutaneous leishmaniasis: involvement of reactive oxygen species and autophagy as a mechanism of action.* PLoS neglected tropical diseases, 2016. **10**(2): p. e0004442.

75. Fonseca-Silva, F., et al., *Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in Leishmania amazonensis.* PloS one, 2011. **6**(2): p. e14666.
76. Fonseca-Silva, F., et al., *Reactive oxygen species production by quercetin causes the death of Leishmania amazonensis intracellular amastigotes.* Journal of natural products, 2013. **76**(8): p. 1505-1508.
77. da Silva, E.R., C. do Carmo Maquiaveli, and P.P. Magalhães, *The leishmanicidal flavonols quercetin and quercitrin target Leishmania (Leishmania) amazonensis arginase.* Experimental parasitology, 2012. **130**(3): p. 183-188.
78. Chen, J., et al., *Insights into drug discovery from natural products through structural modification.* Fitoterapia, 2015. **103**: p. 231-241.
79. Paterson, I. and E.A. Anderson, *The renaissance of natural products as drug candidates.* Science, 2005. **310**(5747): p. 451-453.
80. Chan, K.-F. and Y. Zhao, *Flavonoid dimer as modulator of drug resistance in cancer.* Progress in Nutrition, 2010. **12**(1): p. 327-330.
81. Chan, K.-F., et al., *Flavonoid dimers as bivalent modulators for P-glycoprotein-based multidrug resistance: synthetic apigenin homodimers linked with defined-length poly (ethylene glycol) spacers increase drug retention and enhance chemosensitivity in resistant cancer cells.* Journal of medicinal chemistry, 2006. **49**(23): p. 6742-6759.
82. Chan, K.F., et al., *Flavonoid Dimers as Bivalent Modulators for P-Glycoprotein-Based Multidrug Resistance: Structure–Activity Relationships.* ChemMedChem, 2009. **4**(4): p. 594-614.
83. Wong, I.L., et al., *Modulation of multidrug resistance protein 1 (MRP1/ABCC1)-mediated multidrug resistance by bivalent apigenin homodimers and their derivatives.* Journal of medicinal chemistry, 2009. **52**(17): p. 5311-5322.
84. Chan, K.-F., et al., *Amine linked flavonoid dimers as modulators for P-glycoprotein-based multidrug resistance: structure–activity relationship and mechanism of modulation.* Journal of medicinal chemistry, 2012. **55**(5): p. 1999-2014.
85. Wong, I.L., et al., *Flavonoid dimers as bivalent modulators for pentamidine and sodium stiboglucanate resistance in Leishmania.* Antimicrobial agents and chemotherapy, 2007. **51**(3): p. 930-940.
86. Wong, I.L., et al., *Quinacrine and a novel apigenin dimer can synergistically increase the pentamidine susceptibility of the protozoan parasite Leishmania.* Journal of antimicrobial chemotherapy, 2009. **63**(6): p. 1179-1190.
87. Wong, I.L., et al., *Flavonoid dimers as novel, potent antileishmanial agents.* Journal of medicinal chemistry, 2012. **55**(20): p. 8891-8902.
88. Wong, I.L., et al., *In vitro and in vivo efficacy of novel flavonoid dimers against cutaneous leishmaniasis.* Antimicrobial agents and chemotherapy, 2014: p. AAC. 02425-13.
89. Wong, I.L., et al., *In vitro and in vivo efficacy of novel flavonoid dimers against cutaneous leishmaniasis.* Antimicrobial agents and chemotherapy, 2014. **58**(6): p. 3379-3388.
90. Suman Gupta, N., *Visceral leishmaniasis: experimental models for drug discovery.* The Indian journal of medical research, 2011. **133**(1): p. 27.

91. De Muylder, G., et al., *A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit.* PLoS neglected tropical diseases, 2011. **5**(7): p. e1253.
92. Gonçalves, R. and D.M. Mosser, *The isolation and characterization of murine macrophages.* Current protocols in immunology, 2015. **111**(1): p. 14.1. 1-14.1. 16.
93. Bhuniya, D., et al., *Aminothiazoles: Hit to lead development to identify antileishmanial agents.* European journal of medicinal chemistry, 2015. **102**: p. 582-593.
94. Thompson, T.N., *Optimization of metabolic stability as a goal of modern drug design.* Medicinal research reviews, 2001. **21**(5): p. 412-449.
95. Baranczewski, P., et al., *Introduction to in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development.* Pharmacological reports, 2006. **58**(4): p. 453.
96. Eddershaw, P.J. and M. Dickins, *Advances in in vitro drug metabolism screening.* Pharmaceutical science & technology today, 1999. **2**(1): p. 13-19.
97. Liu, X. and L. Jia, *The conduct of drug metabolism studies considered good practice (I): analytical systems and in vivo studies.* Current drug metabolism, 2007. **8**(8): p. 815-821.
98. Bhoopathy, S., et al., *A novel incubation direct injection LC/MS/MS technique for in vitro drug metabolism screening studies involving the CYP 2D6 and the CYP 3A4 isozymes.* Journal of pharmaceutical and biomedical analysis, 2005. **37**(4): p. 739-749.
99. Janiszewski, J.S., et al., *A high-capacity LC/MS system for the bioanalysis of samples generated from plate-based metabolic screening.* Analytical chemistry, 2001. **73**(7): p. 1495-1501.
100. Rodrigues, A.D., *Preclinical drug metabolism in the age of high-throughput screening: an industrial perspective.* Pharmaceutical research, 1997. **14**(11): p. 1504-1510.
101. White, R.E., *High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery.* Annual review of pharmacology and toxicology, 2000. **40**(1): p. 133-157.
102. Di, L. and E.H. Kerns, *Drug-like properties: concepts, structure design and methods from ADME to toxicity optimization*. 2015: Academic press.
103. Nassar, A.-E.F., A.M. Kamel, and C. Clarimont, *Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability.* Drug discovery today, 2004. **9**(23): p. 1020-1028.
104. Jia, L. and X. Liu, *The conduct of drug metabolism studies considered good practice (II): in vitro experiments.* Current drug metabolism, 2007. **8**(8): p. 822-829.
105. Irurre Jr, J., J. Casas, and A. Messeguer, *Resistance of the 2, 2, 2-trifluoroethoxy aryl moiety to the cytochrome P-450 metabolism in rat liver microsomes.* Bioorganic & Medicinal Chemistry Letters, 1993. **3**(2): p. 179-182.
106. Tollefson, M.B., et al., *1-(2-(2, 2, 2-Trifluoroethoxy) ethyl-1H-pyrazolo [4, 3-d] pyrimidines as potent phosphodiesterase 5 (PDE5) inhibitors.* Bioorganic & medicinal chemistry letters, 2010. **20**(10): p. 3125-3128.
107. Croft, S.L., K. Seifert, and V. Yardley, *Current scenario of drug development for leishmaniasis.* The Indian journal of medical research, 2006. **123**(3): p. 399-410.

108. Faqi, A.S., *A comprehensive guide to toxicology in preclinical drug development*. 2012: Academic Press.
109. Pandey, B.D., et al., *Relapse of visceral leishmaniasis after miltefosine treatment in a Nepalese patient.* The American journal of tropical medicine and hygiene, 2009. **80**(4): p. 580-582.
110. Rijal, S., et al., *Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance.* Clinical Infectious Diseases, 2013. **56**(11): p. 1530-1538.
111. Godinho, J.L.P., et al., *Efficacy of miltefosine treatment in Leishmania amazonensis-infected BALB/c mice.* International journal of antimicrobial agents, 2012. **39**(4): p. 326-331.
112. Fortin, A., et al., *Efficacy and tolerability of oleylphosphocholine (OlPC) in a laboratory model of visceral leishmaniasis.* Journal of antimicrobial chemotherapy, 2012. **67**(11): p. 2707-2712.
113. Kuhlencord, A., et al., *Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice.* Antimicrobial agents and chemotherapy, 1992. **36**(8): p. 1630-1634.
114. Coelho, A.C., et al., *In vitro and in vivo miltefosine susceptibility of a Leishmania amazonensis isolate from a patient with diffuse cutaneous leishmaniasis.* PLoS neglected tropical diseases, 2014. **8**(7): p. e2999.
115. Nicoletti, S., K. Seifert, and I.H. Gilbert, *Water-soluble polymer–drug conjugates for combination chemotherapy against visceral leishmaniasis.* Bioorganic & medicinal chemistry, 2010. **18**(7): p. 2559-2565.
116. Trainor, G.L., *The importance of plasma protein binding in drug discovery.* Expert opinion on drug discovery, 2007. **2**(1): p. 51-64.
117. Smith, D.A., L. Di, and E.H. Kerns, *The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery.* Nature reviews Drug discovery, 2010. **9**(12): p. 929-939.
118. Garnier, T. and S.L. Croft, *Topical treatment for cutaneous leishmaniasis.* Current opinion in investigational drugs, 2002.
119. Van Bocxlaer, K., et al., *Drug permeation and barrier damage in Leishmania-infected mouse skin.* Journal of Antimicrobial Chemotherapy, 2016. **71**(6): p. 1578-1585.
120. Sosa, N., et al., *Topical paromomycin for New World cutaneous leishmaniasis.* PLoS neglected tropical diseases, 2019. **13**(5).
121. El-On, J., et al., *Topical treatment of cutaneous leishmaniasis.* Journal of investigative dermatology, 1986. **87**(2): p. 284-288.
122. Van Bocxlaer, K., et al., *Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse model.* Journal of Pharmacy and Pharmacology, 2016. **68**(7): p. 862-872.
123. Valicherla, G.R., et al., *Pharmacokinetics and bioavailability assessment of Miltefosine in rats using high performance liquid chromatography tandem mass spectrometry.* Journal of Chromatography B, 2016. **1031**: p. 123-130.
124. Ouellette, M., J. Drummelsmith, and B. Papadopoulou, *Leishmaniasis: drugs in the clinic, resistance and new developments.* Drug Resistance Updates, 2004. **7**(4-5): p. 257-266.
125. Tiuman, T.S., et al., *Recent advances in leishmaniasis treatment.* International Journal of Infectious Diseases, 2011. **15**(8): p. e525-e532.