Chapter-III

Part A

Leishmaniasis-A brief review
1. **Introduction**

Leishmaniasis is a major vector-borne disease caused by obligate intra-macrophage protozoan parasite of the genus *Leishmania*. It infects numerous mammal species, including humans.\(^1\) The parasite was first described as early as 1756, by Alexander Russell. In early 1903, L.H. Donovan and W.B. Leishman independently demonstrated the causative parasite in splenic tissue in autopsies from infected patients in India.\(^2\) Leishmaniasis is transmitted by the bite of *Phlebotomine* sandflies and its species are widespread in all continents except Antarctica.\(^3\) However, leishmaniasis is endemic in 88 countries mostly in the areas of tropics, subtropics, and southern Europe, and causes significant morbidity and mortality in Africa, Asia and Latin America. Currently, leishmaniasis has a wider geographical distribution pattern than before and it is considered to be a growing public health concern for several countries.\(^4\)

According to WHO, 147 million people living in the South-East Asia region are at risk, with an estimated 100,000 cases per year and 15000 reported cases. More than 80% of cases are from India.\(^5\) Approximately, 23,000 cases of visceral leishmaniasis are reported every year in India. The present foci of visceral leishmaniasis in India are Bihar, West Bengal, Uttar Pradesh and Jharkhand. Sporadic cases have also been reported from Gujarat, Tamil Nadu, Kerala and sub-Himalayan parts of north India including Uttar Pradesh, Himachal Pradesh and Jammu Kashmir.\(^6\)

2. **Types of leishmaniasis**

Three main clinical forms of leishmaniasis have been reported in humans. These are cutaneous leishmaniasis (CL), which is a less severe form of the disease with usually self-healing ulcers. Visceral leishmaniasis (VL) is the most severe form of the disease which can result in 100% mortality of infected patients if not treated. A third form, mucocutaneous leishmaniasis (MCL), results in extensive disfiguring lesions of the nose, mouth and throat mucous membranes. The diverse clinical manifestations of the disease result from a reaction between the virulence factors of the parasite species and the host’s immune response.\(^7\)

2.1. **Cutaneous leishmaniasis.**

Cutaneous leishmaniasis (CL) is caused by *Leishmania major*, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis*. Cutaneous leishmaniasis often involves only the skin, and may be characterized by one to dozens of lesions. Depending on the
species of Leishmania, ulcers, smooth nodules, flat plaques or hyperkeratotic wart-like lesions may be seen. The initial lesions usually papule, occur on due to expose to sandflies. Many lesions remain localized, but in some cases, the parasites may spread via the lymphatics and produce secondary lesions on the skin, or occasionally the mucosa of other parts of the body. Human Immuno Deficiency Virus (HIV) infected individuals can have unusually severe infection, and the disease is more difficult to cure.

2.2. Visceral leishmaniasis.

Visceral leishmaniasis (VL) also known as kala-azar and it is most serious form of the disease is caused by L. donovani, L. infantum, and L. chagasi. The most common symptoms of visceral leishmaniasis are prolonged undulant fever, weight loss, decreased appetite, signs of anemia, and abdominal distension with splenomegaly and hepatomegaly. Thrombocytopenia may cause bleeding tendencies, including petechiae or hemorrhages on the mucous membranes, and leukopenia can result in increased susceptibility to other infections. Other symptoms may include coughing, chronic diarrhea, darkening of the skin, lymphadenopathy, and in many cases, signs of chronic kidney disease.

2.3. Mucocutaneous leishmaniasis.

Mucocutaneous leishmaniasis (MCL) usually occurs in Latin America, where it is caused by L. braziliensis and, less often, by L. panamensis or L. guyanensis. Mucocutaneous leishmaniasis tends to occur 1 to 5 years after healing of CL caused by these organisms, but it can also be seen while skin lesions are still present. The initial signs are erythema and ulcerations at the nares, followed by destructive inflammation that can spread to involve the nasal septum, and in some cases, the pharynx or larynx. Frequent nosebleeds can be an early sign. The inflammation may perforate the nasal septum, cause severe disfigurement of the face, or block the pharynx or larynx. In some cases, the genitalia may also be involved.

3. The Leishmania parasite

Leishmania is an intracellular protozoan parasite, belonging to the family Trypanosomatidae (order Kinetoplastida), genus Leishmania. Approximately, 30 species have been described, and at least 20 of these organisms are pathogenic for mammals. The parasites exist in two main morphological forms, the amastigotes and promastigotes, which are found in vertebrate and invertebrate hosts, respectively. The promastigotes are phagocytosed by the host’s macrophages, and subsequently, the parasite
evolves into amastigote form which is spherical intracellular form without flagellum, and reproducing by binary fission. The multiplication of the parasites occurs inside the macrophages, which are their main targets. The macrophage lyses and the cycle continue when other hosts’ phagocytes are being infected.\(^3\)

4. The vector

The protozoa transmitted by the bite of tiny 2 to 3 millimeter long insect vector, the *phlebotomine* sand fly. Among 500 known *phlebotomine* species, only approximately 30 of them have been positively identified as vectors of the disease. Only the female sand fly transmits the protozoa, infecting itself with the *Leishmania* parasites contained in the blood it sucks from its human or mammalian host in order to obtain the protein necessary to develop its eggs. During a period of 4 to 25 days, the parasite continues its development inside the sand fly where it undergoes a major transformation. When female sand fly feeds on a fresh source of blood, its painful sting inoculates its new victim with the parasite, and the transmission cycle is completed.\(^12\)

5. Life cycle of *leishmania*

The life cycle of *Leishmania* involves two forms, the promastigote which develops and lives extracellularly in the sandfly vector and the amastigote which multiplies intracellularly in the reticulo-endothelial cells of the host (Figure 1).\(^13\) The female sandfly picks up amastigote infected macrophages from the skin with blood meal. The amastigotes are released in the midgut of the insect, transform to the promastigotes and start multiplying and differentiating actively through a serious of intermediate stages including, numerous procyclic, nectomonad and heptomonad forms, before emerging as infectious metacyclic promastigote. A single flagellum extends from flgellar pocket at anterior pole and pulls the parasite forwards. When mature, metacyclic promastigotes migrate to the proboscis of sandfly. From proboscis the metacyclic promastigotes are ousted to the new mammalian host. The metacyclic promastigotes enter the skin of the vertebrate host when the infected sandfly takes its next blood meal (Figure 1).\(^14a\)

6. Diagnosis of leishmaniasis

There are several diagnostic methods of leishmaniasis. These are like microscopic examination, animal inoculation, DNA detection method (polymerase chain reaction), immunodiagnosis (ELISA, *Immuno-chromatographic strip test* (rK39), western blotting and intradermal leishmanin test (Montenegro test).\(^14b\) However, because of the diverse
epidemiology of leishmaniasis across countries and regions, the most appropriate diagnostic method to detect infection and disease can vary.

6.1. Microscopic examination.

The demonstration of parasites in relevant tissues such as spleen, bone marrow, lymph nodes, liver or the buffy coat of peripheral blood is commonly used in the diagnosis of VL. Normally the diagnosis is based on finding amastigotes in monocytes or macrophages (seldom are they found free in the tissues except in HIV positive patients). The analysis is done using a special logarithmic scale ranging from 0 (no parasites in the microscope field) to +6 (>100 parasites per field). This technique requires an experienced set of eyes behind the microscope as they must recognize an oval shape of 2 to 3 μm inside a monocyte or a macrophage. The two most commonly used tissues are bone marrow and spleen. The sensitivity of this approach using bone marrow is about 60-85% and in spleen
is more than 95%, making direct detection of parasites one of the most powerful techniques and the spleen one of the more sensitive tissues to detect *Leishmania*.

6.2. Serological test.

Serological tests based on indirect fluorescence antibody (IFA), enzyme-linked immunosorbent assay (ELISA) or western blot have shown high diagnostic accuracy in most studies. Two serological tests have been specifically developed for field use and have been sufficiently validated. The first one is the Direct Agglutination Test (DAT)\textsuperscript{15b} and second is rK39-based immunochromatographic test (ICT).\textsuperscript{15b} The DAT is a semi-quantitative test that uses microtitre plates in which increasing dilutions of patient’s serum or blood are mixed with stained killed *L. donovani* promastigotes. If specific antibodies are present, agglutination is visible after 18 hours with the naked eye. This test has been extensively validated in most endemic areas.\textsuperscript{16} The immunochromatographic test using rK39 antigen (39 amino-acid-repeat recombinant leishmanial antigen from *L. chagasi*) has become popular in recent years. It is a qualitative membrane-based immunoassay with nitrocellulose strips impregnated with recombinant K39 *Leishmania* antigen. A drop of blood or serum is smeared over the pad of the strips and dipped in a small amount of buffer; the results are ready within a few minutes.\textsuperscript{17}

7. Treatment

The focus of this section is to discuss the drugs already in use for the treatment of VL. These include pentavalent antimonials, pentamidine, various formulations of amphotericin B, paromomycin, miltefosine and some others significant molecules (Figure 2).\textsuperscript{18} Some of the same drugs are used for treatment of both CL and MCL. Treatment of VL varies from one endemic region to another. Here, some of most popular antileishmanial drugs are summarized. In general, the current treatment options are inadequate and or cytotoxic and new chemical entities are urgently needed.

7.1. Pentavalent antimonial.

Antimony has been used as a therapeutic agent for a long time. The first use of the trivalent antimonials for the treatment of CL was reported by Vianna and for VL by Di Cristina and Caronia in Sicily and Rogers in India in 1915.\textsuperscript{18-21} Later the drug was found to be highly toxic and showed several side effects like as cough, chest pain, and depression. The key breakthrough in the use of antimony for the treatment of leishmaniasis was achieved in 1925 by Upendranath Brahmachari, who synthesized the pentavalent antimony
compound (urea stibamine) as an effective chemotherapeutic agent against VL. This discovery saved millions of lives in India, especially in Assam state, where many villages were suffered due to VL epidemics. Further progress in antimony therapy of VL was achieved through synthesis of antimony gluconate (Solustibosan) in 1937 and sodium stibogluconate (Pentostam) in 1945.

Currently, there are two formulations of pentavalent antimonials in use: sodium stibogluconate and meglumine antimoniate. Both formulations have poor oral absorption and are given via intramuscular injections or intravenous infusions. There are many side effects associated with use of pentavalent antimonials include prolonged QTc interval, ventricular tachycardia, arthralgia and myalgia, elevated hepatic enzymes and pancreatitis. It also causes more toxicity and mortality in HIV positive patients.

7.2. Pentamidine.

Pentamidine was first introduced in for treatment of sleeping sickness. The first use of pentamidine for VL treatment was reported in India in 1949. Most regimens are based on intramuscular injection or intravenous infusion of 4 mg/kg of pentamidine (isethionate or methanosulfonate) per day and up to 30 days. The prominent side effect is the development of insulin-dependent diabetes mellitus which is irreversible. Other side effects include fever, hypoglycemia, hypotension, myocarditis and renal toxicity.

Pentamidine was used as the second line therapy for treatment of antimony-refractory cases of VL in India. However, due to its toxicity and rapidly emerging pentamidine resistant strains, use in India was abandoned in the 1990s and replaced with amphotericin B deoxycholate as the recommended treatment. More recently, pentamidine was successfully used in several cases of HIV positive patients to prevent VL relapse following the initial treatment with an alternative drug.

Pentamidine is still the first option for treatment of CL caused by *L. guyanensis* and is recommended as the first-line treatment in French Guiana, and in Suriname, where it is the only available antileishmanial drug. The typical treatment consists of a single intramuscular injection of 7 mg/kg of pentamidine isethionate which may be repeated 48 h later in complicated cases. In one study these regimens yielded 78.8 and 83.6% cure rates, respectively.
7.3. Amphotericin B.

Amphotericin B is an antifungal polyene antibiotic isolated from *Streptomyces nodosus* in 1955. In vitro activity of amphotericin B on Leishmania was first time reported in 1960 and the first successful treatment of patients with VL was reported in 1963 in Brazil. The Amphotericin B increases membrane permeability by binding to ergosterol present in the plasma membrane of leishmania. Amphotericin B is used in complex with deoxycholate or various lipids and all formulations are administered by intravenous infusion. The deoxycholate form of the drug has many adverse effects including infusion reactions, nephrotoxicity, hypokalemia, and myocarditis, and needs close monitoring and
hospitalization for 4-5 weeks. Lipid formulations of amphotericin B are efficacious at lower doses and have reduced toxicity, but the high cost complicates treatment of the low income patients.43

In India, amphotericin B was traditionally a second line treatment for VL, but due to decreased efficacy of antimonials and pentamidine led to recommendation for use as a first-line treatment starting in 1990s in Bihar. Amphotericin B deoxycholate has been used with different dosing regimens, with a total dose ranging from 7 to 20 mg/kg, and treatment administered on alternate days or daily for up to 43 days at either constant or incremental dosing. Amphotericin B regimens typically produce high cure rates (close to 100%) for both antimony-sensitive and refractory infections.44 Several lipid formulations of amphotericin B (liposomal-AmBisome, lipid complex-Abelcet, colloidal dispersion-Amphocil, lipid emulsion - Amphomul) have also been tested; all enabling regimens with ~100% cure rates.45 Lipid formulations lead to the rapid concentration of the drug in organs such as liver and spleen.46 This greatly reduces adverse effects including nephrotoxicity and allows delivery of large doses of the drug over short periods of time. In an open label study in Bihar in 2010, a single dose of 10 mg/kg of AmBisome produced 96.3% cure rate.47 The outcome prompted the WHO to recommend this regimen as the first line treatment for VL in South Asia.48

7.4. Paromomycin.

Paromomycin is a broad-spectrum amino-glycoside antibiotic, first isolated in the 1950s from Streptomyces krestomuceticus. Paromomycin inhibits protein synthesis by binding to 16S RNA.49 It was shown to be efficacious for the treatment of CL in 1966 and for VL in 1990 in Kenya.50,51 The most common adverse event with paromomycin is injection site pain. This typically does not lead to the discontinuation of therapy. A small fraction of patients experience reversible ototoxicity (2%) and a rise in hepatic transaminases (6%).52 The cure rate among those previously treated with SbV4 or miltefosine was 98%. The cure rate in pediatric patients was 96% and in females 95%. The main advantage of paromomycin is its affordability and the cost of the treatment is nearly $10 per patient.

7.5. Ketoconazole.

Azoles are oral antifungal drugs that inhibit fungal ergosterol biosynthesis at the lanosterol demethylase step resulting in the accumulation of 14 α-methyl sterols. As Leishmania parasites rely on ergosterol for their sterol needs and share this biosynthetic
pathway with fungi, azoles have been explored for their therapeutic potential against Leishmania infections. For CL, the efficacy of compounds varies depending on species.\textsuperscript{53,54}

Ketoconazole was tested for a month in both adults and children on CL caused by \textit{L. braziliensis} (either 600 mg or 100 mg daily, respectively, for 28 days) and resulted in a 76\% cure with mild side effects.\textsuperscript{55} Similar testing in patients afflicted with CL caused by \textit{L. mexicana} resulted in 89\% cure in another study completed by Navin and colleagues.\textsuperscript{56}

Another ergosterol biosynthesis inhibitor, fluconazole (200 mg daily for 6 weeks), was also previously tested in patients with CL originating from \textit{L. major} and resulted in 59\% cure and shorter healing time for patients residing in Saudi Arabia.\textsuperscript{57}

In the case of itraconazole, minimal response rates were observed in cases of CL resulting from \textit{L. major} and in MCL originating from \textit{L. braziliensis}.\textsuperscript{58} Among the several azole drugs tested (fluconazole, itraconazole, ketoconazole), only ketoconazole was found to be consistently efficacious and is now used for treatment of CL infections caused by \textit{L. mexicana} (600 mg per day for 28 days).

7.6. Miltefosine.

Miltefosine belongs to the class of alkylphosphocholine drugs, which are phosphocholine esters of aliphatic long-chain alcohols. These alkylphosphocholine compounds are structurally related to the group of alkyl-lysophospholipids, which are synthetic analogues of lysophosphatidylcholines or lysolecithins, but lack their glycerol backbone. The chemical name of miltefosine is hexadecyl 2-(trimethylazaniumyl) ethyl phosphate, also known as hexadecylphosphocholine. Miltefosine is an amphiphilic and zwitterionic compound due to the positively charged quaternary amine group and negatively charged phosphoryl group. The crystalline compound is a white to off-white hygroscopic powder.\textsuperscript{59}

7.6.1. Anticancer and antileishmanial activity.

Westphal and Munder first described the \textit{in vitro} and \textit{in vivo} anticancer activity of the new class of substance, alkyl lysophospholipids.\textsuperscript{60} It was Hansjoerg Eibl from the Max-Planck-Institute who led to the discovery of miltefosine as a new type of anticancer agent.\textsuperscript{61} Miltefosine was selected for clinical development for oral use against solid tumours and for the topical treatment of cutaneous metastases in breast cancer patients. After successful clinical development the topical treatment was approved as Miltex in
several countries in Europe.\textsuperscript{62} Pendergast and Chan had synthesized several molecules among them, hexadecylphosphocholine (miltefosine), and one alkyl phosphoethanolamine were reported active at <10 µg/ml against \textit{L. donovani} amastigotes and promastigotes.\textsuperscript{63} Miltefosine had an activity of 5.0 µg/ml in the mouse macrophage model, not the highest activity but the best tolerated by host cells.

\textbf{7.6.2. Mechanism of action.}

The exact mechanism of drug activity against the parasite is not fully understood, some of literature studies have reported that the direct involvement of a putative miltefosine transporter (LdMT), B-subunit LdRos3 and P-type ATPase in miltefosine and phospholipids translocation in \textit{Leishmania}.\textsuperscript{64} On the basis of the other published scientific researches, impairment of lipid metabolism and alteration of the plasma membrane permeability could be involved, resulting in fast drug metabolism and efflux of the drug, in turn trigger parasite apoptosis.\textsuperscript{64,65} Miltefosine also stimulates IFN-gamma-dominated antileishmanial immune response via production of inducible nitric oxide synthetase 2 (iNOS2) of the host cell, and therefore catalyzes the generation of nitric oxide (NO) which kills the parasite within the macrophage.\textsuperscript{66} It has been hypothesized that miltefosine causes programmed cell death in \textit{Leishmania} via mitochondrial dependent pathway through its interaction with membrane constituents, affecting cell signaling pathways that cause modulation of cell surface receptors, inositol metabolism, inhibition of protein kinase C and other mitogenic pathways, and consequently trigger the apoptotic pathway.\textsuperscript{65,67-69}

\textbf{7.6.3. Adverse effects.}

Miltefosine commonly induces mild gastrointestinal side effects such as anorexia, nausea, vomiting, and diarrhea; however, these reactions are typically brief and usually resolve as treatment of VL continues.\textsuperscript{70} Renal insufficiency and increases in levels of hepatic transaminases are much less frequent and reversible once use of the drug is discontinued. Miltefosine is teratogenic and cannot be used in pregnant women. Moreover, adequate contraception should be assured in women of childbearing age during and after treatment as new findings detected subtherapeutic concentrations of miltefosine even 5 months post-treatment.\textsuperscript{71} Miltefosine showed its long half-life (150–200 h), which might encourage the development of resistance\textsuperscript{72} and relapse when used as a monotherapy, especially in incomplete courses. In addition, oral administration and early symptomatic improvement predisposes it for incomplete treatment without strict supervision. Relapse of VL after miltefosine treatment has been reported.\textsuperscript{1,73}
7.6.4. Dosages.

The drug is registered with the Drug Controller General of India. According to National Vector Borne Disease Control Programme (NVBDCP) there are some guidelines regarding the administration of dose. After enrollment oral miltefosine treatment will be administered as per following dosage schedule:

i. Adults (>12 years) weighing more than 25 kg: 100 mg miltefosine daily as one capsule (50 mg) in the morning and one capsule in the evening, after meals for 28 days.

ii. Adults (>12 years) weighing (less than 25kg) 50mg, miltefosine daily as one capsule (50 mg) in the morning, after meals for 28 days.

iii. Children (2-11 years): miltefosine will be given at 2.5 mg/kg daily after meals for 28 days.

iv. The drug is not to be used in the case of children below 2 years of age.
8. References
4. Stockdale1, L.; Newton, R. PLOS Neglected Tropical Diseases. 2013, 7(6), e2278-e2292.
5. apps.who.int/iris/bitstream/10665/185042/1/9789241509497_eng.pdf?ua=1


Chapter-III

Part B

Synthesis and characterization of receptor specific mannose linked miltefosine derivative, and evaluation of their antileishmanial activity
1. Introduction

Leishmaniasis is a vector borne,\textsuperscript{1} neglected and tropical infectious disease caused by unicellular protozoan parasites of the genus Leishmania.\textsuperscript{2} The cutaneous leishmaniasis, mainly caused by \textit{Leishmania major}, can lead to scarring\textsuperscript{3} and disfiguration, whereas, visceral leishmaniasis (VL), mainly caused by \textit{Leishmania donovani} and \textit{L. infantum},\textsuperscript{4} are most fatal\textsuperscript{5} due to failure of the host immune system to counter infection. The disease is endemic in 88 countries\textsuperscript{6} and mostly located in tropical and subtropical regions of the world. Leishmania also locally known as Kala-azar is transmitted by the bite of \textit{Phlebotomine} sandflies.\textsuperscript{7}

Several drugs are recommended for the treatment of leishmaniasis like pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) but they have several drawbacks. These drugs are highly toxic, injectable and painful to administer, have poor oral absorption and require long treatment regimens.\textsuperscript{8} In many cases the patients die due to toxic effects of the drugs. Most alarmingly, resistance strains of Leishmania against these antimonials have been reported in India.\textsuperscript{9} Pentamidine was recommended as the second line therapy for treatment of VL in India. However, due to its toxicity and rapidly emerging resistant Leishmanial strains it’s use in India was abandoned in the 1990s.\textsuperscript{10} After this, some progress was made towards the development of safer, more easily applied therapeutics such as lipid formulations of amphotericin B, orally active miltefosine, and paromomycin.\textsuperscript{2} However, these drugs also showed side effects, require prolonged medication and emergence of resistant strains, are still a problem.\textsuperscript{11}

Since, there is no safe drug available, sodium stibogluconate and meglumine antimonite are still used for the treatment of VL. Miltefosine (Figure 1, 1) have been recently developed as anti-leishmanial drug and is the current drug of choice for the treatment of VL or kala-azar.\textsuperscript{12} These drugs require high dose and are very expensive. Patients often discontinue the treatment once they start feeling better. As a result there are several reports for drug resistance against miltefosine\textsuperscript{13} also.

Therefore, new drugs and other strategies for improved treatment against leishmaniasis are essential. Targeted drug delivery strategy is one such viable option. Till today, there is no report for specific targeted drug delivery except liposomal formulations of amphotericin B (AmBisome, Abelcet) with limited success.\textsuperscript{14} Vaccine against VL is also not available. Therefore, there is a scope for the development of new drugs with appropriate target specificity.
Mannose binding protein (MBP) receptors are present in macrophages, spleen and liver cells, where the parasite (*Leishmania donovani*) colonize. Therefore, MBP could be a valuable target for effective anti-leishmanial drug delivery. Keeping all these in mind, we envisaged synthesis of the target specific miltefosine derivatives such as α-D-manno-miltefosine derivative (Figure 1, 2). This miltefosine derivative is expected to selectively bind with MBP on macrophages of various organs such as spleen, liver etc. where the parasites anchors and multiplies.\(^{15}\)

![Figure 1](image)

**Figure 1.** Orally active antileishmanial drug, miltefosine and its derivative

Therefore, we decided to synthesize mannose linked miltefosine derivative. Since, the active site of miltefosine is the region of phosphocholine group, we decided to link the mannose at the terminal alkyl side chain (Figure 1, 2) so that the active site remains unaltered. Also, since, miltefosine is administered orally; the O-linked sugar is expected to be prone to enzymatic cleavage in the stomach. Therefore, we synthesized S-glycosylated miltefosine derivative which is likely to be more stable when administered orally. It is envisaged that the drug will first selectively bind with the MBP and then internalized. Selective drug targeting will not only require very small quantity of drug but also reduce side effects, cost, and chances of developing drug resistance.

2. **Experimental procedure**

2.1. **General Information.**

All solvents used in this study were distilled and/or dried as required before use. Evaporations were done below 40 °C under reduced pressure in rotary evaporator. Analytical thin layer chromatography was performed on silica gel 60 F\(_{254}\) coated on aluminium plates. The spots were visualized by charring with 10% (v/v) H\(_2\)SO\(_4\) in EtOH or detected using UV light. Column chromatography and flash column chromatography were performed on 60-120 and 230-400 mesh silica gel respectively. \(^1\)H and \(^{13}\)C NMR spectra were recorded in Bruker DPX 300/600 MHz and Bruker DPX 75/150 MHz spectrometer respectively using tetramethylsilane (δ=0.00) as internal standard at 25 °C. ESI-MS (positive) was conducted using LC-ESI-Q-TOF micro Mass spectrometer.

Hexadecanedioic acid 3 (572 mg, 2.0 mmol) was first stirred with NaOH (80 mg, 2.0 mmol) in water (10 mL) for 5 h at r.t. Water was removed under vacuum. Traces of water were removed by azotropic co-distillation with toluene (5 mL x 2) until dry. To this sample dry toluene (50 mL) was added and mixed with tetrabutyl ammonium bromide (64 mg, 0.2 mmol) and benzyl bromide (0.236 mL, 2.0 mmol). The mixture was then stirred at reflux temperature for 15 h. TLC (ethyl acetate:petroleum ether::1:5) indicated the formation of a product ($R_f = 0.55$) with complete consumption of the starting material. It was evaporated to dryness and partitioned between brine and dichloromethane (25 mL x 3). The combined organic layer was once washed with brine. The solvent was evaporated to dryness and the residue was purified by column chromatography on silica gel.

Yield: 62 %; white fluffy solid; $^1$H NMR (CDCl$_3$ 300 MHz): δ 7.37- 7.34 (5H, m, Ar), 5.11 (2H, s, benzyl proton), 2.37-2.32 (4H, m), 1.66-1.58 (4H, m), 1.36-1.24 (20H, m); $^{13}$C NMR (CDCl$_3$, 75MHz): δ 180.03 (C_OOH), 173.71 (CO$_2$Bn), 136.01, 128.44, 128.06, 65.99 (CH$_2$Ph), 34.25, 33.98, 29.51, 29.34, 29.14, 29.03, 24.86, 24.58. HRMS m/z: calcd for C$_{23}$H$_{36}$O$_4$Na [M+Na]$^+$: 399.2511, found 399.2489.

2.1.2. Synthesis of benzyl 16-hydroxyhexadecanoate, compound (5).

To a stirred solution of compound 4 (200 mg, 0.5 mmol) in dry THF (3 mL) at 0 °C a 2 M solution of borane-methyl sulphide complex in THF (220 µL, 8 equiv.) was added drop wise for 30 min and the mixture was stirred at r.t. under nitrogen atmosphere. After 15 h TLC (ethyl acetate:petroleum ether::1:5) indicated formation of a product ($R_f = 0.55$) with complete consumption of the starting material. The reaction mixture then quenched by adding methanol (1 mL) drop wise and stirring was continued for an additional 1 h. The solvent was then removed in a rotary evaporator. The residue was dissolved in dichloromethane (10 mL), brine (15 mL). The organic layer was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. The residue was then purified by column chromatography on silica gel.

Yield: 77 %; white amorphous solid; $^1$H NMR (CDCl$_3$ 300 MHz): δ 7.37- 7.34 (5H, m, Ar), 5.12 (2H, s, benzyl proton), 3.67-3.62 (2H, m, CH$_2$OH), 2.38-2.33 (2H, m), 1.67-1.55 (6H, m), 1.26 (20H, s); $^{13}$C NMR (CDCl$_3$, 75MHz): δ 173.75 (CO$_2$Bn), 136.08, 128.51, 128.13, 66.06 (CH$_2$Ph), 62.89 (CH$_2$OH), 34.31, 29.62, 29.57, 29.45, 29.35, 29.11, 25.76, 24.94. HRMS m/z: calcd for C$_{23}$H$_{38}$O$_3$Na [M+Na]$^+$: 385.2719, found 385.2744.
2.1.3. Synthesis of benzyl 16-(tosyloxy)hexadecanoate (6).

To the compound 5 (100 mg, 0.27 mmol) in dichloromethane (3 mL) at 0 °C pyridine (26 µL, 0.3 mmol) was added. To this, a solution of tosyl chloride (63 mg, 0.3 mmol) in DCM (1 mL) was added dropwise. The reaction mixture was stirred first at 0 °C for 30 min. and then at r.t. After 5 h, TLC was performed (ethyl acetate:petroleum ether::1:10) which indicated the formation of a product ($R_f = 0.5$) with complete consumption of the starting material. Water (5 mL) was added to the reaction mixture, and the organic phase was washed with saturated solution of sodium bicarbonate (5 mLx 2), followed by brine. The organic layer was collected and dried over anhydrous sodium sulfate. Solvent was evaporated under vacuum and purified by column chromatography on silica gel.

Yield: 71 %; glass; $^1$H NMR (CDCl$_3$ 300 MHz): δ 7.79 (2H, d, J= 8.4 Hz, Ar), 7.35-7.33 (7H, m, Ar), 5.11 (2H, s, benzyl proton), 4.02-3.99 (2H, m, $CH_2$OTs), 2.45 (3H, s, PhCH$_3$), 2.35 (2H, m), 1.64-1.60 (4H, m), 1.57 (8H, m), 1.27-1.21 (14H, m); $^{13}$C NMR (CDCl$_3$, 75MHz): δ 173.58 ($CO_2$Bn), 144.52, 136.04, 133.11, 129.70, 128.43, 128.05, 127.78, 70.61 ($CH_2$OTs), 65.94 ($CH_2$Ph), 34.22, 29.52, 29.40, 29.15, 28.47, 28.82, 28.70, 25.21, 21.53. HRMS m/z: calcd for C$_{30}$H$_{44}$NaO$_5$ [M+Na]$^+$: 539.2807, found 539.2797.

2.1.4. Synthesis of benzyl 16-iodohexadecanoate (7).

Compound 6 (80 mg, 0.15 mmol) was stirred with sodium iodide (27 mg, 0.18 mmol) in dry dimethyl formamide (5 mL) at 80-100 °C for 3 h. After that TLC (ethyl acetate:petroleum ether::1:10) indicated formation of a product ($R_f = 0.6$) with complete consumption of the starting material. The solvent was removed under vacuum and the residue was dissolved in ethyl acetate (60 mL), washed with brine (2 x10 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was further purified by column chromatography to produce yellowish viscous liquid.

Yield: 79 %; $^1$H NMR (CDCl$_3$ 300 MHz): δ 7.39-7.33 (4H, m, Ar), 5.13 (2H, s, benzyl proton), 3.23-3.18 (2H, m, $CH_2$I), 2.39-2.35 (2H, m), 1.86-1.81 (2H, m), 1.66-1.57 (8H, m), 1.40-1.27 (16H, m); $^{13}$C NMR (CDCl$_3$, 75MHz): δ 173.55 ($CO_2$Bn), 136.05, 128.44, 128.06, 65.95 ($CH_2$Ph), 34.24, 33.49, 29.54, 29.50, 29.35, 29.17, 24.87, 7.24. HRMS m/z: calcd for C$_{23}$H$_{37}$INaO$_2$ [M+Na]$^+$: 495.1736, found 495.1722.

2.1.5. Synthesis of benzyl 16-(diethoxyphosphoryl)hexadecanoate (8).

A mixture of compound 7 (90 mg, 0.19 mmol) and triethyl phosphite (2 mL) in dry DMF (5 mL) was refluxed at 150-160 °C under nitrogen. After 5 h, TLC was performed (ethyl acetate:petroleum ether:: 1:2.5) which indicated the formation of a product ($R_f = 0.5$)
with complete consumption of the starting material. Excess triethyl phosphite was removed under reduced pressure and the residue was dried in vacuum. The residue was then purified by column chromatography on silica gel to get pure 8.

Yield: 74%; yellowish viscous liquid; \(^1\)H NMR (CDCl\(_3\) 300 MHz): \(\delta\) 7.37-7.34 (5H, m, Ar), 5.11 (2H, s, benzyl proton), 4.12-4.06 (4H, m), 2.38-2.33 (2H, m), 1.76-1.62 (8H, m), 1.37-1.24 (26H, m); \(^{13}\)C NMR (CDCl\(_3\), 75MHz): \(\delta\) 173.55 (CO\(_2\)Bn), 135.98, 128.38, 128.01, 65.89 (CH\(_2\)Ph), 63.53, 63.45, 61.28 (POCH\(_2\)CH\(_3\)), 61.19 (POCH\(_3\)CH\(_3\)), 34.17, 30.58, 29.48, 29.43, 29.24, 29.10, 28.97, 24.80, 16.29, 16.04. HRMS m/z: calcd for C\(_{27}\)H\(_{47}\)NaO\(_5\)P [M+Na]\(^+\): 505.3059, found 505.3066.

2.1.6. Synthesis of 16-(diethoxyphosphoryl)hexadecanoic acid (9).

Compound 8 (90 mg, 0.18 mmol) was stirred with aqueous methanolic (5 mL, 50%) sodium hydroxide (9 mg, 0.22 mmol) solution at r.t. After 16 h, TLC was performed using ethyl acetate:petroleum ether (3:2) which indicated formation of a product with complete consumption of the starting material. It was neutralized (pH 5.5) with dilute HCl and solvent was removed in a rotary evaporator. The residue that was then dissolved in ethyl acetate (30 mL), washed with brine (10 mL x 2), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was finally purified by column chromatography on silica gel to get pure compound 9.

Yield: 92%; white solid; \(^1\)H NMR (CDCl\(_3\) 300 MHz): \(\delta\) 4.13-4.07 (4H, m), 2.36-2.34 (2H, m), 1.74-1.59 (14H, m), 1.35-1.22 (20H, m); \(^{13}\)C NMR (CDCl\(_3\), 75MHz): \(\delta\) 176.21 (CO\(_2\)H), 61.15 (POCH\(_2\)CH\(_3\)), 61.12 (POCH\(_3\)CH\(_3\)), 33.28, 30.16, 30.05, 29.26, 28.87, 28.75, 28.68, 28.66, 28.59, 28.56, 28.46, 28.42, 25.59, 24.66, 24.28, 23.00, 21.84, 21.80, 16.04, 16.00. HRMS m/z: calcd for C\(_{20}\)H\(_{42}\)O\(_5\)P [M+H]\(^+\): 393.2770, found 393.2782.

2.1.7. Synthesis of diethyl 16-hydroxyhexadecylphosphonate (16).

To a stirred solution of compound 9 (100 mg, 0.5 mmol) in dry THF (3 mL) at 0°C, a 2 M solution of borane-methyl sulfide complex in THF (190 µL, 8 equiv.) was added drop-wise for 30 min and the mixture was stirred at r.t. under nitrogen atmosphere. After 15 h TLC was performed with ethyl acetate/petroleum ether (4:1) which indicated formation of a product \((R_f = 0.5)\) with complete consumption of the starting material. The reaction mixture then quenched by adding methanol (1 mL) drop-wise and stirring was continued for an additional 1 h. The solvent was then removed in rotary evaporator. The residue was dissolved in dichloromethane (10 mL), washed with brine (10 mL x 2) and finally dried
over anhydrous sodium sulphate, filtered, and evaporated to dryness. The residue was purified by silica gel column chromatography (ethyl acetate-petroleum ether) on silica gel.

Yield: 72 %; yellowish viscous liquid; $^1$H NMR (CDCl$_3$ 300 MHz): $\delta$ 4.12-4.06 (4H, m), 3.70 (1H, s, OH), 3.66-3.62 (2H, m, CH$_2$OH), 1.69-1.57 (18H, m), 1.34-1.25 (18H, m); $^{13}$C NMR (CDCl$_3$, 75MHz): $\delta$ 63.01 (CH$_2$OH), 61.49 (POC$_2$H$_3$), 61.41 (POC$_2$H$_3$), 32.81, 30.73, 29.62, 29.44, 29.09, 26.57, 25.76, 16.51, 16.43. HRMS m/z: calcd for C$_{20}$H$_{43}$NaO$_4$P [M+Na]$^+$: 401.2797, found 401.2768.

2.1.8. Synthesis of 16-(diethoxyphosphoryl)hexadecyl 4-methylbenzenesulfonate (17).

Compound 16 (80 mg, 0.21 mmol) and pyridine (21 µL, 0.26 mmol) was added to dichloromethane (3 mL) at 0 °C. A solution of tosyl chloride (49 mg, 0.26 mmol) in DCM (1 mL) was then added to it drop-wise. The reaction mixture was stirred at 0 °C for 30 min then at r.t. After 5 h, TLC (ethyl acetate:pentane ether:: 2:3) showed the formation of a product ($R_f$ = 0.45) with complete consumption of the starting material. Water (5 mL) was added and then organic phase was washed with saturated solution of sodium bicarbonate (5 mL x 2), followed by brine. The organic layer was collected and dried over anhydrous sodium sulphate and evaporated to dryness. The residue was purified by silica gel column chromatography (ethyl acetate-petroleum ether) to yield yellowish viscous compound 17.

Yield: 76 %; $^1$H NMR (CDCl$_3$ 300 MHz): $\delta$ 7.92 (2H, d, $J$ = 8.4 Hz), 7.34 (2H, d, $J$ = 8.1 Hz), 4.14-3.99 (6H, m), 2.45 (3H, s, tosyl CH$_3$), 1.77-1.59 (18H, m), 1.34-1.22 (18H, m); $^{13}$C NMR (CDCl$_3$, 75MHz): $\delta$ 129.77, 129.64, 127.86, 125.20, 64.65, (POC$_2$H$_3$), 61.32 (POC$_2$H$_3$), 30.71, 29.61, 29.57, 29.37, 29.23, 29.08, 28.57, 25.88, 22.40, 16.49, 16.41. HRMS m/z: calcd for C$_{27}$H$_{49}$NaO$_6$PS [M+Na]$^+$: 555.2885, found 555.2888.

2.1.9. Synthesis of 1,2,3,4,6-penta-O-acetyl-α-D-mannopyranose (20).

To a stirred solution of d-mannose (2 g, 11.1 mmol, 1 equiv.) in pyridine ( mL), acetic anhydride (6 mL) was added slowly and the reaction mixture was stirred at r.t. temperature for 24 h under nitrogen atmosphere. TLC (ethyl acetate:pentane ether (60-80)::1:1) indicated the formation of a product ($R_f$=0.6). The solvent was removed in a rotary evaporator. Traces of pyridine and acetic anhydride were removed by co-evaporation with dry toluene (2 mL x 3). The residue was then dissolved in ethyl acetate (10 mL), washed with water (10 mL x2), dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. The yellowish viscous product was used as it is for the next reaction.
2.1.10. Synthesis of 2,3,4,6-tetra-O-acetyl-1-S-acetyl-1-thio-α-D-mannopyranoside (18).

To a solution of 1,2,3,4,6-penta-O-acetyl-α-D-mannoopyranose (0.21 g, 2.7 mmol, 1 equiv.) in DCM at 0 °C under an atmosphere of nitrogen was added thiolacetic acid (165 mg/152 µL, 0.5 mmol, 1.1 equiv.). To this BF₃·Et₂O (465mg/404 µL) was added drop wise and reaction mixture were stirred at r.t. for overnight. TLC (ethyl acetate:petrol::1:1) indicated the formation of product with almost complete consumption of starting material. The reaction mixture was neutralized with aqueous sodium bicarbonate and partitioned between DCM (50 mL) and H₂O (50 mL) and the organic phase was re-extracted with H₂O (3 x 50 mL). The combined aqueous layers were once extracted with DCM (50 mL). The combined organic layer was finally washed with water (50 mL), dried over sodium sulphate, filtered and concentrated. The resulting residue was purified by silica gel (230-400) chromatography using ethyl acetate-petroleum ether.

Yield: 72 %; yellowish viscous glass; ¹H NMR (CDCl₃ 300 MHz): δ 5.96 (1H, d, J₁,₂ = 1.8 Hz, H-1), 5.35-5.30 (2H, m, H-2, H-4), 5.10 (1H, dd, J₂,₃ = 3.3 Hz, J₃,₄ = 10.2 Hz, H-3), 4.29 (1H, dd, J₅,₆ = 4.8 Hz, J₆,₆ = 12.3 Hz, H-6'), 4.07 (1H, dd, J₅,₅ = 2.4 Hz, J₆,₆ = 12.3 Hz, H-6), 3.96-3.90 (1H, m, H-5), 2.43 (3H, s, SOCH₃), 2.19, 2.09, 2.05, 2.00 (12H, 4 X s, 4 X COCH₃), ¹³C NMR (CDCl₃, 75MHz): δ 190.34, 170.60, 169.86, 169.78, 169.48, 80.10, 72.37, 70.93, 69.79, 65.59, 62.08, 31.21, 20.83, 20.66, 20.55, 20.48. HRMS m/z: calcd for C₁₆H₂₂NaO₁₀S [M+Na]⁺: 429.0831, found 429.0832.

2.1.11. Synthesis of diethyl 16-(2,3,4,6-tetra-O-acetyl-1-thio-α-D-mannopyranoside) hexadecyl phosphonate (13).

The compound 17 (15 mg, 0.03 mmol) and compound 18 (18 mg, 0.044 mmol) were dissolved in dry DMF and the reaction mixture was stirred at 0 °C for 1 h and then at r.t. After 24 h, TLC (ethyl acetate:petroleum ether::2:3) showed formation of a product (Rf = 0.45) with complete consumption of the starting material. The solvent was then removed in a rotary evaporator. The product was dissolved in ethyl acetate (30 mL), washed with brine (10 mL x 2). The organic layer was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (230-400) using ethyl acetate-petroleum ether.

Yield: 70 %; yellowish viscous glass; ¹H NMR (CDCl₃ 300 MHz): δ 5.35-5.25 (4H, m), 4.35-4.29 (2H, m), 4.11-4.05 (4H, m), 2.62-2.58 (2H, m), 2.16, 2.10, 2.05, 1.99 (12H, 4 X s, 4 X COCH₃), 1.72-1.60 (16H, m), 1.34-1.24 (20H, m); ¹³C NMR (CDCl₃, 75MHz):


To a stirred solution of the phosphonate 13 (40 mg, 0.055 mmol) in dry CH_{2}Cl_{2} (2 mL) at 0 °C under nitrogen atmosphere was added TMSBr (0.08 mL, 0.605 mmol, 11 equiv.) drop wise via syringe and then the reaction mixture was allowed to warm at r.t. The reaction mixture was then stirred at r.t. for 48 h. Another porting of TMSBr (0.03 mL, 0.227 mmol, 4 equiv.) was added and stirring was continued for another 48 hours. The reaction mixture was then cooled to 4 °C and water (300 µL) was added and stirring continued for another 1 h. To this methanol (500 µL) and pyridine (500 µL) was added and stirred for 2 h. Pyridine was added to stabilize the free phosphonic acid. The volatile reagents were then removed in a rotary evaporator. The residue was co-evaporated with methanol three times to give a dark yellow residue. TLC of the product with 15% methanol in DCM indicated formation of a product with complete consumption of the starting material. The compound could not be purified by silica gel column chromatography using various solvent combinations. However, the compound 14 as its pyridinium salt could be extracted with toluene. Since, the product was not pure enough yield could not be calculated and also, ^1H and ^13C NMR could not be done. The product was directly used for the next step.


The phosphonic acid derivative 14 (30 mg, 0.045 mmol) was dissolved in dry pyridine (10 mL) by heating (oil bath) at 50 °C for 30 min. Choline tosylate (123 mg, 0.44 mmol, freshly dried over P_{2}O_{5}), and trichloroacetonitrile (2 mL) were added, and the reaction mixture was stirred for another 15 h at 50 °C. After that, solvent was removed under reduced pressure. The residue was directly purified by flash chromatography on silica gel (230-400) using 20% methanol in DCM to give the desired phosphocholine 15.

Yield: 55 % (overall yield of previous and this steps); yellowish viscous liquid; ^1H NMR (CDCl_{3} 600 MHz): δ 5.33-5.32 (2H, m), 5.26 (1H, J= 9.6 Hz, t), 5.195 (1H, ddd, J= 3.0 Hz, ), 4.39-4.36 (1H, m), 4.34 (1H, s), 4.29-4.26 (1H, m), 4.104 (1H, ddd, J= 2.7 Hz), 4.02-4.00 (1H, m), 3.69 (1H, s), 3.52-3.50 (1H, m), 3.26 (9H, s), 3.23 (4H, s), 2.72-2.63
(2H, m), 2.15, 2.07, 1.97 (12H, 3 X s, 4 X COCH₃), 1.68-1.59 (4H, m), 1.44-1.39 (4H, m), 1.34-1.29 (18H, m); ¹³C NMR (CDCl₃, 150 MHz): δ 170.85, 170.19, 170.10, 82.16, 70.85, 69.70, 68.91, 66.11, 62.27, 55.68, 53.30, 53.28, 53.25, 30.73, 29.42, 29.37, 29.32, 29.25, 29.13, 29.10, 28.83. HRMS m/z: calcd for C₃₅H₆₄N₂NaO₁₉PS [M+Na]⁺: 776.3785, found 776.3787.


To a solution of 15 (20 mg) in dry MeOH (1 mL), 25% aqueous ammonia solution (200 µL) was added and stirred in r.t. for 5 h. TLC with 30% methanol in DCM indicated the formation of a product with complete consumption of the starting material. The reaction mixture was then evaporated to dryness. The product was then purified by preparative HPLC using RP C-18 (50% aqueous acetonitrile) to give purified compound 2.

Yield: 85 %; yellowish viscous liquid; ¹H NMR (CDCl₃ 600 MHz): δ 5.22 (1H, s), 4.63 (1H, s), 4.26 (2H, bs), 3.90 (2H, s), 3.81 (1H, dd, J= 1.8 Hz), 3.76-3.73 (1H, m), 3.59 (2H, d, J= 5.4 Hz), 3.62-3.61 (2H, m), 3.23-3.22 (11H, m), 2.70-2.66 (1H, m), 2.62-2.57 (1H, m), 1.67-1.58 (6H, m), 1.41-1.39 (4H, m), 1.30 (17H, m); ¹³C NMR (CDCl₃, 150 MHz): δ 85.01, 73.46, 72.40, 71.79, 67.42, 66.39, 61.32, 57.16, 53.29, 53.26, 53.24, 30.95, 30.83, 30.44, 29.35, 29.33, 29.29, 29.26, 29.06, 28.90, 28.47, 23.33. HRMS m/z: calcd for C₂₇H₅₈N₂NaO₉PS [M+Na]⁺: 608.3362, found 608.3366.

2.2. Biological Study

2.2.1. Culture of Leishmania donovani.

The L. donovani strain AG83 (MHOM/IN/1983/AG83), originally isolated from an Indian kala-azar patient, was maintained by serial passage in hamsters. L. donovani amastigotes periodically recovered from the spleens of infected hamsters were transformed into promastigotes through amastigote culture in M199 supplemented with 10% FCS, 2 mM glutamine, penicillin G (100 U/mL) and streptomycin sulfate (100 μg/mL) at 22 °C. Promastigotes were used at the log phase of growth, approximately 2 to 3 days after subculture. Parasites were kept in culture by weekly passaging.

2.2.2. In vitro anti-promastigote activity.

Anti-parasitic activity of the synthesized compound 2 against L. donovani (AG83) promastigotes was determined by a quantitative colorimetric assay using Alamar blue reagent (Life Technologies). When cells are alive they maintain a reducing environment within their cytosol. Resazurin/AlamarBlueH (7-Hydroxy-3H-phenoxazin-3-one 10-
oxide), the active ingredient of AlamarBlue reagent, is a nontoxic, cell-permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. This assay has been reported to be more sensitive than MTT assay when compared for efficacy in estimating cell viability against a cancer cell line treated with more than 100 different drugs.\textsuperscript{16a} So we have used this assay for assessing the cell viability of cells treated with our compounds.

Assays were performed in sterile 96-well plates using 100 μL of log-phase promastigotes adjusted to 2×10^6 cells/mL. These cells were incubated in the absence (control) and presence of 1.562 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25.0 μM, 50.0 μM, 100.0 μM, and 200.0 μM of compounds and the equivalent volume of the solvent (DMSO) for 24 h. After completion of treatment, 10 μL of the resazurin dye (0.01%) was added, and plates were incubated for a further 4 h at 37 °C. After incubation, cells were analyzed in a microplate reader at a wavelength of 570 nm, using 600 nm as a reference wavelength (normalized to the 600 nm value).\textsuperscript{16b} Absorbance in the absence of any compound or solvent alone was set as the 100% control. Cell viability was evaluated based on a comparison with untreated control cells, and the effective dose (ED) concentration of the compounds or the solvents that are necessary to reduce the growth of promastigotes by 50% (ED\textsubscript{50} values) and 90% (ED\textsubscript{90} values).

3. Result and Discussion

Synthesis of the compound 16-(1-thio-α-D-mannopyranoside) hexadecyl phosphocholine (2) was achieved using multistep procedure as shown in scheme 1. Hexadecanedioic acid (3) was selectively esterified\textsuperscript{17} to give mono benzylated hexadecanoic acid 4 (yield 62%). The free carboxylic acid of compound 4 was reduced with BH\textsubscript{3}.Me\textsubscript{2}S\textsuperscript{18} in dry THF to give the compound 5 (yield 77%). For this reduction, we tried with several reagents like sodium cyanoborohydride, sodium borohydride, and BH\textsubscript{3}.THF. The reaction failed with sodium cyanoborohydride and sodium borohydride. The BH\textsubscript{3}.THF produces compound 5 in low yield 15%. The compound 5 was then tosyolated\textsuperscript{19} with tosyl chloride in DCM and pyridine to give compound 6 in 71 % yields which was easily converted to compound 7 (yield 79 %) using sodium iodide\textsuperscript{20} in DMF.
Scheme 1. Synthesis of targeted α-D-manno-miltefosine derivative (2).

Scheme 1: Reagents and condition: (a) NaOH, TBAB, Toluene, Benzyl bromide reflux, 5 h; (b) BH₃·Me₂S, in dry THF 0 °C- r.t., 15 h; (c) Tosyl chloride, DCM, Pyridine, 0 °C, r.t., 5 h; (d) NaI, DMF, 80-100 °C, 3 h; (e) Triethyl phosphite, 150-160 °C, 6 h; (f) NaOH, Methanol:H₂O (1:1), r.t., overnight; (g) Lawesson’s reagent, DCM, r.t., overnight; (h) BH₃·Me₂S, in dry THF 0 °C- r.t. 15 h; (i) NIS-TfOH, DCM, 15 min; (j) TMSBr, CH₂Cl₂, 0 °C to r.t., 96 h, then MeOH; (k) HOCH₂CH₂N⁺(CH₃)₃ OTs', Cl₃CCN, Py, 50 °C, 15 h; (l) BH₃·Me₂S, in dry THF 0 °C- r.t. 15 h; (m) Tosyl chloride, DCM, Pyridine, 0 °C- r.t., 5 h; (n) diethyl amine, DMF, 0 °C-r.t., 24 h; (o) NH₃, MeOH, r.t., 5 h.

Compound 7 when treated with triethyl phosphite₂¹ at about 150 °C gave compound 8 in 74 % yield. De-esterification of 8 with sodium hydroxide in methanol:water produced 9 in high yield (92 %). Our original scheme was to synthesize the phosphonate compound 13.
Chapter-III; Part-B: Synthesis of mannose linked miltefosine

via the compounds 10, 11. But, unfortunately, the reaction of 9 with lawesson’s reagent gives the compound 10 and their reductive product 11 in very low yield. Therefore, we modified the synthetic route and the target compound 13 was synthesized via compounds 16, 17. The compound 9 was reduced to alcoholic compound 16 (yield 72%) by borane\(^{17}\) (BH\(_3\).Me\(_2\)S) in dry THF. The generated hydroxyl group of 16 was tosylated\(^{18}\) by using tosyl chloride in DCM and pyridine to give 17 (yield 76%). The glycosylation reaction of 17 and 18 in DMF and diethyl amine\(^{22}\) gave the phosphonate compound 13 in 70% overall yield. The compound 18 was obtained in 72% overall yield by reacting mannose pentaacetate 20 with thiolacetic acid in BF3 etherate\(^{23}\) (Scheme 2).

**Scheme 2.** Synthesis of 2,3,4,6-tetra-O-acetyl-1-S-acetyl-1-thio-α-D-mannopyranoside

Reagents and conditions. (a) acetic anhydride, pyridine, 0°C-r.t., 24 h; (b) thiolacetic acid, BF\(_3\).Et\(_2\)O, DCM, 0 °C - r.t. 5 h.

For the synthesis of compound 14 from 13, we tried several reagents like HBr, TMSCl, and BBr\(_3\) at different temperature and molar concentration, but could not get the desired product in acceptable yield. Yield varied form 20-40% only. Finally we tried TMSBr\(^{24}\) at different molar concentration and temperature for the removal of diethyl group of phosphonate and got compound 14 in reasonable yield. Since the free phosphonic acid derivative was unstable it was converted to pyridinium salt which was stable at low temperature. We were unable to calculate the exact yield of the reaction as it was extremely difficult to purify 14 from various salts and polymeric compounds of TMSBr formed during the reaction. Attempt was made to purify 14 by normal silica gel and flash chromatography, and also by RP column chromatography but without success. Various combinations of solvents were used for this purpose. Finally semi pure compound 14 was obtained by direct extraction of the reaction mixture with dry toluene. Tentative yield of the compound 14 was 60%. ESI-MS proved the formation of the compound 14. NMR of the compound 14 could not be obtained.

The crude compound 14 was then treated with choline tosylate\(^{25}\) and trichloro acetonitrile in dry pyridine for 48 h to give the compound 15 (yield 55%) which was purified by flash column chromatography. Deacetylation of 15 with 25% aqueous ammonia and methanol gave the final product 2 (yield 85%).
3.1. Antimicrobial Activity

The *in vitro* antileishmanial activity of the mannose linked miltefosine derivative 2 was investigated using Alamar-blue assay system. The ED$_{50}$ and ED$_{90}$ values were calculated for the compound. Miltefosine was used as positive control. The mannose linked miltefosine derivative 2 showed enhanced activities against *L. donovani* Promastigotes in comparison to miltefosine. The results are shown in table 1.

**Table 1.** *In vitro* anti-leishmanial activity of miltefosing and compound 2.

<table>
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<th>ED$_{90}$</th>
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In future we will carry out assay for *in vitro* activation of macrophage and suitable cell lines by the compound 2 and also relevant *in vivo* assay to evaluate the potential of the mannose-miltefosine conjugate 2 for targeted drug delivery.

4. Conclusion

Till today there is no effective and receptor specific drug for the treatment of leishmaniasis. Most of the current drugs are costly and highly toxic. Moreover, there are many reports of the occurrence of resistant VL strains against these drugs. Keeping these in mind, we synthesized the target specific miltefosine derivatives such as α-D-manno-miltefosine. The miltefosine derivatives is expected to be selectively bind with mannose binding proteins on macrophages of various organs such as spleen, liver etc. where the parasites anchors and multiplies. Since, miltefosine is administered orally; the O-linked sugars are likely to be degraded by glycosidases. Therefore, instead of O-glycoside, we synthesized S-glycosylated miltefosine derivative which are likely to be more stable when administered orally. The compound may also be administered via i.v. route.
In this thesis we have reported detail synthesis of the mannose linked miltefosine and also in vitro anti-leishmanial activity. The mannose linked miltefosine derivative showed enhanced anti-leishmanial activity in comparison to miltefosine. In future we shall carry out in vitro assay for macrophage targeting as well as suitably designed in vivo experiments to ascertain function and efficacy of the synthesized molecule.

Selective drug targeting will not only require very small quantity of drug but also reduce chances of drug resistance due to likely reduced treatment regime. Miltefosine was originally developed as a drug against cancer. The mannose-miltefosine compound may also help towards targeted drug delivery against cancer in spleen or liver.
5. References
Spectroscopic Data
Chapter-III: Part B: Spectroscopic data
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[Image of a spectroscopic chart with molecular structures and peak assignments]

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Chapter III: Part B: Spectroscopic data
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