

# Slow pace of antileishmanial drug development

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## Review

**Cite this article:** Kumar A, Pandey SC, Samant M (2018). Slow pace of antileishmanial drug development. *Parasitology Open* 4, e4, 1–11. <https://doi.org/10.1017/pao.2018.1>

Received: 5 November 2017

Revised: 21 December 2017

Accepted: 28 December 2017

### Key words:

Challenges; drug; leishmaniasis; slow progress; target

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## Abstract

The protozoan parasite *Leishmania* is endemic in large parts of the world which causes leishmaniasis. Its visceral form is fatal if not treated and is caused mostly by *Leishmania donovani*, *Leishmania infantum* and *Leishmania chagasi*. Given the difficulties linked to vector (sandfly) control and the lack of an effective vaccine, the control of leishmaniasis relies mostly on chemotherapy. Unfortunately, the prevalence of parasites becoming resistant to the first-line drug pentavalent antimony (SbV) is increasing worldwide. Few alternative drugs are available that includes amphotericin B, pentamidine and miltefosine (oral). Already, decreases in efficacy, resistance and toxicity have been noted against these drugs. Dry antileishmanial pipeline further indicates the slow pace of drug discovery in this field where resistance as a major barrier. Full understanding of the genetic and molecular basis of the parasite is lagging. Since leishmaniasis is a neglected disease and occurs predominantly in the developing world largely, therefore, it is unaddressed. The pharma industry argues that development of the new drug is too costly and risky to invest in low return neglected diseases is very high. Research is also needed to identify new and effective drug targets. The lack of drug research and development for neglected diseases will require some new strategies. We have discussed the various cause of slow pace of antileishmanial drug discovery in this review to pay attention of researchers and also take the public and private initiative to make the process fast for new antileishmanial drug development.

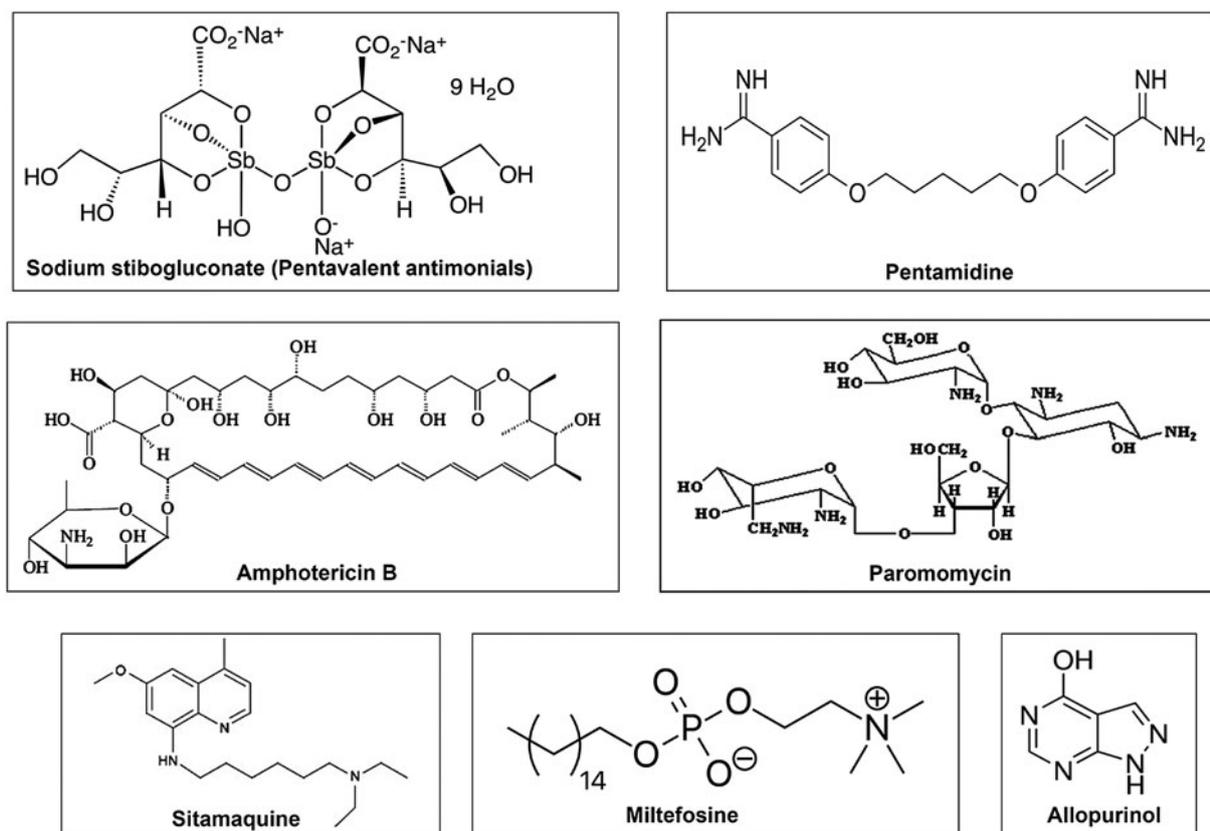
## Introduction

Leishmaniasis, with three basic clinical manifestations of the disease: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL) caused by over 15 different species of the intracellular protozoan parasite *Leishmania*. With an estimated 12 million cases of leishmaniasis worldwide and 1.5–2 million new cases reported each year (Herwaldt, 1999). The parasite is primarily found in South America, Asia, southern Europe and Africa (Guerin *et al.* 2002). Since there is no antileishmanial vaccine is clinically available, control of Leishmaniasis relies almost exclusively on chemotherapy. For the past 7 decades pentavalent antimonials constituted the standard antileishmanial treatment worldwide, however, the last 20 years their clinical value was imperiled due to the widespread emergence of resistance to these agents in Bihar, India, where more than 50% of VL cases occur globally (Sundar *et al.* 2007). The availability of molecular methods, genome sequence, proteomic methods to the study *Leishmania* biology is practical and quite possible. Researchers should have to apply these methods and effective tools to the study of resistance mechanisms/novel drug targets and speed up the process of antileishmanial drug discovery.

Laboratory-induced drug resistance and field isolate resistance mechanism is partly understood in *Leishmania*. The high-level resistance to antimony observed in *Leishmania* can be due to decreased drug uptake and simultaneous selection of loss in metal reduction (Faraut-Gambarelli *et al.* 1997; Rojas *et al.* 2006). In contrast to *in vitro* selected strains, resistance to Sb(V) in *Leishmania* field isolates is not well understood (Carrío *et al.* 2001; Hadighi *et al.* 2006). Other available antileishmanial drugs are also showing resistance in different part of the world and resistance mechanism is unclear. Current studies with field isolates of *Leishmania* (Lira *et al.* 1999; Kumar *et al.* 2010) suggest similarities and differences with laboratory-resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates. Understanding how antileishmanial drugs work and why they sometimes fail is fundamental to the optimal use of the existing formulations and is also likely to be instructive in the development of new therapies. It, therefore, appears quite clear that the cadence of antileishmanial drug development has not kept pace with the clinical needs. In this review, we further discuss the antileishmanial drug developed in past decades, current scenario of those drugs, emerging/novel drug targets, and challenges of antileishmanial drug development.

## Journey of antileishmanial drugs development

The therapeutic options for *Leishmania* infections are quite limited and include only a few drugs (Fig. 1). The timeline of these drug discoveries or uses is explained in Fig. 2 that



**Fig. 1.** Chemical structure of few drugs used as therapeutic options for *Leishmania* infections. Sodium stibogluconate is first line antileishmanials. Pentamidine, Amphotericin B and Paromomycin are second-line drugs, exhibited to be effective against CL and VL. Miltefosine, allopurinol and sitamaquine have shown oral activity against Leishmaniasis and are in phase III/IV clinical trial.

indicated the slow pace of antileishmanial discovery. Only one new class of antileishmanial drugs in terms of oral formulation has been developed in the last 20–30 years but they are also showing resistance now a day. Route of administration, mechanism of action and side effect of available antileishmanials are summarized and discussed in Table 1. Due to unavailability of an effective vaccine against leishmaniasis, drugs are only the important available means for the treatment.

### Preliminary/initial antileishmanials

Urea stibamine (Urea salt of Stibnic acid) was used as a first effective drug against *Leishmania donovani* in 1920. It saved thousands of lives in India from 1920 to 1930. After that potassium antimony tartrate (Tarter emetic) was started to use against *Leishmania* in Vianna. Stibamine and stibacetin were also used for the treatment of *Leishmania* but showed few side-effects. Then pentavalent antimonials were developed as the first line of drug for the treatment of Leishmaniasis with reduced side-effects at that time.

### First-line antileishmanials

The pentavalent antimonials were first introduced in 1945 and remained the standard drugs for about 6 decades. The two

compounds currently available for clinical use are sodium stibogluconate [(SSG) also known as sodium antimony gluconate (SAG) and its trade name is Stibanate/Pentostam)] and Meglumine antimoniate (also known as N-methylglucamine antimoniate and its trade name is Glucantime/Prostib). Stibogluconate {Sb(V)} under the brand of Pentostam has been manufactured by GlaxoWellcome which contains SSG. Using intracellular susceptibility testing, parasites isolated from Sb(V) unresponsive patients were found to be more resistant to Sb(V) as compared with parasites isolated from Sb(V) responsive patients (Kumar *et al.* 2009). Several side-effects have decreased the use of antimonials.

### Second-line antileishmanials

The methanesulphonate or isethionate salts of pentamidine have been used as second-line drugs in the treatment of VL refractory to antimonial treatment. However, the effectiveness of pentamidine is rapidly declining in India, suggesting that *Leishmania* parasites are becoming resistant (Sundar, 2001). Amphotericin B (polyene macrolide antifungal antibiotic) was discovered in the 1960s, from a bacterium of genus *Streptomyces* and given to patients who are failed to treat with SSG/SAG or pentamidine. More effective lipid-associated formulations of Amphotericin B are also available. Its instability at high temperature, painful

**Fig. 2.** The timeline of antileishmanial drug discoveries from early 1920 to 2017, indicating the slow pace of antileishmanial discovery.



**Table 1.** Current antileishmanial drugs and their associated information

Antileishmanial drugs	Route of administration	Mechanism	Side-effect	Resistance reports	References
Pentavalent antimonials	Intravenously or intramuscularly	<ul style="list-style-type: none"> <li>• Pentavalent form converted to a lethal trivalent form in amastigote.</li> <li>• Activated within the amastigote, but not in the promastigotes.</li> <li>• Destroy <i>Leishmania</i> species by DNA degradation, suggesting the role of apoptosis.</li> <li>• Inhibits phosphorylation of ADP to ATP and citric acid cycle.</li> <li>• Interference of glycolysis and beta-oxidation of fatty acids in amastigotes.</li> <li>• Decreased energy and reducing equivalents for antioxidant defence within amastigote</li> </ul>	<ul style="list-style-type: none"> <li>• Venous thrombosis</li> <li>• Acute electrocardiographic changes</li> <li>• Cardio, nephro and hepatotoxicity</li> <li>• Abdominal cramps, headache</li> <li>• Arthralgias, myalgias,</li> <li>• Sterile abscesses at intramuscular sites</li> </ul>	Resistance was developed in the Bihar state of India and reported from another province	(Carter <i>et al.</i> 2006; Sereno <i>et al.</i> 2001; Perry <i>et al.</i> 2015)
Pentamidine	Intravenous or intramuscular	<ul style="list-style-type: none"> <li>• Inhibit parasite polyamine synthesis or binds with DNA helix through electrostatic attraction between negative phosphate groups and positive centre of amidine.</li> <li>• Damage mitochondria and kinetoplast-DNA core of both promastigotes and amastigotes.</li> <li>• Inhibit RNA polymerase and biosynthesis of nucleic acid, proteins, phospholipids, polyamines</li> </ul>	<ul style="list-style-type: none"> <li>• Rashes, fever, phlebitis, sterile abscesses</li> <li>• Hypotension</li> <li>• Nephrotoxicity</li> <li>• Hypoglycaemia</li> <li>• Leucopenia, anaemia, thrombocytopenia</li> <li>• ECG changes</li> </ul>	Patients showed unresponsiveness in India	(Kandpal and Tekwani, 1997)
Amphotericin B	Intravenously by infusion	<ul style="list-style-type: none"> <li>• Binds to cell wall sterols but preferentially to ergosterol, which is the major cell membrane sterol of <i>Leishmania</i>, but not mammalian cell membranes causing pores which alter ion balance, loss of low molecular weight components such as glucose, amino acids and result in cell death</li> </ul>	<ul style="list-style-type: none"> <li>• Acute fever, chills, pain</li> <li>• Anaphylaxis</li> <li>• Hypotension, convulsions</li> <li>• Hypokalaemia, hypomagnesemia</li> <li>• Nephrotoxicity</li> </ul>	Resistance reported in Bihar, India and different part of the world	(Bern <i>et al.</i> 2006; Laniado-Laborin and Cabrales-Vargas, 2009)
Paromomycin	Intramuscular injections for VL and as ointments for CL	<ul style="list-style-type: none"> <li>• Paromomycin inhibits protein synthesis by binding to 30S subunit ribosomes.</li> <li>• Misreading and premature termination of mRNA translation and causes the low concentration of <math>Mg^{2+}</math> which induce dissociation.</li> <li>• It also induces respiratory dysfunction in <i>L. donovani</i> promastigotes</li> </ul>	<ul style="list-style-type: none"> <li>• Skin rashes, burns</li> <li>• Local pruritus</li> <li>• Ototoxicity (eighth cranial nerve toxicity)</li> <li>• Nephrotoxicity</li> </ul>	Laboratory strain of <i>Leishmania tropica</i> and <i>L. donovani</i> , has developed resistance	(Jhingran <i>et al.</i> 2009)

(Continued)

Table 1. (Continued.)

Antileishmanial drugs	Route of administration	Mechanism	Side-effect	Resistance reports	References
Sitamaquin	Oral	<ul style="list-style-type: none"> <li>Affect mitochondrial electron transport chain.</li> <li>At higher concentration sitamaquine affects parasite growth, morphology and motility</li> <li>It is rapidly metabolized, forming desethyl and 4-CH<sub>2</sub>OH derivatives, which might be responsible for its activity</li> </ul>	<ul style="list-style-type: none"> <li>Higher doses showed nephrotoxicity</li> <li>Mild methemglobinaemia</li> </ul>	<i>In vitro</i> resistance has been reported in <i>Leishmania donovani</i> promastigote	(Jhingran et al. 2009; Bories et al. 2008)
Miltefosin	Oral	<ul style="list-style-type: none"> <li>Inhibition of ether remodelling. phosphatidylcholine biosynthesis.</li> <li>Interfere with signal transduction and calcium homeostasis.</li> <li>Interfere with cellular membranes without interacting with DNA.</li> <li>Modulates membrane permeability and fluidity</li> <li>Alter membrane lipid composition.</li> <li>Induces apoptotic cell death and necrosis</li> </ul>	<ul style="list-style-type: none"> <li>Vomiting, diarrhoea in 30–40% patients</li> <li>Teratogenic (toxic effects on reproductive capacity in female)</li> <li>Not to be used in pregnant women</li> <li>Anorexia, nausea</li> <li>Hepatotoxicity</li> </ul>	Resistance in laboratory strains of <i>L. braziliensis</i> , <i>L. guyanensis</i> , and <i>L. Mexicana</i>	(Castelo Branco et al. 2016)
Allopurinol	Oral	<ul style="list-style-type: none"> <li><i>Leishmania</i> spp. are unable to synthesize purines. Allopurinol is hydrolysed to allopurinol riboside, an analogue of inosine that is incorporated instead of ATP into leishmanial RNA. It interferes with the normal protein synthesis (purine salvage cycle) of the parasite</li> </ul>	<ul style="list-style-type: none"> <li>Toxicity very low</li> <li>Skin rashes</li> <li>Neutropenia</li> <li>Thrombocytopenia</li> <li>Anaemia</li> <li>Can result in renal stones</li> </ul>	Resistance in <i>L. infantum</i> and to associate it with disease relapse in the canine host	(Chawla and Madhubala, 2010; Baneth and Shaw, 2002; Yasur-Landau et al. 2016; Wyllie et al. 2012)

delivery and high cost limits its usefulness. Paromomycin (aminoglycoside antibiotic) another second-line drug, exhibited to be effective against CL in clinical studies in Russia, and in clinical trials with VL in the early 1990s. Paromomycin in combination with stibogluconate was used successfully for treating SSG unresponsive VL in India and Kenya (Moore and Lockwood, 2010).

### Oral antileishmanials

Few agents (miltefosine, allopurinol, sitamaquine) have shown oral activity against Leishmaniasis. They are in phase III/IV clinical trial. Miltefosine was the first oral drug for VL. It is originally developed as an anticancer agent for topical treatment of skin metastases in 1992 but started to use for VL after 2000. Its activity against *L. donovani* was discovered in 1987 (Croft *et al.* 1987). Allopurinol (Hypoxanthine analogue) was used for CL in 1991 first time and its efficacy against Indian Kala-azar was first reported (Saenz *et al.* 1989). Allopurinol is ineffective as monotherapy against VL or CL. Now it is used with SSG for combination therapy. Sitamaquine (8-aminoquinoline and primaquine analogue) was developed by Walter Reed Army Institute of Research, the USA in 1994 for treatment. It is under development for over 8 years by Glaxo Smithkline, UK and Walter Reed Army Institute of Research, USA but the progress is very slow.

### Current scenario of available drugs

#### Pentavalent antimonials

Despite the concern of proliferative resistance and various side-effects, pentavalent antimonials (SbV), the generic SSG (pentosam), N-methyl-glucamine antimoniate (Glucantime) and branded meglumine antimoniate, etc. are being used as a drug of choice for the treatment of Leishmaniasis over more than 5 decades (Singh *et al.* 2006), but during the last two decades, antimonial resistance and therapeutic failures reached epidemic dimensions. Inadequate treatment in terms of dosing and duration and poor compliance promote the widespread antimonial resistance in an endemic area (Maltezou, 2010). Pentavalent antimonials (SbV) considered as a prodrug, which should convert to trivalent antimonials (SbIII) in order to demonstrate their antileishmanial activity (Carter *et al.* 2006). The reduction of pentavalent to trivalent compound takes place either in macrophages or in the parasite, however, it is still a dilemma (Shaked-Mishan *et al.* 2001). Parasite-mediated reduction has been found to be associated with the loss of reductase activity of parasite, which may also lead to drug resistance. Though the exact mechanism of action is unknown (Serenio *et al.* 2001), but both forms (SbIII and SbV) of antimonials destroy *Leishmania* species by DNA degradation, suggesting the role of apoptosis, adenosine diphosphate phosphorylation and oxidation of fatty acid. Furthermore, the antimonials are also concerned with increasing the efflux of intracellular thiols by multi-drug-resistant protein A (MRPA), glycolysis inhibition and ABC (ATP-binding cassette) transporters (El Fadili *et al.* 2005). In natural antimonial resistance, the impaired thiol metabolism results in inhibition of SbV activation and decreased uptake of the active form SbIII by amastigotes; this is accomplished by the lower expression aquaglyceroporin 1 gene, which is involved in the uptake of SbIII (Decuypere *et al.* 2005). The major cause of acquired resistance was the mismanagement of drug (Perry *et al.* 2015), due to its easy availability in its endemic regions along with the loss of drug activation by parasites. The transporters of pentamidine-resistant protein 1 (PRP1) MRPA and ABC family that act as an efflux pump for antimonials, are also associated with antimony resistance (Coelho *et al.* 2003).

#### Pentamidine

A drug which can be used as a second-line drug against Leishmaniasis is pentamidine, an aromatic diamine. It contains two main salts methansulphonate and isothionatethatare mainly used for the treatment of VL. In India, initially it was used to treat SbV refractory patients, but due to its drawbacks of decreased efficiency and high resistance, it was no longer be used. Later on, this drug was also used in combination with other drugs. In a study, patients which were unresponsive to antimony revealed better response for a combination of a low dosage of pentamidine and allopurinol as compare to the full dosages of pentamidine (Das *et al.* 2001). Furthermore, a comparative study in Peru on meglumine antimoniate (glucantime) and pentamidine against CL due to *Leishmania braziliensis* shows that glucantime was much more operational than pentamidine (Andersen *et al.* 2005). Although, its precise mode of action is not known, in a study (Kandpal and Tekwani, 1997) it is reported that in *L. donovani* promastigote the drug enters through polyamine and arginine transporters. Pentamidine is a highly toxic drug causes serious side-effects such as nephrotoxicity, hypoglycaemia and hypotension, etc. Although the mechanism of pentamidine resistance is not very well understood, ABC protein PRP1 can be associated with the resistance of pentamidine in the intracellular stage of *Leishmania* (Coelho *et al.* 2007).

#### Amphotericin B

Another drug which was previously being reported against Leishmanial infection is Amphotericin B (AmB), a macrolide polyene antifungal drug widely used to treat systemic fungal infections (Marcondes *et al.* 2011). AmB is the drug of choice in the endemic areas of Bihar, India where antimonials resistance is common (Bern *et al.* 2006). AmB binds to membrane ergosterol, the predominant sterol of the fungal and leishmanial cell membrane. Even though it is highly effective but AmB also shows toxicity and its side-effects have been reported (Laniado-Laborin and Cabrales-Vargas, 2009). With its three clinical formulations, Ominous effects of plain AmB have been circumvented in which deoxycholate have been replaced by other lipids. These formulations are AmB colloidal dispersion (ABCD: Amphocil), liposomal AmB (L-AmB: Ambiosome) and AmB lipid complex (ABL: Abelcit). These lipid formulations of AmB retain their antifungal activity and show very high efficacy to cure this fatal disease and are less toxic. In VL cases, liposomal AmB has been proved as an efficient drug but its high cost limits its use to a common man suffering from this lethal disease. In a study, it was found that the success of AmB treatment mainly depends on immunity status of the patient and indicates that consecutive relapse could increase exposure of AmB resistant isolates (Di Giorgio *et al.* 1999). Some investigations have shown that resistance to AmB was found to be associated with gene TarII 64.4 and TarII 512.2 amplification in *L. tarentolae* mutant cell lines (Singh *et al.* 2001) and overexpression of L-asparaginase gene in *L. donovani* (Singh *et al.* 2017). This finding warrants the possibility of resistance against the most successful drug.

#### Paromomycin

Paromomycin is broad-spectrum antibiotic produced by the bacterial spp. *Streptomyces riomusus* was originally developed as an oral drug for the treatment of intestinal protozoans in the 1960s. Later on, in 2002, it was first introduced as an antileishmanial drug in the form of PM sulphate (Williams *et al.* 1998). Due to its low cost, better efficacy, shorter duration of administration and fewer side-effects it became very popular and thought to be a candidate for the first line therapy for VL patients. However, VL

and CL both can be cured by paromomycin but its finite accessibility limited its use in endemic regions (Thakur, 2003). Due to its limited use resistance is not yet recorded in patient treatment but reported *in vitro* in *L. tropica* and *L. donovani* (Jhingran *et al.* 2009). To overcome these problems PM is being reported in combination with liposome and albumin microsphere and thereby exhibiting better results (Wiwanitkit, 2012).

### Sitamaquine

Sitamaquine was developed for the treatment of VL, chemically known as 4-methyl-6-methoxy-8-aminoquinoline. The main fore-deal of this drug is its oral administration. The Kenyan phase II trial of sitamaquine gave an opinion about its efficacy, against VL and was well endured (Wasunna *et al.* 2005). However, despite its efficacy, some side-effects such as a headache, abdominal pain and kidney dysfunctioning were observed. The repercussion of Indian phase II trial was different from Kenyan trial (Jha *et al.* 2005). The Indian trial showed somewhat equal efficacy but the side-effects observed were vomiting, dyspepsia, cyanosis, nephritic syndrome and glomerulonephritis. At higher concentration sitamaquine affects parasite growth, morphology and motility (Duenas-Romero *et al.* 2007). The mechanism involves electrostatic interaction between positively charged sitamaquine and phospholipid anionic polar head group and the insertion of the drug within the biological membrane is facilitated by phospholipid acyl chain (Coimbra *et al.* 2008). In clinical practices, the resistance against this drug has not been reported yet but *in vitro* resistance has been reported against *L. donovani* promastigote by selecting the drug pressure of sitamaquine at the concentration of 160  $\mu\text{M}$  (Bories *et al.* 2008). Due to less activity and efficacy, its further clinical trials were restricted seriously (Garnier *et al.* 2006).

### Miltefosine

The recently introduced drug for the antileishmanial activity is miltefosine, an alkylphosphocholine also known as hexadecylphosphocholine which was originally developed as anticancerous agent (Croft and Coombs, 2003). Being used as a first oral drug for the treatment of Indian VL it was considered a major breakthrough in antileishmanial chemotherapy (Sundar *et al.* 2006). Most of the studies (Sundar *et al.* 2000) shown that >95% of patients were cured regularly at this dosing pattern. Later on, it was observed that few patients were falling back again after 9–12 months of successful treatment with miltefosine. After that this drug was also used in combination with ambisome and found working effectively, but some of its side-effects (Castelo Branco *et al.* 2016) raised questions about the effectiveness of this combination drug (Sundar *et al.* 2011). The clinical resistance is not yet clear but being an oral drug it was used improperly in endemic countries like India, that became the reason for its resistance. It is reported that single point mutation at LdRos3 and LdMT or overexpression of multidrug-resistant gene MDR1 rendered the parasites remarkably less sensitive to miltefosine, and this resistance persisted *in vivo*; cross-resistance with other antileishmanial was not detected (Perez-Victoria *et al.* 2001; Pérez-Victoria *et al.* 2006; Seifert *et al.* 2007). In all miltefosine resistant *Leishmania* lines decreased drug accumulation is the common phenomenon, and this may be due to decreased uptake, increased efflux, faster metabolism or altered plasma membrane permeability; the first two mechanisms have been described in experimental models of miltefosine resistance (Pérez-Victoria *et al.* 2006; Seifert *et al.* 2007). Further, its long half-life and teratogenic properties (Sundar and Olliaro, 2007) are the two major drawbacks that became the reason of its clinical resistance and eventually lead to the end of its use as a regular drug.

### Allopurinol

Allopurinol is second line drug used for the treatment of leishmaniasis due to unresponsiveness or resistance of first-line antileishmanial drugs. The antileishmanial activity of allopurinol was first reported in 1974 (Pfaller and Marr, 1974). It is an analogue of purine and administered by oral route. Allopurinol is used alone or in combination with other drugs to the control of infection. Metabolization of allopurinol results into interruption of the pathogen's protein synthesis process, as they are not able to synthesize purines (Chawla and Madhubala, 2010). Specifically, it is attributed to the inhibition of the leishmanial enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). HGPRT takes part in the purine salvage pathway of the parasite. It converts dephosphorylated purines to nucleoside monophosphate leading to disrupted protein translation as well as the death of the parasite. Leishmaniasis has been treated for many years with allopurinol in domestic dogs and humans (Baneth and Shaw, 2002). Recently some researchers have reported resistance of allopurinol in *L. infantum* and associated it with disease relapse in the canine host dog because the dog is the main reservoir for this zoonotic infection (Yasur-Landau *et al.* 2016).

Beside above mentioned antileishmanial drugs few other compounds also been shown anti-*Leishmania* potency, e.g. the anti-trypanosome drug fexinidazole showed potential for treating VL (Wyllie *et al.* 2012), Nitroimidazo-oxazole compound DNDI-VL-2098: an orally effective preclinical drug candidate (Gupta *et al.* 2014) and the R enantiomer of the antitubercular drug PA-824 as a potential oral treatment for VL (Patterson *et al.* 2013). The main point of concern of antileishmanial drug discovery is the stepwise increase in resistance. The current scenario is worst and research going on *Leishmania* cannot rule out about the future availability of the candidate leishmanial antigens/immunogens. So further it needs extensive study and validation of emerging potent and novel drug targets of *Leishmania*.

### Emerging and novel drug targets in Leishmania

Discovery of new potential drug targets mainly depends on biochemical, metabolic pathways and their enzymes, which are substantive for the survival of the parasite. Particular enzymes which are present in the parasite and absent from mammalian cells are of great interest. However, still, mammalian enzymes, which have structural homology, might also provide objectives for antileishmanial therapy because report showed favorable interactions of inhibitors with the active pocket of parasite enzyme, without adversely affecting human enzyme (Singh *et al.* 2017) and this is the rationale of the listed potential target in *Leishmania*. They could be interesting and promising for future antileishmanial drug development.

### Enzymes of Trypanothione synthesis pathway

Trypanothione (bis-glutathionyl spermidine) is an indispensable molecule, which protects *Leishmania* against oxidative stress. Two enzymes namely trypanothione reductase (TR) and trypanothione synthetase (TS) catalyse the synthesis of trypanothione. TS catalyses the synthesis of trypanothione from two molecules of spermidine and glutathione, then in the presence of NADPH, trypanothione is upheld in its reduced form by the enzyme TR (Fairlamb *et al.* 1985). Reduced trypanothione, in turn, reduces tryparedoxin (TX) followed by reduction of TX recycling enzyme tryparedoxin peroxidase (TP). Since, in *Leishmania*, this is the only pathway that is decisively implicated in maintaining oxidative stress, therefore, both TP and TR have been

shown to be important drug targets. Null mutation of TR show attenuated infectivity (Dumas *et al.* 1997). The role of TR as potential targets for rational drug design has also been reviewed recently (Kumar *et al.* 2014). Thus, enzymes of Trypanothione synthesis pathway provide a promising drug target in trypanosomatids.

### Metacaspases

Another kind of molecule which can be used as a drug target in *Leishmania* is metacaspase which is believed to cause apoptosis (Lee *et al.* 2007). *Leishmania major* metacaspase has been found to be essential for proper segregation of nucleus and kinetoplast of the parasite (Denise *et al.* 2006). It is reported that under peroxide stress, metacaspase causes apoptosis of parasite (Gonzalez *et al.* 2007). Since metacaspase are associated with chromosomal separation and survival of the parasite they can directly be used as a drug target (Meslin *et al.* 2011). So the molecules that can target on the biosynthetic machinery of metacaspase might be used as the effective antileishmanial agent. However, more studies are required to understand the entire role of leishmanial metacaspases.

### Enzymes of folate biosynthesis

The key enzyme which is responsible for the production of thymidine in folate metabolism is DHFR (Booth *et al.* 1987). Using NADPH as cofactor it reduces dihydrofolate to tetrahydrofolate. As a result, DHFR inhibition stops thymidine biosynthesis and as a consequence, biosynthesis of DNA. Auspiciously DHFR has been crystallized from *Trypanosoma cruzi* and *L. major* and structural data between parasite and human enzymes may be achieved so that inhibitors of DHFR can be designed (Senkovich *et al.* 2009). An enzyme dihydrofolate reductase-thymidylate (DHFR-TS) is also associated with the survival of parasite and without this enzyme parasites are not able to survive in animal hosts (Veras *et al.* 1999). In addition, an approach using database mining to explore novel inhibitors of DHFR has also been made but it needs more focus to use DHFR as a drug target (Zuccotto *et al.* 1998). Despite these advantages, various resistance mechanisms to DHFR have been uncovered such as overexpression of the enzyme PTR-1 and DHFR-TS (Wang *et al.* 1997). The enzyme PTR-1 not only causes the reduction of biopterin to dihydrofolate and tetrahydrofolate but also reduces dihydrofolate to tetrahydrofolate. Therefore DHFR and PTR-1 can be used as more efficient drug targets.

### Topoisomerase

DNA topoisomerases are involved in various biological processes like DNA replication, transcription, recombination, and repair and classified as type I and type II. DNA topoisomerases have been used as chemotherapeutic targets against *Leishmania* (Das *et al.* 2004). Camptothecin, a plant alkaloid which is an inhibitor of eukaryotic topoisomerase-I shows some inhibitory properties against *T. brucei*, *T. cruzi* and *L. donovani* (Bodley *et al.* 1995). A pentacyclic triterpenoid, Dihydrobetulinic acid (DHBA) which is a derivative of betulinic acid shows inhibitory properties against both Topoisomerase I and topoisomerase II of *L. donovani* (Chowdhury *et al.* 2003). Three isoflavanoids, smiranicin, lysaperin H and 8-prenylmucronulatol have been found to be observed with anti-leishmanial attribute and this activity has been correlated to inhibition of topoisomerase II and kDNA linearization (Salem and Werbovetz, 2005). Thus these enzymes should be structurally analysed so that specific inhibitors against *Leishmania* can be designed.

### Protein kinases

Cyclin-dependent kinases (CDKs) are reported to play a significant role in cell division cycle, differentiation, transcription, and apoptosis. For their own activation, few CDKs requires active cyclins and for functional activation, few require phosphorylation which could be accomplished at their conserved threonine residues (t-loop) by cdc2 activating kinases (Affranchino *et al.* 1993). So in *Leishmania*, these cdc-2 activating kinases which are essential for the progression of the cell cycle can be used as potential drug targets. Two putative CDKs, LmexCRK3 and LmexCRK1 (Hassan *et al.* 2001) have also been found to be essential to the promastigotes form. CRK3 belongs to indirubin class, is found to be active throughout the life cycle in *L. mexicana* (Grant *et al.* 1998; Hassan *et al.* 2001). The chemical inhibitors of CRK3 deteriorate parasitic viability within macrophage, thus CRK3 can be used as a potential drug target. In another study on *L. donovani*, recently it was found that glycogen synthase kinase (LdGSK3) is also associated with the control of cell cycle and apoptosis based on the indirubin test (Xingi *et al.* 2009). Thus, LdGSK3 in combination with CRK3 can be used as possible drug targets. Similarly, other cdk's can also be diagnosed as potential drug targets.

### Mitogen-activated proteins kinases (MAPK)

Mitogen-activated protein (MAP) kinases play a significant role in signal transduction. Moreover, they act as a regulator of cell differentiation, proliferation and apoptosis. Until now, 15 MAP kinases have been identified in *L. mexicana* (Wiese, 2007) and the most studied are LmxMPK, LmxMPK9 and LmxMCK (Bengs *et al.* 2005). These MAP kinases can further be evaluated as a potential drug target.

### Proteinases

Proteinases are of four main types: serine, aspartate, cysteine and metalloenzyme. In case of parasitic protozoans most identified and characterized are cysteine proteinases (CPs). Because of structurally different from mammalian homologue and their role as a putative virulence factor in parasite–host cell interaction CPs have become of great interest as a potential drug target. More than 65 CPs have been observed from the genome sequence analysis of *L. major* and are further categorized into various types viz. CPA, CPB and CPC. Further gene replacement studies of *L. mexicana* revealed the role of CPA, CPB and CPC in host–parasite interaction. In a study when multicopy CPB gene array (Dcpb) was replaced by *L. mexicana*, BALB/c mice was observed with poor lesion growth with reduced virulence (Alexander *et al.* 1998). Some highly potent inhibitors (Semicarbazones, Thiosemicarbazones and Triazine Nitriles) of *L. mexicana* cysteine protease CPB have been reported that may also have useful efficacy against other important cysteine proteases (Schröder *et al.* 2013).

### Methionine aminopeptidase 2 (MetAP-2)

MetAP-2 is a cellular metalloprotease, participates in the late hydrolysis of the initiator methionine of protein synthesis (Walker KF, 1998). Fumagillin which is an inhibitor of MetAP-2 prevent the replication of *L. donovani* (Zhang *et al.* 2002).

### Microtubule-associated protein (MAP2)

In past, parasitic microtubules have been identified as a potential target but due to the high toxicity of antimicrotubule drugs, its further use in regular practice was restricted. MAP2, an ethyl

3-(chloroacetamido)-benzoate that is effective on diverse species of *Leishmania*, alters the organization of microtubule in the parasite. Its *in vivo* activity was observed in an *L. major* mice model by the important reduction of the parasite load in the liver, spleen and lymph node (Hiam *et al.* 2006).

### Enzymes of sterol biosynthesis

The major growing sterols in *Leishmania* are ergosterol and stigmasterol, which is different from mammalian complimentary cholesterol. This can be used as an effective drug target. Ergosterol has two main functions: (1) it is a construction unit of the cell membrane and (2) it might play a significant role in the hormonal activity. Because of sterol based mechanism, AmB reveals its effectiveness against Leishmaniasis but due to AmB resistance for *Leishmania*, it orders investigators to search an alternative drug of AmB. Azasterol, a class of *s*-adenosyl-L-methionine performs antileishmanial activity by inhibiting 24-methyltransferase (the vital enzyme in ergosterol biosynthesis) (Lorente *et al.* 2005). Other compounds such as azole and triazole inhibit 14 $\alpha$ -methylsterol 14-demethylase. Thus are effective against *Leishmania*. During the development of the novel drug, it must be in consideration that when the sterol profile of *Leishmania* get alters it metabolizes host sterol for its survival (Roberts *et al.* 2007).

### DEAD BOX RNA helicase

DEAD-box RNA helicases are important in tumorigenesis and viral infection (Ariumi, 2014) so it represents a potent novel therapeutic target (Xu *et al.* 2011). In the case of *Leishmania*, ATP-dependent DEAD-box RNA helicase plays important role in RNA metabolism and cellular signalling. Recently it has been reported that a 67 kDa DEAD-box ATP-dependent RNA helicase preventing antisense rRNA cleavage, and protecting rRNA from degradation and *Leishmania* from cell death (Padmanabhan *et al.* 2012). Further *Leishmania* DDX3 homologue of DEAD-box RNA helicase plays a central role in mitochondrial protein quality control under normal growth conditions and particularly upon stress by preventing ROS-mediated damage and polyubiquitinated protein accumulation in the mitochondrion (Padmanabhan *et al.* 2016). Our unpublished QSAR study also suggested many inhibitors for DEAD-box RNA helicase.

### Casein kinase 1 isoform 2 (CK1.2)

Casine kinase is a member of the highly conserved Ser/Thr protein kinase family which play important role in signal transduction (Knippschild *et al.* 2005). CK1 contains six isoforms in *Leishmania*. LmjF35.1010 (LmCK1.2), the major isoform, has been validated pharmacologically as a drug target because CK1 shown to be essential for intracellular parasite survival and infectivity (Durieu *et al.* 2016). Two lead compounds have also been identified and selected as LmCK1.2 inhibitors based on their specificity, antileishmanial activity, the absence of cytotoxicity (Durieu *et al.* 2016). There is a further need to understand their mode of action and these compounds could also be used as pharmacological tools to study parasite-specific signal transduction.

### L-asparaginase

L-asparaginases enzyme belongs to amidohydrolases family that catalyse the conversion of L-asparagine into L-aspartic acid and ammonia (Vimal and Kumar, 2017). The presence of genes coding for putative L-asparaginase enzymes in the *L. donovani* genome has given a hin towards the specific role of this enzyme in the survival of

the parasite. Singh *et al.* (2017) have advocated that L-asparaginases could be a potential drug target against the pathogen *Leishmania*.

### Challenges in antileishmania drug development

The current pace of antileishmanial drug development is very slow and it should be kept up with the clinical needs, particularly as resistance to current agents is being reported frequently and drug resistance mechanisms should be explored. The further slow pace of new antileishmanial drug development is due to: (1) different clinical manifestations, (2) a deficient market, (3) failure of public-health policy, (4) lack of public-private partnerships (PPPs) and (5) lack of technology transfer.

### Clinical problem

Leishmaniasis is caused by 21 of 30 species of *Leishmania* that infect mammals (<https://www.cdc.gov/dpdx/leishmaniasis/index.html>). These include VL caused by the *L. donovani* complex; MCL is caused by parasite belongs to the braziliensis complex, such as *L. braziliensis*, *L. panamensis*, *L. peruviana*; and CL is caused by *L. major* in the Old World but in America this clinical form could be caused by parasite belongs to the subgenus *Leishmania* and *Viannia*. The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, DNA sequence analysis or monoclonal antibodies (Hijawi *et al.* 2016). Among CL, MCL and VL, VL is a fatal form in which the parasites have migrated to the vital organs (liver, bone marrow and spleen) of the body (Kumar *et al.* 2015). Up to 90% of the world's cases of VL are found in Bangladesh, Brazil, India, Nepal and Sudan. Wide and vast clinical manifestation of this disease is one major challenge in antileishmanial drug development (Pace, 2014).

### Deficient market

Food and Drug Administration (FDA), USA approved thousands of new chemical entities from 1995 to 2016, but only a few were for infectious diseases. Among these anti-infectious agents, Miltefosine was approved as the only antileishmanial agent in 2014 in last 21 years (<http://www.centerwatch.com/drug-information/fda-approved-drugs/year/2016>). There is a more than the 10-fold greater chance of a drug being brought to market for cancer, neurological disorders than for infectious disease. This number is negligible and matter of sorrow for the neglected disease leishmaniasis. Because the pharmaceutical industry argues that drug development is risky and too costly to invest in the low-return area such as leishmaniasis. This a most prominent reason for a limited number of new antileishmanial drugs marketed. The drugs for the neglected diseases working group is currently exploring the feasibility of not-for-profit initiative internationally that would focus on drug development projects for the most the neglected diseases like leishmaniasis. Such an initiative would remove the process of life-saving drugs development from a market-driven logic.

### Lack of strong public-health policy

Public-health policy has an intense impact on health status. Good policies are not in use in developing and underdeveloped countries. Most of the developing countries spent <\$20 on all health programme per year per person (Sachs, 2002). In these circumstances, the market is too small to attract investors in the development of the drug for the diseases that mainly affect developing countries. The chronic neglected disease crisis calls for a substantial health policy and long-term response worldwide that and will

definitely create a paradigm shift in health, research and development. The evidence sheds light that the public-health policy issues are limited in developing and underdeveloped countries. There should be an international pharmaceutical policy for all neglected diseases and a PPP sector come up and promote not-for-profit research and development capacity.

### Public-private partnerships

Measures should be taken to overcome the above-discussed constraint and PPP could be a solution. It attempts to fill the gaps in the health needs of developing countries through the establishment of public-private networks and collaboration. The public sector includes aid agencies, international organizations, academia and government bodies and the private sector includes pharmaceutical industries, foundations, philanthropic institutions, and charities. Recently, some PPPs have started drug development activities in the pharmaceuticals sector, as a new paradigm. The PPP concept has recently evolved into product-based and more structured collaborations. The tropical disease research (TDR), WHO and the Walter Reed Army Institute of Research (WRAIR), Maryland, USA is a good example of public institutions working in partnership with the private sector on drug development programmes. The antimalarial drug mefloquine was discovered by the WRAIR and later developed jointly with industry and TDR (Gelband *et al.* 2004). However, company engagement is limited and doing initiative for AIDS, Malaria and Tuberculosis, because these diseases rank higher in the public-health priorities of developed countries than other neglected diseases and represent a potential market for the industry. For the neglected diseases such as leishmaniasis, PPP engagement and effort should be more and more.

### Technology transfer

Capacity building and technology transfer have been promoted for many years by TDR, United Nations Development Programme, and Drugs for Neglected Diseases Initiatives (DNDI). FDA, NIH and several US university technology transfer offices have compiled a list of their technologies that relate to these specific neglected diseases (Allarakhia, 2013). This list is expected to function as an additional tool to facilitate the transfer of such technologies to potential licensee institutions worldwide for the development of effective medicine against neglected disease. Adopting approaches to transfer technologies offers the broadest benefit to populations in need. Practical issues confronted by technology transfer would be helpful in the development of products in neglected disease areas.

### Conclusion

Despite impressive advances in science and technology we have failed to allocate sufficient resources to fight the diseases leishmaniasis. There are a deficiency and sluggishness of research and development into neglected diseases to control the re-emergence, develop more effective drugs, and overcome resistance for leishmaniasis to shorten the treatment. Only a few drugs are available for the treatment of leishmaniasis and the appearance of drug resistance is further complicating the control of this disease. A wide range of biological assays of the possible compounds should be performed with several strains and different parasite forms, and active compound should be reached to clinical trials immediately. To meet these challenges, a firm commitment by the pharmaceutical industry partnering with academics, novel chemical libraries and combining innovative screening strategies is required to achieve victory. This suggests that there is a need to come up with newer hit/leads which may help furthermore

to combat with leishmaniasis and slow down the potential emergence of resistant *Leishmania* parasites.

**Acknowledgements.** Authors are thankful to Kumaun University SSJ campus, Almora (Uttarakhand), India and National Institute of Technology (NIT), Raipur (CG), India for providing a facility for this work.

**Financial support.** This study was financially supported by the Science and Engineering Research Board (SERB, India) young scientist grant YSS/2014/000773 awarded to M.S.. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of interest.** The authors have declared no conflict of interest.

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