Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Leishmaniasis: Current status of available drugs and new potential drug targets

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ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 15 March 2012
Accepted 15 April 2012
Available online 20 June 2012

Keywords:
Leishmaniasis
Drug resistance
Drug targets
Drugs

ABSTRACT

The control of *Leishmania* infection relies primarily on chemotherapy till date. Resistance to pentavalent antimonials, which have been the recommended drugs to treat cutaneous and visceral leishmaniasis, is now widespread in Indian subcontinents. New drug formulations like amphotericin B, its lipid formulations, and miltefosine have shown great efficacy to treat leishmaniasis but their high cost and therapeutic complications limit their usefulness. In addition, irregular and inappropriate uses of these second line drugs in endemic regions like state of Bihar, India threaten resistance development in the parasite. In context to the limited drug options and unavailability of either preventive or prophylactic candidates, there is a pressing need to develop true antileishmanial drugs to reduce the disease burden of this debilitating endemic disease. Notwithstanding significant progress of leishmanial research during last few decades, identification and characterization of novel drugs and drug targets are far from satisfactory. This review will initially describe current drug regimens and later will provide an overview on few important biochemical and enzymatic machineries that could be utilized as putative drug targets for generation of true antileishmanial drugs.

1. Introduction

The leishmaniases are a wide spectrum of vector born disease with great epidemiological and clinical diversity. It is caused by more than 20 species of protozoan parasite that belongs to family kinetoplastida and genus *Leishmania*. The disease is spread by more than 30 species of *Phlebotamine* sand fly in old world and *Leutzomia* in new world[1]. The digenetic life cycle of *Leishmania* consists of motile, flagellated, extracellular promastigotes form in the gut of sand fly vector that infects mammalian host and transform into nonmotile, nonflagellated amastigotes form, which survive and multiply within phagolysosomal compartment of macrophages. Leishmaniasis has traditionally been classified into three major clinical forms: visceral (VL), cutaneous (CL), and mucocutaneous leishmaniasis (MCL), which differ in immunopathologies and degree of morbidity and mortality. Most VL caused by *Leishmania donovani* is fatal if untreated, whereas CL caused by *Leishmania major*, *Leishmania mexicana*, *Leishmania braziliensis*, and *Leishmania panamensis*, frequently self cures within 3-18 months, leaving disfiguring scars[2].

Over more than 90% cases of VL ensue in five countries: India, Bangladesh, Nepal, Sudan and Brazil and 90% of CL cases occur in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria[3]. However, these numbers only reflects the reported cases and there is gross under reporting of cases in endemic areas that hide the actual severity of the disease[4]. Although, spread of disease in endemic and non-endemic regions is multi-factorial but lack of effective control measures for both, parasite and its vector are main factors. The poor knowledge about the disease and lack of effective health policies are the primary hurdles in the elimination of leishmaniasis from every

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Foundation prject : The financial supports received from Department of Biotechnology, New Delhi (BT/PR11177/MED/29/99/2008) is greatly acknowledged. This article is dedicated to all those poor people who lost their lives during drug trials in various parts of the world.

corner of the world is far from reality.

Soon after realization that *Leishmania* causes this disease, generic pentavalent antimonials have been the cornerstone of leishmanial chemotherapy in disease endemic countries especially in Indian subcontinent[5]. In addition, branded sodium stibogluconate and meglumine antimoniate are the alternatives of generic antimonials. The other second line drugs like amphotericin B, its liposomal formulations and miltefosine are being used in the treatment with more efficacies and dramatic potential for curing leishmaniasis however, they are comparably costlier than the generic antimony[6]. Other drugs like paromomycin and pentamidine have shown some usefulness and could be a potential supplement in the drugs regimen but their use and availability in disease endemic regions is limited[6-8].

Identification and characterization of cellular targets and answering the problem of drug resistance in leishmaniasis has always been the main thrust of protozoan research worldwide. The recent advancements in innovative animal models and parasites with reporter gene constructs have provided rapid and high through output drug screening methods in both, *in vivo* and *in vitro*[9,10]. Unfortunately no vaccine candidates either prophylactic or preventive are under animal or clinical trials[11-13]. Thus it becomes significantly important to search an effective drug/s and/or a prophylactic vaccine/s for an early and effective control of leishmaniasis. This review gives an overview of 1) drugs that are being used and/or are still in phase of clinical trials, 2) mechanisms of drug action and resistance, 3) and finally elaborates biochemical machineries and enzymes as new potential drug targets.

2. Current scenario of available drugs

2.1. Pentavalent antimonials

Pentavalent antimonials, the generic sodium stibogluconate (pentosam) and branded meglumine antimoniate *etc*, are being used in the treatment of leishmaniasis over more than five decades and still they are the first line drugs of choice where resistance is not reported[14]. The growing incidence of resistance has raised serious concern for its use in disease endemic area[15]. The pentavalent antimoniate (Sb^{V}) considered as a pro-drug, which is further converted to trivalent antimonite (Sb^{III}), an active form of the drug albeit to the parasite is also susceptible to Sb^{V}[16]. The reduction of pentavalent to trivalent compound takes place either in macrophages or in the parasite however, it is still a dilemma[17]. Parasite mediated reduction has been found to be associated with the loss of reductase activity of parasite, which may also lead to drug resistance. This is supported by the observation that Sb^{V} resistant *Leishmania donovani* amastigotes lose their reductase activity. The recent finding of a parasite thiol dependent reductases (TDR) 1 enzyme, that catalyze the conversion of Sb^{V} to Sb^{III} using glutathione as a reductant also supports this possibility[18]. In addition, arsenate reductase 2 (ACR2) a new antimoniate reductase characterized in *Leishmania* sp. increases sensitivity of parasites to Sb^{V}[19]. It has also been reported that this reduction takes place primarily in macrophage rather than parasite[20]. The supporting evidences that come from organisms like bacteria and yeast, where the metal reduction is mediated by host specific enzymes suggests that this conversion is host specific[21].

The routes of antimonials entrance into *Leishmania* and macrophages are not known. However, parasitic aquaglyceroporin, aquaporin 1 transporter is supposed to be responsible for the transport of antimonials into amastigotes[22]. In addition, the transport of Sb^{V} via phosphate transporters is based on the fact that pentavalent arsenate, a metal related to Sb^{V}, is able to enter the parasite[21]. Both form of antimonials Sb^{V} and Sb^{III}, kills *Leishmania* species by DNA fragmentation, suggesting the role of apoptosis, β-oxidation of fatty acid and adenosine diphosphate phosphorylation. However, the exact mechanisms of action are still unexplored[23-25]. In addition, the antimonials inhibit glycolysis and metabolic pathways and increases efflux of intracellular thiols by a ATP binding cassette (ABC) transporter, multi drug resistant protein A (MRPA)[26]. Pentamonials are also known to inhibit trypanothion reductases, an enzyme responsible for protection from host reactive oxygen and nitrogen species to parasites[27].

The widespread misuse of drug, as it was easily available over the counters in endemic regions; along with loss of drug activation by parasites are the major causes of acquired resistance. The *in vitro* studies on Sb^{V} resistant *Leishmania* axenic amastigotes and promastigotes indicate their diminished ability to reduce Sb^{V} to Sb^{III}[10]. A study on amastigote and promastigote forms of Sb^{III} resistant *Leishmania*, have shown reduction in accumulation of metals due to either reduced uptake or increased efflux[28]. Overexpression of a heat shock protein (HSP70) gene has been found to be associated with the antimonial resistance[29]. The transporters of ABC family, MRPA and pentamidine resistant protein 1(PRP1) that act as efflux pump for antimonials,are also linked to antimony resistance[15,30]. Further, various genes identified in antimonial unresponsive clinical isolates suggests the multifactorial mechanism of resistance[31-34].

2.2. Amphotericin B (AmB)

It is a polyene antifungal drug widely used to treat systemic fungal infections[35]. In endemic areas of Bihar where antimonials resistance is common, AmB is the drug of choice[36]. AmB shows high affinity for ergosterol, the predominant sterol of fungal and leishmanial cell membrane. Despite its high efficiency, AmB is also toxic and its side effect has been reported[37,38]. Adverse effects of plain AmB have been circumvented with its three clinical formulations in which deoxycholate have been replaced by other lipids. These formulations are liposomal AmB (L-AmB: Ambiosome), AmB colloidal dispersion (ABCD: Amphocil) and AmB lipid complex (ABL: Abelcit). These lipid formulations of AmB retain their antifungal activity and show very high efficacy to cure this deadly disease and

are less toxic. In VL cases, liposomal AmB has been proved as an efficient drug with more than 95% efficacy but high cost limits its use to common man suffering from this deadly disease.

The antileishmanial activity of AmB and its lipid formulation is due to its interaction of both sterols *i.e.* ergosterol of *Leishmania* and cholesterol of host macrophages. Since cholesterol is complexed by AmB, it markedly inhibits binding *Leishmania donovani* promastigotes to macrophage[39]. Further, at higher concentration (<0.1 M), it induces the formation of aqueous pores in leishmanial promastigotes cell membrane that result in osmotic changes leading to the cell lysis[40]. Inspite of excellent efficacy the administration of AmB is also associated with the toxicity and emergence of parasitic resistance. The damaging effect of AmB in kidney tubular cell is mainly due to increased salt and Ca^{2+} concentration, H^{+} permeability across the aqueous pores that lead to sustained collapse of pH and Ca^{2+} gradient across the membrane, a mechanism responsible for apoptosis in eukaryotic cells. The *in vitro* studies demonstrate that resistant *Leishmania* lacks ergosterol, the main target of AmB[41]. In *Leishmania donovani* AmB resistant strain, parasitic cell membrane lacks C-24 alkylated sterols that might be due to inactivation of enzyme S-adenosyl methionine transferase which is responsible for alkylation at C-24 position in ergosterol moieties leading to resistance[42]. Another study has shown that resistance to AmB was found to be associated with gene TarII 64.4 and tarII 512.2 amplification in *Leishmania tarentolae* mutant cell lines[43]. Till date clinical resistance against AmB is not reported but the relative nonspecific mode of action of AmB at the level of membrane may be a factor for its infrequent resistance. It has been shown in a study that success of AmB treatment greatly depends on patient immunity status and indicate that successive relapse could enhance emergence of AmB resistant isolates[44,45]. These finding warrants the possibility of resistance against the most successful drug.

2.3. Miltefosine

Miltefosine is originally developed as anticancerous agent, which is an alkylphosphocholine (hexadecylphosphocholine) moiety[46]. It is the first oral drug used for the treatment of VL and was considered a major breakthrough in anti-leishmanial chemotherapy[47,48]. Its phase I/II/III trials provoked a storm of protection against VL that was followed by phase IV trial, which also proved its relevance in outpatient setting in those areas where VL is endemic[49,50]. The combination of miltefosine and ambisome has also been evaluated, and has been found effective with good tolerability but unfortunately side effects raise questions against extreme efficacy of this combinatio[51]. The main adherence of the drug is compromised by its long terminal residence, time and teratogenicity. Miltefosine has a median long half-life of approximately 152 hours, which could encourage development of clinical resistance. Further, its teratogenic and abortifacient nature limits its use in pregnancy.

The activity of miltefosine is due to intracellular accumulation of drug, which is regulated by two transporters, LdMT and its β-subunit LdRos3, a P-type ATPase, belonging to aminophospholipid translocase family[52]. In studies on resistant mutant of *Leishmania*, it is observed that due to decreased influx, accumulation of miltefosine inside the parasite is greatly hampered[52]. Although, the exact mode of antileishmanial action is still unclear but it has been found that it causes apoptosis like processes in *Leishmania donovani* as observed in amastigote but how it happens, still unknown[53]. Miltefosine also reduces the lipid content in promastigotes membrane and enhances the phosphatidylethanolamine content suggesting a partial inhibition of phosphatidylethanolamine-N-methyltransferase that leads to decreased parasite proliferation[54].

The clinical resistance is not yet reported but being an oral agent its improper use in endemic countries like India increases the probability of resistance and spread of resistance parasites where prevalence of infection is significantly high. This is now being observed that few patients after successful treatment with miltefosine relapsed after 9-12 months. However, more studies are required to understand whether these are relapse or reinfection or resistance. Broadly, decreased drug efflux is the main reason for miltefosine resistance in *Leishmania*, though many mechanisms are reported for this decreased intracellular drug concentration. It has been reported that single point mutation at LdMT and LDRos3 in experimental leishmaniasis may lead to resistance. In addition to LdMT, over expression of multidrug resistant MDR1 gene which encodes a glycoprotein is also responsible for drug resistance[55]. Furthermore, miltefosine resistance is also correlated with lipid content in parasite membrane. It has been observed that the amount of unsaturated phospholipid alkyl chains was lower in miltefosine resistance parasites[56].

2.4. Paromomycin

Paromomycin is chemically an aminoglycosidic antibiotic and has both antileishmanial and antibacterial activity. Paromomycin cures both, VL and CL (more effectively) but limited availability restricts its use in endemic regions[57,58]. A controlled study on the efficacy of topical paromomycin sulfate and methylbenzethonium chloride in CL has shown total elimination of parasite within the first 10 days of treatment[59]. The Indian and Sudanese trials further demonstrate its high efficacy and excellent tolerability, which have also been comparable to AmB[60,61]. A phase II study in Tunisia and France has shown that WR279, 396, a formulation of paromomycin and gentamicin was found safe and highly effective in treatment of CL. It offers great potential as a new, simple, easily applicable and expensive topical therapy for CL[62].

The mechanism of paromomycin action is largely unclear. Its *modus operandi* in *Candida krusei* supports cytochrome C inhibition but in *Leishmania* requires further elucidation. Recently it has been shown that cationic paromomycin binds to the negatively charged leishmanial

glycocalyx suggesting mitochondria as a primary target[63]. In addition, paromomycin inhibits translocation and recycling of ribosomal subunits and hence protein synthesis. Paromomycin in *Leishmania donovani* promotes association of 50S and 30S subunits of both, cytoplasmic and mitochondrial ribosomes and stops their recycling that eventually inhibits protein synthesis[64]. Further exploration came from the study of Hirokoma *et al* that proves that paromomycin interacts with both 30S and 50S subunits without inhibiting the association of translation initiation factor-3 (IF3) to the 30S ribosomal subunit[65]. Due to its limited use resistance is not yet reported in outpatient treatment but resistance has been reported *in vitro* in *Leishmania donovani* and *Leishmania tropica*[63,66]. However, rapid emergence against paromomycin due to its aminoglycosidic nature cannot be over ruled.

2.5. Sitamaquine

Sitamaquine, chemically 8-aminoquinoline, is the only drug that was developed for treatment of VL. It was originally developed in collaboration with GlaxoSmithKline and Wlater Reed Army Institute[49]. The advantage of this drug is its oral administration. The phase II trial of sitamaquine in India demonstrated its efficacy against VL and was well tolerated[67]. However, despite efficacy few side effects like vomiting, dyspepsia, cyanosis, nephritic syndrome and glomerulonephritis were also observed. The consequences of Kenyan phase II trial were different from Indian trial[68]. The Kenyan trial showed somewhat equal efficacy but observed side effects were abdominal pain, headache and kidney dysfunctioning.

Sitamaquine at high concentration affects parasite motility, morphology and growth[69]. Mechanism of its action involves electrostatic interaction between phospholipid anionic polar head groups and positively charged sitamaquine and then with phospholipid acyl chains leading to drug insertion within biological membranes[70]. After binding to the membrane, sitamaquine accumulate in *Leishmania* cytosolic acidic compartments, acidocalcisome. However, correlation between its action and accumulation is not clear[71]. There is transient affinity between sitamaquine and membranes and also energy dependent efflux was demonstrated, suggesting the presence of an uncharacterized transporter[70]. Although, resistance against this drug has not been reported yet in clinical practices but, *in vitro* resistance against *Leishmania donovani* promastigote has been reported by selecting drug pressure of sitamaquine at 160 μm concentration[72]. In one of the study, conducted on cutaneous leishmaniasis caused by *Leishmania major* on BALB/c mice, sitamaquine dihydrochloride did not reduce the parasite burden and lesion progression was continued. The lack of its efficacy and activity seriously restricted further clinical trials[73]. However, with available status of knowledge, more studies are required to understand its efficacy, mode of action as well as toxicity.

2.6. Pentamidine

Pentamidine is an aromatic diamine used to cure leishmaniasis as a second line drug. Its isothionate and methansulphonate salts are mainly used for the treatment of VL. It was initially used to treat Sb^V refractory patients in India but its declining efficacy and high resistance risk has led to its closure in India. Some combinational strategies have also been tried with this drug. A study on antimony unresponsive patient revealed that combination of low dosage of pentamidine and allopurinol as compare to the full dosages of pentamidine are more effective and less toxic with and ultimate cure of 73% and 58%, respectively[74]. However, its efficacy is questionable in comparison to other drugs. A comparative study on pentamidine and meglumine antimoniate (glucantime) against CL due to *Leishmania braziliensis* in Peru shows that glucantime was more effective than pentamidine[75]. However, it has been found more effective to curb CL caused by *Leishmania panamensis* and *Leishmania guyanensis*[76,77].

Although, its precise mode of action is not known, it is reported that the drug enters inside *Leishmania donovani* promastigote through arginine and polyamine transporters[78,79]. In a biochemical study it was found that in pentamidine resistant, *Leishmania donovani* and *Leishmania amazonensis* promastigote clones, drug resistivity is due to decreased uptake followed by increased efflux of drugs. There is alteration in polyamine carrier that might be responsible for the alteration in surface protein nature and content leading to decreased influx of drug. Furthermore, this drug gets accumulated in mitochondria and enhances efficacy of mitochondrial respiratory chain complex II inhibitors suggesting its leishmanicidal activities due to decreased mitochondrial membrane potential. It is also reported that it inhibit mitochondrial topoisomerase II[80]. Pentamidine is highly toxic; causes hypoglycemia, nephrotoxicity and hypotension *etc*. Pentamidine resistance mechanism is not well understood, but intracellular ABC protein PRP1 can confer resistance to pentamidine in intracellular stage of *Leishmania*[81].

Despite the significant progress that has been made during last few years in chemotherapies for leishmaniasis, all drugs of this regimen have some limitations like price, feasibility, safety, efficacy, toxicity, side effects and probability of growing resistance. The most important consideration of present therapeutics is gradual increase in resistance to antimonials in particular and others in general, predominantly in Indian subcontinents. In recent years, clinical trials of combination therapies are operational[6]. Combination of two or more drugs could reduce treatment duration and drug doses and consequently drug toxicity but probabilities of resistance development against current available drug regimen cannot be denied. The main combination drugs currently under consideration are LAmB (Liposomal amphotericin B) and miltefosine, LAmB and paromomycin, LAmB and antimonials and paromomycin and antimonials in India and also being evaluated in other part of the world (www.clinicaltrials.gov). There is no vaccine candidate available, and current recent progress

on leishmanial research does not guaranty about future availability of candidate leishmanial antigens/immunogens. Hence, identification of novel drug targets and development of true antileishmanial agents should be the priority area of research.

3. New potential drug targets

Leishmaniasis is the only tropical disease, which is being treated by non-leishmanial drugs. Moreover, the exact mechanisms of action of these drugs are not clearly understood. The recent research funding from various organizations like WHO/TDR, Tropical Medicine Research Centre, USA and European Commission *etc*, to disease endemic countries like India and Sudan, only encourage clinical trials and diagnostic evaluation studies. Recent publications in leishmanial research reveals that focus is being made only on drug trials/combination therapy of available non-leishmanial drugs, evaluation of diagnostic and prognostic capability of available tools, and very little emphasis is being paid on other aspects by leishmanial biologist and researchers. In the past several decades very little emphasis has been made on novel control strategies in terms of new drug targets and vaccine candidates.

Search of new potential drug targets mainly focus on biochemical and metabolic pathways essential for parasite survival (Figure 1). The target enzymes of these pathways should have significant structural and functional difference from its mammalian counterparts for selective inhibition of target sites. Further, strategies to target more than one enzyme of a metabolic pathway simultaneously may prove more usefulness and effectiveness.

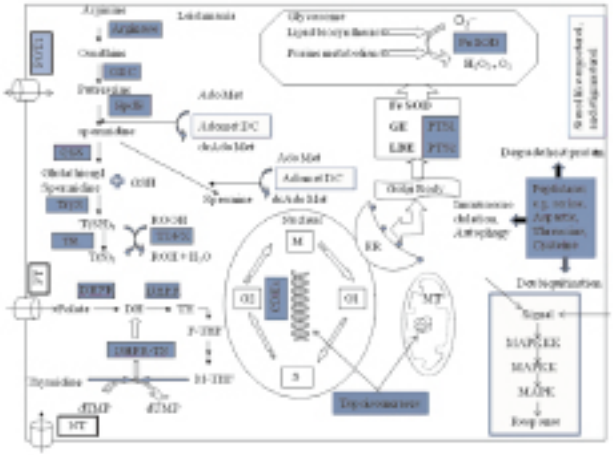

Figure 1. An overveiw of potential drug targets in *Leishmania* species. The coloured boxes represent the potential drug targets. ODC: ornithin decarboxylase, SpdS: spermidine synthase, TR: trypanothion reductase, TDPX: trypanodoxin peroxidase, DHFR: dihydrofolate rudutase, DH: dihydrofolate, TH: tetrahydrofolate, DHFR-TS: dihydrofolate rudutase thymidylate-synthase, M-THF: methylene tetrahydrofolate, dTMP: deoxy thymidine monophosphate, dUMP: deoxy uridine mono phosphate, MAPKKK: mitogen activated proteins kinase kinase kinase. MAPKK: map kinase kinase, MAPK: map kinase, CDKs: cyclin dependent kinase, NT: nucleotide transporter, FT: folate transporter. MT: mitochondria, ER: endoplasmic reticulam.

3.1. Enzymes of polyamine biosynthesis

The putrescine, spermidine and spermine, like polyamine play important role in growth and differentiation of parasite from promastigote to amastigote stage[82]. Polyamines not only involves in parasite growth and differentiation but also down regulates lipid peroxidation generated by oxidants compounds and makes the environment compatible for survival[83]. There is reduced polyamine metabolism during initial phase of parasite adaptation in varying environment from vector to host[84]. Parasites overexpress arginase, ornithine decarboxylase, s-adenosylmethionine decarboxylase (Adomet DC) and spermidine synthase, the enzymes involved in polyamine metabolism. However, polyamine pool remains unchanged or marginally affected during their growth and metabolism that imply existence of regulatory mechanisms[85]. Any of these regulatory mechanisms offer a greater possibility for a future drug target.

In *Leishmania*, arginine is converted to L-ornithine by enzyme arginase. L-ornithine is further converted to putrescine through decarboxylation by enzyme ornithine decarboxylase, which in turn converted to spermidine and spermine, the substances responsible for cell growth and proliferation of *Leishmania* as well as Th2 type response that is responsible for pathogen survival in mammalian host[86]. Being first step of polyamine metabolism, targeting arginase pathway will be highly beneficial. Inhibitors of polyamine biosynthetic pathway have shown antileishmanial activity. Adomet DC inhibitor cures animal leishmaniasis but have not been tested on humans and seeks further experimental studies[87]. However, failure of alpha-difluoromethylornithine, a polyamine inhibitor used to treat trypanosomiasis, to cure leishmaniasis proves some differences between two closely related parasites. Therefore, more efforts are needed to discover *Leishmania* specific polyamine inhibitors[88]. The polyamine transporters (*Lm*POT1) that transport both putrescine and spermidine are also good targets that regulate the intracellular polyamine level. Hence, development of inhibitors to stop polyamine biosynthesis and transportation may be quite useful as novel antileishmanial therapies.

3.2. Peptidases

Peptidases are increasingly being seen as potential drug targets[89]. Therapeutically, peptidase inhibitors have been successfully introduced to treat HIV, hypertension, pancreatitis and multiple myeloma. A total of 154 peptidases were found to be present in the *Leishmania major* genome, with example of serine, cysteine, aspartic, threonine and metallopeptidases. Two aspartic peptidases were found in the *Leishmania major* genome sequence, one with sequence similarity to presenlin1 (PS1), which is a multipass membrane peptidase and the other with intramembrane signal peptide peptidase (SPP)[90]. PS1 is potentially involved in autophagy while SPP cleaves the transmembrane domain

of signal peptidase that may be vital drug target[91].

Twenty-one threonine peptidases have been found in the *Leishmania major* genome, though all are classified as proteasome subunit[91]. The proteasome is a multisubunit, multicatalytic peptidase responsible for degradation of ubiquitinated proteins in the cytosol[92]. Similar to plasmodial and trypanosomatids proteasome, the proteasome of *Leishmania* is a potential therapeutic target, as the use of specific inhibitors has shown the proteasome to be necessary for growth of *Leishmania maxicana* promastigotes and amastigotes *in vitro*[93].

The cysteine peptidase of the papain family are extensively studied peptidase of *Leishmania*, comprising the cathepsin-L like peptidases CPA and CPB, and the cathepsin-B like peptidase CPC, which are lysosomal. Neither CPA nor CPC are essential for survival of *Leishmania maxicana* in the host but, CPB has been found to be a virulence factor and may be targeted in initial phase of disease[94]. In addition, the use of an inhibitor specific to cathepsin-L like cysteine peptidase, K11777, has shown that these peptidases are necessary for the growth of parasites that indicates potential of the peptidase as a drug target[95].

Among several serine peptidases of protozoan, subtilisin-like serine peptidase, a part of the secretary endosomal system, which participate in processing of secreted proteins may be very useful as drug target. It has been shown that the subtilisin- like peptidases of *Plasmodium* are potential drug targets[96]. Investigation of the effect of serine peptidase inhibitors on the survival of *Leishmania* has shown that TPCK(N-tosyl-l-lysyl-chloromethylketone) and benzamidine both reduces viability and induce morphological changes in the *Leishmania amazonensis* promastigotes, suggesting serine peptidases could be useful potential drug targets[97].

3.3. Enzymes of glycosomal machinery

Leishmania like other trypanosomatids depends solely on their host for carbon source to fulfill its energy requirements. The amastigotes uptake blood glucose from mammalian blood stream and other essential component like fatty acids, amino acids receives from phagolysosome of macrophages. In trypanosomatids glycosomes, peroxisome like organelle, are found. These glycosomes plays important role in many metabolic activities like glycolysis, oxidation of fatty acid, lipid biosynthesis, and purine salvage pathways *etc*. Due to the result of these metabolic activities superoxide radicals are generated as side products in large amount. To protect glycosomal enzymes from superoxide radical toxicity Fe-superoxide dismutase (FeSOD´s) are evolved in *Leishmania* species. In *Leishmania chagasi* two different types of FeSOD, Lcfesodb1 and Lcfesodb2 have been characterized, which are differentially expressed in stationary promastigotes, amastigotes and early logarithmic promastigotes stage, respectively. These are responsible for survival and protection from lethal superoxide radicals within glycosomes[98]. More importantly, FeSod is absent in mammalian counterpart, so it could be used as effective drug target.

Moreover, inside glycosome first seven steps of glycolysis occur whereas rest three occurs in cytosol. Enzymes hexokinase (HKK), phosphofructokinases (PFK) are autocatalytic enzymes and their hexose phosphate intermediates accumulation are lethal for parasite, thus in trypanosome unique mechanism has been evolved to escape this lethality. The first seven steps of glycolysis have no net gain of ATP and rest steps occur in cytosol and that result in ATP production. This unique compartmentalization regulates HXK and PFK autocatalysis, which depend on ATP[99]. These glycolytic enzymes are essential for parasite survival and enzymes of glycosomes may be targeted due to presence of unique targeting signal sequences, PTS1& PTS2 present either on N-terminal or C-terminal end of these enzyme. If the targeting sequences are disrupted may lead to mistargeting and degradation of essential enzymes of glycolysis that creates lethality[100]. Thus unique organization of glycolytic pathway and evolutionary distance from mammalian host posing leishmanial glycolytic intermediate enzymes could be potential drug targets, however due to conserved glycolytic machinery single enzymatic inhibition will not be sufficient to marshal with parasite survival or death.

3.4. Enzymes of thiol metabolic

The *Leishmania* parasite survives and proliferates in hazardous environment of macrophage in mammalian host. This remains a puzzle that how parasite escape from reactive oxygen and reactive nitrogen species that is generated by host as immune effectors mechanisms against parasite. Recent reports indicate that parasite has developed unique defense mechanism to marshal respiratory burst activities of macrophages. Cascades of three antioxidant enzyme of trypanothion metabolism are required to counteract mammalian antioxidant glutathione metabolism. Trypanosomatids contain trypanothion [$T(SH)_2$], a dithiol instead of a glutathione as a main reductant and replaced the ubiquitous GSH/glutathione reductase redox couple with its $T(SH)_2$ /trypanothion reductase(TR) system[101,102]. Why $T(SH)_2$ is evolved inside parasitic system remain an enigma.

TR is a flavoenzyme that reduces thioredoxin and tryparedoxin and some short chain protein like dithiol. These reactions delivers reducing equivalent to peroxidase for detoxification of toxic radicals produced during hydroperoxide and deoxyribonucleotide synthesis. Trypanothion enable metabolic pathways by maintaining low molecular mass thiols trypanothione and monoglutathionyl spermidine, glutathiol and ovathiol in their reduced state *i.e.* important for the uninterrupted progression of metabolic pathways[103].

In *Trypanosoma brucie* and *Leishmania infantum* it is reported that $T(SH)_2$ is capable of reducing NO and Fe into a harmless stable dinitrosyl iron complex with 600 time more affinity than mammalian GSH reductase system, that

protects parasites from potentially lethal nitric oxide (NO) molecule. The absence of this pathway in mammalian host and trypansomatids sensitivity towards oxidative stress, trypanothione reductase and enzymes of trypanothione metabolism are an attractive drug targets for antileishmanial drug designing[104]. Homology modeling of *Leishmania infantum*, TR and mammalian glutathione reductase show remarkable difference in their three dimensional and catalytic active sites. Hence specific inhibitors designed against TR may be an ideal drug that will stop parasite growth without altering host glutathion reductase (GR) activity.

3.5. Cyclin dependent kinases

Cyclin dependent kinases (cdks) play crucial role in cell division cycle, transcription, apoptosis and differentiation. The genomic analysis has reported 10 orthologous cyclin in *Leishmania* species. In *Leishmania major* one additional mitotic like cyclin, CYCA is also found[105,106]. Cdk requires active cyclin for its own activation while few cdks requires activation by the phosphorylation for their functional activity. This phosphorylation could be achieved by cdc2 activating kinases, at their conserved threonine residues (t-loop)[107]. Cdk related kinase3 (CRK3 gene) encodes cdc2 related protein kinase with activity matching to eukaryotic histone H1. CRK3 is active at G2/M phase of *Leishmania* cell cycle. In *Leishmania mexicana*, disruption of CRK3 leads to change in cell ploidy though it was avoided when extra copy of CRK3 was expressed from episome ensuring that CRK3 is essential[108]. The chemical inhibitors of CRK3 impair the parasite viability within macrophage, thus validating CRK3 as potential drug target. The most potent inhibitor of CRK3 belongs to indirubin class, which provides pharmacophores for further drug development[105]. In *Leishmania donovani*, it was recently shown that glycogen synthase kinase (LdGSK3) is also involved in cell cycle control and apoptosis based on indirubin test[109] exploiting the LdGSK3 as potential drug target in combination with CRK3. Likewise, other cdk may also be explored as possible targets.

3.6. Mitogen activates proteins kinases (MAPK)

In mammals MAPK play important role in all aspects of immune response from initiation of innate immunity to activation of adaptive immunity. In addition, they also regulate cell differentiation, proliferation and apoptosis. MAPK receiver molecules, receive external stimuli and signals, and through cascades of intermediates regulate transcriptional, proliferative and differentiation status of a cell[110]. In *Leishmania mexicana*, 15 MAPK have been identified[110,111]. *Leishmania mutant* lacking MAPK gene has shown their significance in transformation and cellular growth. The MAPK gene deleted promastigotes after differentiation into amastigote lose proliferative capacity, also peritoneal macrophages were able to cope with infection, which their importance for amastigotes[112]. MAPKs are not only important to amastigotes but also for promastigotes. The direct evidence came from the *in vitro* study on overexpression of *Leishmania major* MAPK shows stage specific phosphotransferase activity and accumulation in axenic amastigotes not in promastigotes[113]. Identification of *Leishmania* specific sequences of MAPK and their targeting offers great probability for an effective drug. *Leishmania* MAPK inhibitors should have same effect on amastigote survival as in case of gene deletion. Hence efforts are required to identify leishmanial MAPK related targets. These kinases may be potential drug target not only for the development of novel drug regimen but also for therapeutic immunomodulation.

3.7. Enzymes of sterol biosynthesis

In *Leishmania* species the main endogenous sterols are ergosterol and stigmasterol, which differs from mammalian counterpart cholesterol, therefore can be used as potent drug targets. Ergosterol has two important functions: first, it is a structural component of cell membrane and second, it might play hormonal role. AmB sounds strongly against leishmaniasis because of sterol based mechanism of action, but AmB resistance for leishmaniasis dictates researchers to find an alternative drug of AmB deliberately. Azasterols, a known class of s-adenosyl-L-methionine, show antileishmanial activity and inhibits 24-methyltransferase, which is a vital enzyme in ergosterol biosynthesis[114]. Other sterols like azol and triazole are also effective against *Leishmania*, which inhibits 14 α -methylsterol 14-demethylase. However, *Leishmania* has potential to survive in altered sterol profile, and also have ability to utilize and metabolize host sterol[85]. This consideration must be accounted during novel drug development. The most appropriate way will be the inhibitors of sterol biosynthesis with other metabolic pathway combined together. Along with the combinational therapy, to find some another potential sites in sterol biosynthetic pathway that can be used as attractive drug targets.

3.8. Dihydrofolate reductase (DHFR)

DHFR is a key enzyme in folate metabolism, linked to the production of thymidine[115,116]. DHFR reduces dihydrofolate to tetrahydrofolate using NADPH as cofactor. Therefore, inhibition of DHFR prevents biosynthesis of thymidine and as a consequence, DNA biosynthesis. Fortunately, this enzyme from *Leishmania major* and *Trypanosoma cruzi* have been crystallized and the structural data may be exploited to observe structural difference between parasite and human enzymes that may help to design selective DHFR inhibitors[117,118]. An approach to discover novel parasite DHFR inhibitors using database mining has also been made to search the Cambridge structural database but DHFR as drug target requires further attention[119,120]. In addition, enzyme dihydrofolate reductase-thymidylate (DHFR-TS) that catalyzes conversion of dihydrofolate from methyhylene

tetrahydrofolate(M-THF) and thymidine, has been shown to be related to parasite survival and parasite lacking this enzyme are not able to survive in animals[121]. Inspite of these advantages several potential resistance mechanisms to DHFR have been discovered including over-expression of the enzyme DHFR-TS and enzyme ptrI[122,123]. The enzyme ptr1 predominantly involved in reduction of biopterin to dihydrobiopterin and tetrahydrobiopterin but also capable of reducing dihydrofolate to tetrahydrofolate. Hence, a combined strategy to target both DHFR and ptrI in will be more effective as a true antileishmanial drug.

3.9. Topoisomerases

DNA topoisomerases are ubiquitous enzymes needed to remove torsional stress in DNA by introducing transient protein-bridge DNA breaks either on one (type I) or both (type II) DNA strands. Topoisomerases are major targets in cancer and bacterial chemotherapy[124,125]. In parallel protozoan parasites are not distinct, they require topoisomerase specially topoisomerase II due to the presence of complex intercatenated network of thousands of minicircles as well as maxicircles in kinetoplastids mitochondria. Topoisomerase II has been reported to be overexpressed and shows increased activity in arsenite resistance *Leishmania donovani*[126]. The topological problem associated with mitochondrial DNA presents potential sites for topoisomerase II activity. Antibacterial and anticancerous drugs like novobiocin, etoposide, and fluoroquinolones can be used to target topoisomerase II in order to inactivation of genetic integrity and cell survival[127]. More efforts are required towards topoisomerase targeted drug interaction and development of anti-topoisomerase chemicals against drug resistant *Leishmania* parasites.

3.10. Metacaspases

Metacaspases are orthologous to caspases that play crucial role in apoptosis but imperfectly understood in protozoa. It has been found that metacaspases may be possible candidates to induce programmed cell death in trypasomatids[128]. In *Leishmania donovani* two metacaspases: LdMCA1 and LdMCA2 are reported showing 98% homology with each other and contain characteristic C terminal proline rich domain and both are expressed in promastigotes and amastigotes form[129]. A metacaspase from *Leishmania major* (*Lm*jMCA) has found to be essential for the proper segregation of the nucleus and kinetoplast[130]. Metacaspase gene of *Leishmania major* is actively expressed in amastigotes and procyclic promastigotes but surprisingly at a lower level in metacyclic promastigotes. Metacaspases in *Leishmania*, on treatment with H_2O_2 trigger process of programmed cell death of parasites. It has also been found that parasites, which over express metacaspases are more sensitive to H_2O_2 induced programmed cell death[131]. Molecules that can target to metacaspase biosynthetic machinery and induces their early expression might prove as efficient antileishmanial agents. In addition, since they are required for chromosomal segregation and parasite survival, they can also be directly targeted[132]. However, more studies are required to understand the complete function of leishmanial metacaspases.

3.11. Leishmanial antigens that modulates host immune functions

Leishmania survival and proliferation within parasitophorous vacuole primarily depends on its strategy to evade macrophage effector molecules and diverting its immune response. Macrophage arsenal is full of its potent armatures such as reactive nitrogen and oxygen species, lysozymes, and other microbicidal molecule, production of variousTh1 cytokines for parasitic inhibition[133]. On parasitic infection the chase begin, macrophage attack on parasite with its reactive nitrogen and oxygen molecules as a result of induction of respiratory burst activity, while parasite has to defend itself for its survival, it has to produce something that will counterattack and suppress this activity or develop a strategy to skip from this attack. *Leishmania* survival strategy includes: (1) its glycoprotein (gp63: a metalloproteinase) facilitates binding of metacyclic promastigotes to macrophages via CR3 receptor without eliciting oxidative burst in macrophages, (2) It inactivates complement components C3, C5, C9 by phosphorylation, (3) The parasitic superoxide dismutase scavenges reactive oxygen intermediates, (4) It suppresses iNOS expression of host macrophages, and (5) It induces disease exacerbating Th2 cytokines production such as TGF-β and IL-10 and suppresses the production of Th1 cytokines *viz.* IL-4, IL-12 *etc*[134]. Further, *Leishmania* parasite also alters various signaling cascades by induction of PTPase activity of host leading to down regulation or inactivation of various signaling cascade intermediates like NF-κβ, AP1, STAT1 *etc* that ultimately lead to the down regulation RNI, ROS, Nramp1 and other immune responses of macrophage[135].

One of the other survival strategies is the down regulation or dysfunctioning of ion transporters recruited on the cell membrane of phagolysosomes. The divalent cationic transporters creates Fe^{2+}, Mn^{2+}, Zn^{2+} deprived environment inside the phagosomes by pumping them out. These ions specifically iron is required for various crucial processes like antioxidant defense (Fe-Sod), mitochondrial respiration and DNA replication that are required for parasitic growth and survival[136]. The *Leishmania* parasite effectively down regulates or blocks the activity of these transporters but the mechanisms are not known. However, a divalent cations transporter natural resistance associated with macrophage protein1 (NRAMP1) now referred as SLC11A1 that belongs to a divalent cations transporter solute carrier family, play an important role in host resistance against diverse pathogenic

organism such as *Salmonella*, *Mycobacterium* and *Leishmania* by maintaining cations deficient environment inside the phagolysomes[137]. NRAMP1 is exclusively expressed on professional phagocytes and pumps out cations, required for parasitic growth and survival, from phagosomal milieu to cytosolic compartment of macrophage (Figure 2). The parasite expresses *Leishmania* iron transporter ZIP family one (LIT1) transporter, which transport Fe^{2+} inside parasite[138]. It seems that some parasitic proteins, soluble, surface or excretory-secretory, modulate Nramp1 pump function during leishmanial pathogenesis. These leishmanial antigens might work as potential drug targets but they are needed to be identified and characterized for development of drug and vaccine candidates.

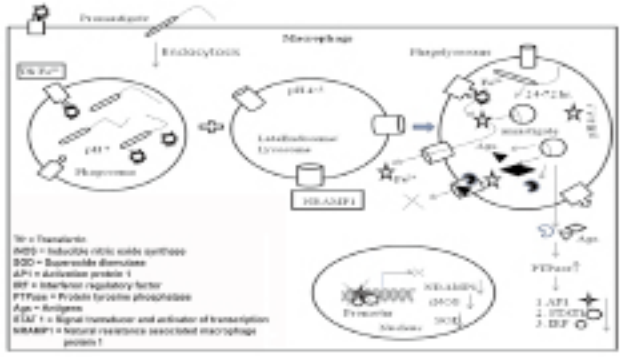

Figure 2. The figure summarizes the role of NRAMP1 in pathogenesis of leishmaniasis. Some leishmanial antigens may also upregulate PTPase functional activity, which further downregulate (inhibit phosphorylation) AP1, STAT1, IRF1 *etc* like transcription factors. Without phosphorylation they are not activated and bind to the promoter of NRAMP1, iNOS, SOD genes *etc* in macrophage and reduce their expression.

4. Conclusion and perspectives

Leishmaniansis is a life threatening disease that affects predominantly to people of developing countries living below poverty line. In disease endemic regions poor sensitizations, malnutrition and unhealthy living environment factors are the main reason for its pathogenesis and manifestations. The migration of population from endemic to nonendemic areas, and tourist activities in endemic regions are primary cause of disease spread in newer areas. To control the spread of disease in endemic countries, effective control measures for both, the vector and parasites are still required. The growing incidences of resistance with available drug regimen warrants the precise use antileishmanial drugs as well as necessitates the development of newer cost effective drugs and vaccine candidates. During past couple of decades the leishmanial research has made significant progress in various research areas but unfortunately identification of novel drug targets and drugs is far from satisfactory. In addition, the unavailability of either prophylactic or preventive vaccine candidates further makes difficult the control of disease. It has now become essential to identify priority research areas to combat disease progression worldwide.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The financial supports received from Department of Biotechnology, New Delhi (BT/PR11177/MED/29/99/2008) is greatly acknowledged. This article is dedicated to all those poor people who lost their lives during drug trials in various parts of the world.

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