Recent Advances in the Chemotherapy of Visceral Leishmaniasis

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6.1 Introduction

Leishmaniases are an infectious disease caused by more than 20 species of the protozoan parasite that have a place to genus *Leishmania* and family trypanosomatidae. It spreads by the bite of a preinfected female sandfly vector belonging to more than 30 species of genus Phlebotomine. Leishmania parasite spends its life cycle in digenetic mode consisting of flagellated and motile promastigote form in the gut of female sandfly vector and when sandfly bites to mammalian host, it converts into aflagellated, nonmotile amastigote form within the phagolysosomal compartment of the macrophage. Leismaniasis manifests mainly three types of clinical spectrum namely cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL). Among them, cutaneous form of leishmaniasis is most common, ranging from self-healing small nodules to giant mucosal tissue damage. It is caused by *Leishmania major, L. maxicana, L. tropica*, *and L. amazonensis*. However, MCL leads to the degeneration of a mucous layer of nasopharyngeal cavity, caused by *L. braziliensis and L. panamensis.* The most severe visceral form of leishmaniasis, also known as kala-azar, black fever, or dumdum fever, causes high fever, weight loss, acute anemia, fatigue, and enlargement of the liver characterized by splenomegaly and hepatomegaly. The immune system of patients gradually decreases and may be lethal, if left untreated. It may be of zoonotic or anthroponotic type based on their transmission characteristics and caused by *L. donovani* and *Leishmania infantum* in Old World and New World, respectively.

Recent epidemiological reports of VL estimate up to 0.4 million cases from 98 countries and 3 territories on 5 continents [1]. Among them, 90% of global VL cases

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present only in six countries namely India, Bangladesh, Nepal, Ethiopia, Sudan, and Brazil [2]. 50% of the visceral leishmaniasis infection burden is present only in India. Rural areas are commonly affected as compared to the urban areas due to the climatic and environmental conditions that play a major role in the growth and development of sandfly vectors. The number of VL patient is increasing continuously due to unavailability of proper treatment options, lack of vaccines, drug resistance into a patient, and uncontrolled increase in sandfly vectors. Over the last few decades, many new drugs and formulations were introduced to treat the VL infection, but most of the drugs have shown toxicity along with resistance condition. Since 1930s, pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) were opted as the first-line treatment option for VL in the endemic countries. But due to extensive drug resistance of more than 60% in few areas of India and Nepal, it was banned since 1990s [3]. In early 1990s, amphotericin B and its liposomal formulation were introduced as a potent antileishmanial drug with high efficacy and negligible resistance. Miltefosine, the first oral therapy for VL, has shown high efficacy of more than 90% with a relapse rate of 20% within 12 months in the clinical trials [4]. Paromomycin is an aminoglycoside antibiotic with antiprotozoal activity. It has shown a cure rate of 94.6% in patients suffering from VL. This drug has made a milestone due to its cost-effectiveness and lack of resistance. The main target of a chemotherapeutic agent against VL is the amastigote form of leishmania, which intracellularly survived and replicated within the macrophages of visceral organ of the vertebrates. This amastigote form exists in the parasitophorous vacuole, which looks like a secondary lysosome with pH ranging between 4.5 and 5.0. The availability of acidic environment has been responsible for the acquisition of nutrient and ion homeostasis in amastigotes of parasite [5]. This process also involves various transporters which are responsible for the drug influx into parasite and they are responsible for the drug susceptibility of the parasite against chemotherapeutic agent. Chemotherapeutic approaches evolve new drugs based on the antileishmanial activity and tissue macrophage target activity systems. A table has been presented here to represent the antileishmanial options for the different faces of leishmaniasis (Fig. 6.1) (Table 6.1).

6.1.1 Diagnosis

In the early twentieth century, classical microbiological diagnostic methods were used after the association of clinical leishmaniasis with Leishmania parasite. Visceral organs like spleen, bone marrow, and lymph nodes are the major shelter for the parasite in the human body. The diagnosis options available for VL are complex as compared to other diseases such as malaria, typhoid, and tuberculosis. Microscopic examination is the gold standard approach to diagnose leishmaniasis apart from biopsy techniques for VL. Splenic aspirate has shown a specificity of >95%, while liver biopsy, bone marrow aspiration, lymph node fine-needle aspiration cytology (FNAC), and blood buffy coat have a sensitivity of 76% only. Lymph gland puncture gives a positive result of 40–50% for kala-azar cases while 58.6% for CL cases. However, the study of a nasopharyngeal swab of visceral leishmaniasis patient

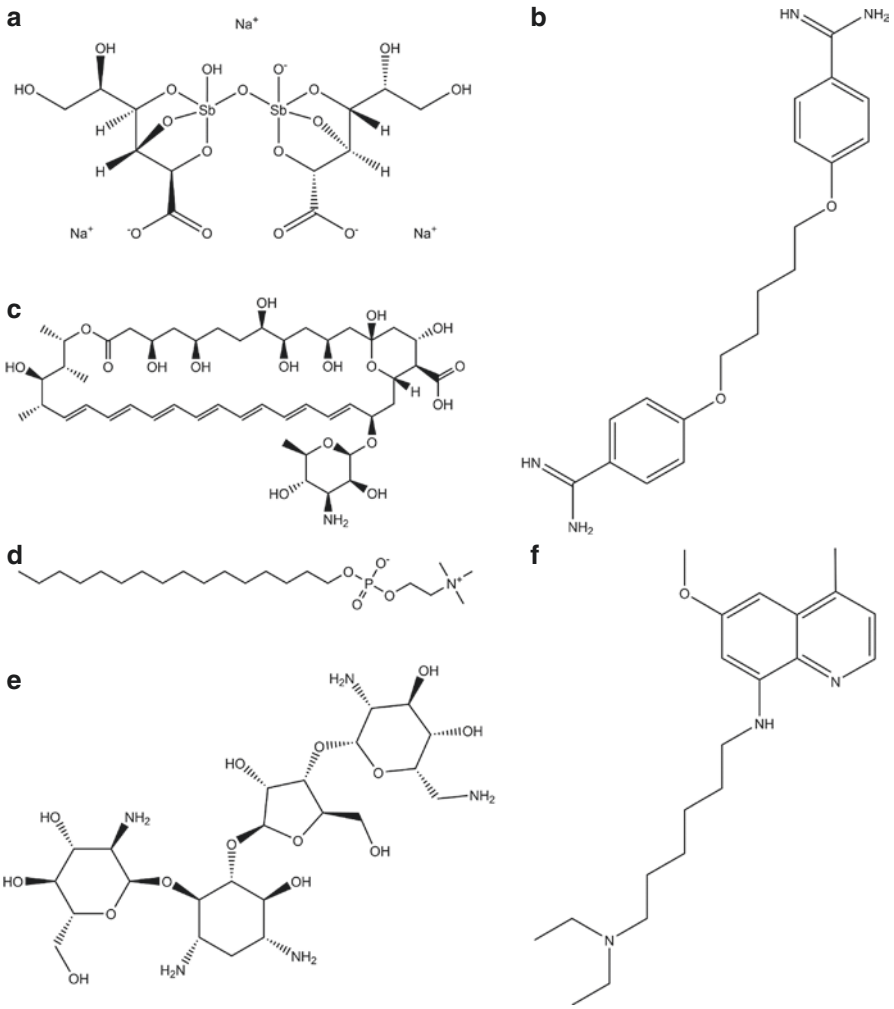

Fig. 6.1 Available chemotherapeutic drugs for the treatment of visceral leishmaniasis. (**a**) Sodium stibogluconate, (**b**) pentamidine, (**c**) amphotericin B, (**d**) miltefosine, (**e**) paromomycin, (**f**) sitamaquine

shows positive result of 28% and 36% with stain and culture, respectively. As compared to aforesaid diagnosis method, immunological approaches such as direct agglutination test, enzyme-linked immunosorbent assay, and rapid antibody detection have shown high sensitivity [6]. According to recent information the molecular diagnostic methods such as polymerase chain reaction-based assays have shown high sensitivity and specificity (up to 100%) for detection of Leishmania parasites. By using these methods we can define the specific features of parasite such as drug resistance or virulence and they provide information to know the disease severity and treatment outcome [7].

Table 6.1 Different clinical forms of leishmaniasis, their geographical distribution, causal leishmania species, vectors, respective reservoirs, and available treatment options

Infection	Geographic distribution	Pathogenic species	Vector	Reservoir	Available treatment
Cutaneous Leishmaniasis (CL)	Semi-deserts in Middle East, North India, Pakistan, North Africa, central Asia	*Leishmania major*	*Phlebotomus papatasi*	Gerbils	Sodium stibogluconate, miltefosine, paromomycin
CL	Sub-Saharan Savanna, Sudan	*L. major*	*P. duboscqi*	Rodents	
CL	Towns in Middle East, Mediterranean Basin, central Asia	*L. tropica*	*P. sergenti*	Humans	
CL	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*	Hyraxes	
CL	Yucatan, Belize, Guatemala	*L. mexicana*	*L. olmeca*	Forest rodents	
CL	Tropical forests of South America	*L. amazonensis*	*L. flaviscutellata*	Forest rodents	
(Mucocutaneous Leishmaniasis) MCL	Tropical forests of South and Central America	*L. braziliensis*	*Lutzomyia spp., L. umbratilis*	Forest rodents, peridomestic animals	Sodium stibogluconate
(Visceral leishmaniasis) VL	North East India, Nepal Bangladesh, Burma	*L. donovani (Asia)*	*Phlebotomus argentipes*	Humans	Sodium stibogluconate, Meglumine antimoniate, Amphotericin B deoxycholate, liposomal amphotericin B, miltefosine (oral), paromomycin, sitamaquine (oral), pentamidine
VL	Mediterranean Basin, Middle East, China, Central Asia	*L. infantum*	*P. perniciosus, P. ariasi*	Dogs, foxes, jackals	
VL	Sudan, Kenya, Horn of Africa	*L. donovani (Africa)*	*P. orientalis, P. martini*	Rodents, canines, humans	
VL	Central America, Northern South America, esp. Brazil, Venezuela	*L. chagasi*	*Lutzomyia longipalpis*	Foxes, dogs, opossums	

6.1.2 Advancement in Visceral Leishmaniasis Chemotherapy

Kala-azar first got attention in 1824 in Jessore, India. However, by 1862 this disease got spread in Burdwan and caused endemic proportions. In 1900s, the agent of Leishmania was firstly isolated by William leishman, who isolated the parasite in a spleen sample of soldiers who died with dum-dum disease, in Calcutta, India. Later on, Upendra Nath Brahmachari, a medical practitioner, firstly discovered urea stibamine, an organic antimonial compound that played a promising role in the treatment of visceral leishamania or kala-azar. Later on, pentavalent antimonials became the traditional treatment option for VL till 1990s. But the extensive resistance of antimonial drugs in endemic areas of India made it banned. Furthermore, amphotericin B, pentamidine, miltefosine, paromomycin, and sitamaquine were introduced.

6.2 Chemotherapies for Visceral Leishmaniasis

Leishmaniasis infection has been included in the list of neglected tropical diseases and has a strong relationship with poverty [8]. At present, there is no vaccine candidate either preventive or prophylactic under the clinical trials for the effective treatment of leishmaniasis diseases. Therefore, the treatment of leishmaniasis only relies on chemotherapy. The first line of the drug used to treat VL was pentavalent antimonials and the second line of drugs are amphotericin B and pentamidine. Miltefosine was introduced as the first oral drug for Leishmania with high efficacy, but the patient has shown resistance in very short time due to point mutation in the genome of leishmanial strain [9]. Paromomycin has also shown high efficacy without any resistance condition. In recent years, many new drugs have been formulated, but no one is considered as an ideal drug for the treatment of VL because of their high toxicity, long duration of the treatment, and development of drug resistance in the patient.

6.2.1 Pentavalent Antimonials

Urea stibamine was the first antimonial introduced in 1930s by U.N. Brahmchari but due to its comparatively high toxicity it was soon after replaced by sodium stibogluconate. Since 1950s, pentavalent antimonials occupied the position of first-line drug for the treatment of visceral leishmaniasis where resistance is not reported. Initialy, antimonials were given in very low dosage of 10 mg/kg/day for only a short period of 6–10 days; later on in 1982 WHO increased the dose quantity to 20 mg/kg/day, but they recommend that total daily quantity of drug should not exceed 850 mg. Antimonials were marketed by two names, generic-form sodium stibogluconate and branded-form meglumine antimoniate. The pentavalent antimonials (Sb^{V}) are basically a prodrug that converts into trivalent antimonite (Sb^{III}), which is an active form of the drug. The reduction of pentavalent antimonials to its trivalent form takes place either in host macrophages or in parasite but it is still a quandary [10]. Apart from its

primary role for the treatment of VL, it was banned in India and Nepal in 2000s due to higher resistance of more than 60% in few districts of Bihar state [11].

If we talk about the entry of antimonial drugs into parasite and macrophage we will get a nonspecific proof, but there may be a possibility that parasitic aquaglyceroporins are responsible for the transport of drugs within the amastigotes. Parasitic phosphate transporter also plays a role in the transport of pentavalent antimonials into amastigotes. The killing effect of antimonials (Sb^{V} and Sb^{III}) against parasite includes DNA fragmentation, inhibition of the glycolytic pathway, apoptosis, inhibition of trypanothione reductase (protects the parasite from the reactive oxygen and nitrogen species of host), β-oxidation of fatty acids, and an increased efflux of thiols.

The responsible factors that evoke to the resistance of antimonial drug in the endemic areas of India include the widespread misuse of the drug due to its easy availability over medical stores and the lack of activation of SbV to SbIII by Leishmania parasite [12]. Resistance to trivalent arsenic was reported in a few districts of Bihar state due to the continuous drinking of arsenic-contaminated water leading to cross-resistance for trivalent antimony. Elevated intracellular trypanothione level also contributes to resistance [13]. However, the increasing level of tryparedoxin peroxidase also contributes to resistance for Sb^{III}. As aquaglyceroporin (AQP1) plays an essential role in influx of drug, modulation of AQP1 expression leads to decrease in the susceptibility of amastigotes for antimonials. Upregulation of various chaperons and stress-related proteins contributes to the repairing of cellular damage induced by antimonials [14].

6.2.2 Pentamidine

After the unveiling of pentavalent antimonials and amphotericin, alternative chemotherapeutic drug pentamidine was introduced in 1980s. Pentamidine is an organic compound of diamine that has gained an attention as a second-line drug against VL with increasing cure rate. Firstly pentamidine showed a cure rate of up to 93%, but after some time cure rate decreased upto 70–80% in some Indian epidemic areas [15]. When pentamidine was combined with antimonials and allopurinol the cure rate increased as compared to single form. After the treatment failure of pentavalent antimonials in Eastern Africa, pentamidine proved as an effective drug.

The mode of action of pentamidine is unknown, but it enters into the promastigotes of *Leishmania donovani* via arginine and polyamine transporter. Pentamidine may be toxic with another disorder like neurotoxicity, hypertension, hypoglycemia, and many others. The drug resistance was also reported, but the exact mechanism is unknown [16].

6.2.3 Amphotericin B

Amphotericin B (AmB) deoxycholate, an antifungal drug, was firstly introduced to treat fungal infection, but later on it was used for the treatment of visceral and mucocutaneous leishmaniasis. The first clinical trials of amophotericin B were

conducted in 1990s for the treatment of visceral leishmaniasis [17]. Amphotericin B deoxycholate has shown high efficacy of 98–100% in those areas where resistance to antimonials has been reported. The initial recommended dose of amphotericin B was 1 mg/kg/day for 20 days leading to a cure rate of 99%, but if the quantity of amphotericin B was changed to 0.5 mg/kg/day for up to 14 days, it also showed very good result. After some years, researchers found that the amount of amphotericin B will be more effective when administered on alternative days as compared to daily doses.

For the effective treatment of leishmaniasis researchers formulated a new alternative drug to increase the efficacy and decrease the toxicity and other side effects and established lipid formulation of amphotericin B. Till date ten forms of liposomal amphotericin B have been tested and various ranges of response were obtained in India [18]. They tested the efficacy at different doses and concludes that the efficacy of liposomal amphotericin B increases along with the increase in the percentage of the dose [19]. The liposomal formulation of amphotericin B proved itself as an efficient drug and increased the cure rate to more than 90%, but the high cost and less availability are wide limitations. The antileishmanial activity of liposomal AmB is due to its activity towards both ergosterol of parasite and cholesterol of infected host macrophage. Amphotericin B makes complexes with cholesterol, therefore inhibiting the binding of promastigotes to the membrane of host cell macrophages. Further higher concentration of AmB makes pore in the parasitic cell membrane leading to increase in osmotic disbalance and free radical formation that ultimately leads to cell lysis. Apart from the good efficacy of amphotericin B, resistance has also been reported from the endemic zone of VL. Other deleterious effects on human body include damaging of kidney tubular cell, an increasing concentration of Ca^{2+} ion, hydrogen ions across the membrane and salt concentration that leads to cell death. The presence of less amount of ergosterol in the cell membrane of leishmania parasite triggers the resistance phenomenon, since ergosterole is the main target of amphotericin B [20].

6.2.4 Paromomycin

Paromomycin was added as an essential medicine in 2007 by WHO. It is an aminoglycosidic antibiotic cultured from *Streptomyces rimosus*, having both antibacterial and antileishmanial activity. Paromomycin effectively cures both visceral and cutaneous leishmaniasis but less availability in endemic areas restricts its use. Paromomycin has been given either alone or in the form of combination with Sb^{v} to treat visceral leishmaniasis. The clinical trials of paromomycin have been done in 1990s in Bihar state of India. The efficacy of paromomycin was found to be excellent as compared with other licensed drugs. This drug could be considered as a cheapest drug for the treatment of visceral leishmaniasis. A patient treated with paromomycin achieved high efficacy of 77–97% as compared with antimonials (66%) along with less toxic effect [21]. The mechanism of action of paromomycin is not clearly known for leishmaniasis while it has been associated with the inhibition of cytochrome C in *Candida krusei.* Recent studies have shown that positively charged

paromomycin is targeted to anionic leishmanial glycocallyx suggesting mitochondria as a crucial target. Paromomycin promotes the association of 50S and 30S ribosomal subunits, thus stopping their recycling process and ultimately inhibiting protein synthesis. This antibiotic also induced respiratory disbalence in the promastigote form of *L. donovani*. Further, it interacts with both 30S and 50S subunits and promotes the association of translation initiation factor 3 (IF3) with the 30S ribosomal subunit [22]. Limited use of paromomycin in the treatment of Leishmania yet does not generate resistance in case of outpatient treatment, but in vitro resistance was reported in the case of *Leishmania donovani* and *Leishamnia tropica.*

6.2.5 Miltefosine

Miltefosine is an alkylphosphocholine, primarily developed as an anticancer drug. This is the first leading oral drug which is used to treat visceral leishmaniasis. Phase I, II, and III clinical trial was conducted and provides a strong evidence of protection against visceral leishmaniasis followed by phase IV trials [23]. The combination therapy of miltefosine and ambisome has been evaluated and has been found to be highly effective with good tolerability, but questions were raised due to the side effect generated due to an extreme combination of these two drugs. Miltefosine has a long half-life of almost 152 h and is therefore responsible for long-term residence and teratogenicity. This situation is responsible for the development of resistance and this abortifacient and teratogenic nature limits its use during pregnancy.

Miltefosine enters into the cell and accumulates to perform its activity. Two signaling transporters namely LdMT and its β-subunit LdRos3, a P-type ATPase, regulated the accumulation of miltefosine within the cell. Both transporters are related to aminophospholipid translocase family. The mode of antileishmanial action is still unclear, but it has been the cause for the apoptosis-like process in the amostigote form of *Leishmania donovani*. Miltefosine reduces the lipid content and enhances phosphatidylethanolamine in promastigote membrane, suggesting a production of phosphatidylethanolamine-*N*-methyl transferase that is responsible for inhibition of leishmanial parasite proliferation process [24]. Miltefosine has yet not shown clinical resistance, but improper use of this oral agent in endemic countries (India) enhances the probability of resistance. The resistance towards miltefosine correlated with the low lipid content in the membrane of Leishmania promastigotes and the amount of phospholipid alkyl chains was lower in miltefosine resistance strains. In India, the efficacy of combination therapy of miltefosine with amphotericin B or paromomycin is very high and it is useful to cure antimony resistance strain of visceral leishmaniasis [25].

6.2.6 Sitamaquine

Sitamaquine is a second oral drug after the discovery of miltefosine for the treatment of visceral leishmaniasis and recently it underwent the clinical trial phase I and II. Sitamaqine is an 8-aminoquinoline that was developed originally with the

collaboration of Walter Reed Army Institute and GlaxoSmithKline [26]. The phase II clinical trial of sitamaquine has shown good efficacy in the Indian subcontinent against visceral leishmaniasis. However, along with its high efficacy, fewer side effects also developed like vomiting, cyanosis, nephritic syndrome, and dyspepsia. The result of Kenyan phase II clinical trial was comparatively different from Indian trials; it showed similar efficacy but somewhat different side effects like abdominal pain, kidney malfunctioning, and headache [27]. Sitamaquine targets succinate dehydrogenase enzyme leading to development of an oxidative stress and ultimately parasitic clearance. The high concentration of sitamaquine affects parasite morphology, motility, and proliferation. Initially, the positively charged sitamaquine interacts with the anionic polar head of phospholipids. After binding, sitamaqine starts to accumulate into cytosolic part of parasite. The drug resistance against sitamaquine is yet not clinically developed, but *Leishmania donovani* promastigote in vitro experiment developed resistance at 160 μL sitamaquine concentration [28].

6.3 Novel Formulation of Anti-leishmanial Drugs

Over the past years, new formulation and alternative drugs of old ones have been available, but at the present time, none of them are ideal drugs due to the very high toxic effect, resistance, long-term treatment, prohibitory price issue, and inadequate mode of insertion not accepted in endemic areas. Therefore, many patients are not capable to complete the whole treatment process due to increasing risk of drug resistance and toxic effect. The combination therapy has shown positive result or short-term solution to delay emerging drug resistance, increasing drug efficacy, and shortening treatment duration [29]. However, this method is limited to the fewest number of FDA-approved drugs and the chances of finding a new mechanism of action compared to parasite are very less.

The discovery of a new formulation of anti-leishmanial drug to treat visceral leishmania should be a very important or long-term objective. In addition, target product profile (TPP) is an important tool for drug discovery management and plays a central role in drug discovery process. Nowadays, the drug discovery process for visceral leishmania mainly follows two approaches, the molecular target-based approaches and the phenotypic target-free approaches [30]. For the target-based approaches, the first step would be the identification and endorsement of potential targets. Recently, many efforts were done to find potential leishmanial targets, initially with the help of TDR database. This database is created on the basis of genetic, biochemical, and pharmacological data related to tropical pathogen additionally associated with computer-based druggability. The phenotypic target-free screening approaches target a particular enzyme or an individual biomolecule, or focuses on a pathway that might be a beneficial solution.

At the present time the combination therapy of anti-leishmanial drug with nanocarriers has been a potential and emerging formulation for the treatment of leishmaniasis. Nanocarriers have a power to penetrate into macrophage cell and are able to release drugs into cell, increasing the local drug concentration and ultimately

arresting the protozoa life cycle. In this strategy nanocarriers target the macrophage cells to treat leishmaniasis infection, which has an ability to overcome all natural biological barriers. Nanocarriers would be less toxic, have high efficacy, enhance selectivity, have high drug solubilization, prevent degradation of the drug, and promote the accurate release of drug at the target site. Nanospheres, polymers, and liposomes have promising nanocarriers for the delivery of anti-leishmanial drug at specific targets. However, this technology is an emerging current treatment option with reduced treatment cost, improved bioviability, and less drug toxicity that definitely enhance the efficacy of the treatment.

Liposomes are small synthetic vesicles in globular shape that can be produced from cholesterol biomolecules and nontoxic phospholipids. Due to small size, hydrophobic and hydrophilic balance, biocompatibility, flexibility, and stability to load various biomolecules as cargo, liposomes are recently used as a mode of drug delivery process. The best example of liposome formulation of amphotericin B is *AmBisome*®; this is discovered in 1981, which has proven to be effective against leishmania parasitic infection.

Functionalized carbon nanotube is a good example of drug carriers as it has been tested as drug transporter against leishmaniasis. In case of *L. donovani*-infected patient, amphotericin B attached with functionalized carbon nanotube has shown high efficacy and good result as compared to conventional amphotericin B [31].

Targeting of the various enzymatic reactions of host and parasite cell pathways, by high-throughput screening, appears to be more beneficial. The use of this phenomenon is a critical step targeted by a specific chemical compound that interferes a specific outcome of the pathway. Recently, these techniques are emerging as tools for the establishment of new drug target against leishmaniasis [32].

Continouosly generating resistance of Leishmania parasitic strain increases the amount of genomic data. Therefore, the genomic sequence provides beneficial information for the discovery of novel vaccine and new chemotherapeutic targets. The development of new molecular tools like microarray and deep sequencing technology and proteomics has an ability to revel these clinical goals. In addition, computational algorithms and emerging bioinformatics with high-throughput virtual screening greatly play a crucial role for identification and formulation of new potent anti-leishmanial drug target. Proteomics and transcriptomics have been used for the identification of stage-specific gene at different stages in Leishmania parasite. These proteomics approaches are capable of finding new chemotherapeutic leishmanial targets and these targets have a potential to control leishmaniasis infection.

6.4 Drug Targets

Several drugs are available to treat VL patients, but the associated toxicity and increase in resistance to kill the Leishmania parasites have urged the need of new compounds to eradicate VL infection. New potential drug targets focus on different enzymes involved in a number of biochemical and metabolic pathways which provides energy for parasitic survival. There are several targets inside the Leishmania

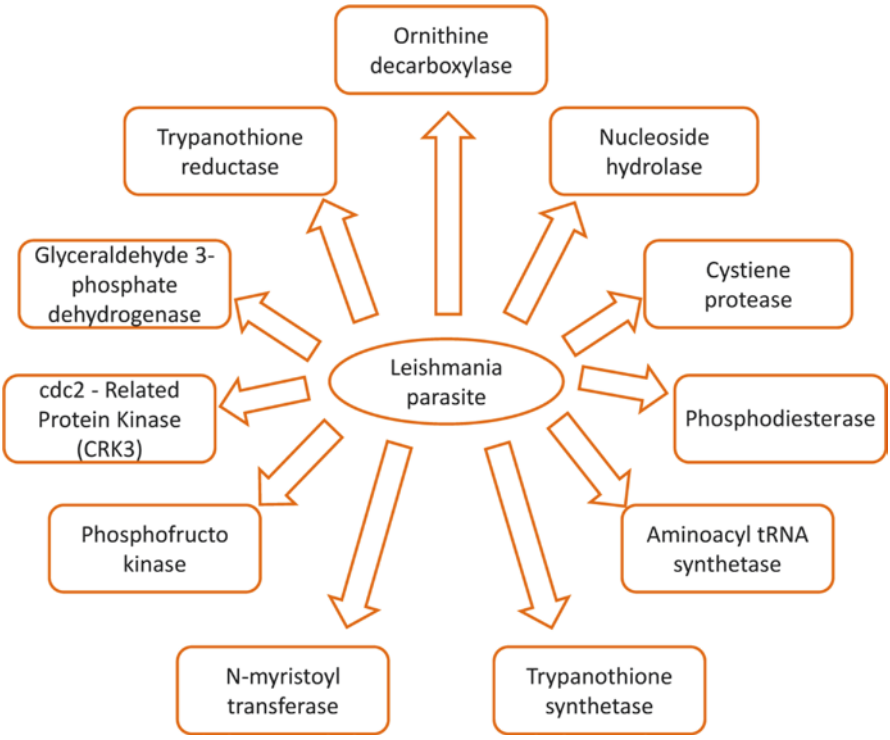

Fig. 6.2 Leishmania drug targets used for screening of anti-leishmanial compounds

parasites, regulating parasite metabolism or redox potential, that can be used to develop new chemically synthesized molecules to kill Leishmania parasites [33] (Fig. 6.2).

6.4.1 Trypanothione Reductase

Trypanothione reductase (TryR) is a flavoenzyme which catalyzes the redox reaction to convert trypanothione (TPT) and NADP into trypanothione disulfide and NADPH, found in the protozoan parasite of genus *Leishmania* and *Trypanosomes*. It is a stable homo-dimer, bounded tightly with each other by FAD molecule. FAD acts as a cofactor and it also requires NADPH as a co-substrate. TryR plays a main role in redox metabolism of parasite; therefore TryR has been regarded as an ideal drug target for leishmania infection. The inhibitions of this enzyme will disrupt the redox balance which may lead to parasite death [34]. The process of selection of drug target depends upon the level of genetic and chemical validation, assay feasibility, toxicity potential, resistance, and drug-ability. TryR has two acive sites; one is found at the FAD-binding site while the second is formed by the interaction of both chains.

6.4.2 Ornithine Decarboxylase

Ornithine decarboxylase (ODC) is found to be involved in polyamine biosynthesis pathway. Polyamines are a component of trypanothione dimer [$T(SH)_2$], useful for *Leishmania* parasite infection. ODC plays a role in housekeeping functions in the amastigote stage of Leishmania parasite. Increased resistance to antimonial drugs has increased the expression of ODC in Leishmania parasites. This enzyme has two active sites and plays a role to increase the virulence property of *L. donovani*. Therefore, it can be taken as a potential drug target for the identification of anti-leishmanial leads [35]. The α-diflu oromethylornithine is an irreversible inhibitor to target ODC while the coenzyme of this reaction is pyridoxal 5′-phosphate. Currently DFMO (α-difluoromethylornithine) is used as a drug for the treatment of patients suffering from protozoan parasitic diseases like visceral leishmaniasis and malaria. DFMO mimics the substrate which binds with the enzyme ODC. The enzyme of ODC with N-terminal extension in *L. donovani* has the future scope to develop the new drugs against leishmaniasis [36].

6.4.3 Nucleoside Hydrolase

The inabilities of protozoan parasites to synthesize purines via de novo pathway make them dependent on salvage pathway for purine synthesis. Nucleoside hydrolase is an enzyme that plays a crucial role in salvage pathway. Leishmania parasites are purine auxotrophs, so for their growth demands purines are necessary. Nucleoside hydrolases have also been identified in other trypanosomatids including *Trypanosoma brucei gambiense*, *L. donovani*, *L. Mexicana*, *L. tropica*, *T. cruzi*, and *T. brucei*. In these protozoan parasites the residues which present in the active site of nucleoside hydrolase are conserved in nature and these conserved residues provide the great promise to design a drug [37]. Another study, on the recombinant enzyme of nucleoside hydrolyses of *L. donovani* (rLdNH), was expressed in bacteria *Escherichia coli* combined with maltose-binding protein (MBP). The recombinant enzyme of rLdNH-MBP showed the capacity to bind with inosine as substrate in in vitro condition [38].

6.4.4 Cysteine Proteases

Cysteine protease is one of the proteases belonging to papain family, present in metazoan and protozoan parasites. As compared to mammalian hosts, leishmania parasites lack redundancy of cysteine protease; it makes them attractive targets for the development of new anti-leishmanial drugs. Cysteine protease plays an important role in infection, metabolism, replication, and development of protozoan parasites. These proteases are essential for survival of Leishmania parasites in macrophage intracellular compartments. These enzymes show virulence along with modulation of host immune responses. Cathapsin-B and cathapsin-L are the

cysteine proteases which are localized in lysosome-endosome compartments, and play a role in intracellular protein degradation [39]. The genome of leishmania parasite consists of multiple copies of cathapsin-L and a single copy of cathapsin-B cysteine proteases [40]. The derivatives of nitric oxide (NO) have the capacity to inhibit the development of parasites; glyceryl trinitrate is one of the releasing drugs of NO, and was used to treat cutaneous leishmaniasis.

The inhibitors of proteases are useful to block the entry of many parasites like *Plasmodium*, *Trypanosoma, Phytomonas serpens*, and *Taxoplasma gondii* into the host [41]. Cysteine protease has also shown its role in the modulation of host immune response, degradation of different host proteins, and autophagy. Lacking of this enzyme in Leishmania parasite or blocking of this enzyme by inhibitors showed reduced virulence activity and infectivity. Therefore, they have identified that the inhibition of this enzyme in different leshmania parasites provides best therapeutic option to combat leishmania parasitic infection [42]. Cystatin is one of the natural inhibitors that have been found to inhibit this enzyme of *L. donovani* [43]. Another natural bioflavonoid has shown anti-leishamnial and anti-proteolysis activity against cysteine protease-B, and the use of this natural inhibitor in specific concentration doesn't give any toxicity towards host cells.

6.4.5 Phosphodiesterases

Phosphodiesterase (PDE) is an enzyme involved in the control of the cellular concentration of secondary messengers like cAMP and cGMP, a key regulator of many physiological processes, and it is ubiquitously expressed [44]. In human beings there are 21 PDE genes, separated into 11 families. These enzymes have been found as therapeutics in many diseases like cancer, pulmonary diseases, and asthma [45]. Leishmania majorly contains five different PDE genes such as LmjPDEA, LmjPDEB1, LmjPDEB2, LmjPDEC, and LmjPDED. Among these five genes LmjPDEB1 and LmjPDEB2 show similarity, but there is a difference among Ala798 and Arg823 of LmjPDEB1 [46]. Etazolate, dipyridamole, and trequinsin are drugs which inhibit the proliferation of *L.major* and *L.infantum* promastigotes with IC_{50} values ranging between 30 and 100 μM [47]. In *L. donovani* promastigotes, flavonoids luteolin and quercetin arrest the cell cycle at G1 phage and eventually lead to increased cell apoptosis, so flavonoids are nonselective inhibitors treated as a specific drug to treat leishmaniasis. Because of the presence of structural similarity between LmjPDEB1 and human PDEs, it provides the chance to design inhibitors to treat leishmaniasis. 3-Isobutyl-1-methylxanthine (IBMX) can bind to the catalytic domain of LmjPDEB1 [48]. Till now, there are so many inhibitors to inhibit PDEs of human beings, but these are not effective to inhibit LmjPDEB1; rolipram is a selective inhibitor of PDE4 with IC_{50} values of 330 μM for the catalytic domain of LmjPDEB1 and also IBMX has shown IC50 value of 580 μM [49]. The other inhibitors like luteolin, a flavonoid, and other nonselective inhibitors of PDEs have shown the inhibition activity of 80% to LmjPDEB1 at 20 μM inhibitor. The structure of PDE provides a template to design drugs to treat leishmaniasis [50]. All three

LmjPDEA, LmjPDEB1, and LmjPDEB2 have the functional complementation; all three are involved in hydrolyzing cAMP. The recombinant of LmjPDEB1 and LmjPDEB2 has been shown to be cAMP specific with a low molar range of Km values. The drugs which are like dipyridamole, trequinsin, and etazolate exhibited the capacity to bind with PDEs; these inhibitors have the capability to inhibit the proliferation of *Leishmania* amastigotes [47].

6.4.6 Aminoacyl-tRNA Synthetase

Aminoacyl-tRNA synthetase (aaRS) is an enzyme, useful in translating the nucleotide-encoded gene sequences into proteins and this enzyme is ubiquitously expressed [51]. This enzyme recognizes a single amino acid and attaches to t-RNA, additionally with the properties of proofreading and editing mechanisms [52]. The charged t-RNA adds further amino acids and it proceeds to elongation process during translation [53]. Mostly eukaryotes carry two genes to each aaRS. They are in the cytoplasmic and mitochondria and are not remunerated to each other if one gene is knocked down. But in trypanosomatids no need of compensation between two genes; only one gene of aaRS per amino acid is enough, except for the amino acids AspRS, LysRS, and TrpRS. The reduction of one gene of aaRS leads to arresting the complete growth of protozoa [54]. These enzymes play a role in cell survival and provide the chance to design drug against them. The *L. major* tRNA synthetase is the first MetRS; its active site is bound with MetAMP and pyrophosphate. The human mitochondrial MetRS sequence shows 30% similarity with LmMetRS catalytic core, but the active site is highly conserved. There are two changes in binding site of anti-codon that is in *L. major* Asn580 and Lys732 whereas in human mitochondrial enzyme Gly and Arg are sufficient to arrive at selective inhibitors to target the enzyme [55].

6.4.7 Trypanothione Synthetase

Trypanothione synthetase is a bifunctional enzyme that catalyzes biosynthesis and involves in hydrolysis of the glutathione-spermidine adduct. Leishmania parasite has shown the unique nature regarding trypanothione, is involved in redox balance, and provides defense against oxidative and chemical stress [56]. This enzyme binds with three substrates such as glutathione, glutathionyl-spermidine, and ATP and then it forms N1, N8-bis (glutathionyl)spermidine, ADP, and phosphate. The main function of this enzyme is to generate the free energy from ATP hydrolysis to conjugate glutathione and spermidine to form glutathionylspermidine intermediate and it leads to form the product of trypanothione. These enzymes are useful to maintain polyamine levels which play a role in cellular proliferation and differentiation. This enzyme consists of C-terminal and N-terminal domains. Among these two, the former is a papain-like cysteine protease useful to catalyze $T(SH)_2$ biosynthesis while the latter is useful to catalyze trypanothione to glutathione [56]. Hecogenin acetate

tubocurarine, tubocurarine chloride, geneticin, dihydrostreptomycin, ribostamycin sulfate, tomatine, beta-carotene, geneticin, dihydrostreptomycin, paromomycin sulfate, capreomycin sulfate, spermine, 18-alpha-glycyrrhetinic acid, and mundulone are in silico anti-lishmanial drugs specifically against *L. major* [57]. The inhibitors of *L. donovani* LdTryS are tomatine, conessine, uvaol, and butelin. Few amino acid sequence alignment of glutathionylspermidine synthase reveals that it is derived from *E. coli* and the enzyme TryS from *Leishmania infantum* [58]. The structural difference between these enzymes provides the opportunity to design novel drugs against leishmaniasis.

The energy metabolism in trypanosomatids only depends on carbon sources of host. Glycolysis pathway is not only to provide ATP but also to produce glycoprotein coats to protect themselves from the immune system. So this pathway is so essential for surviving protozoan parasites. Therefore, this pathway promises the target for drug discovery against Leishmania parasite. In this pathway hexokinase converts glucose into glucose-6-phosphate by the transfer of the γ-phosphate of ATP to glucose. It is present in peroxisome-like organelles called glycosomes while in other organisms these enzymes are cytosolic. There are four types of hexokinases, hexokinase I–IV. Hexokinase IV is called glucokinase; it is present in liver [59]. This enzyme is the first enzyme which is involved in glycolysis; it plays a critical role in the biosynthesis, so it may have the potential to become a drug target. The potent FPPS inhibitors are useful to cure cutaneous as well as visceral leishmaniasis; these inhibitors have shown the ideal activity against *T. cruzi both* in vivo and in vitro. The study related to drugs has been done only on *T. cruzi* and *T. brucei* but not that much on Leishmania strains.

6.4.8 *N*-Myristoyl Transferase

N-Myristoyl transferase (NMT) is an enzyme, useful for the attachment of myristate. It is 14-carbon saturated fatty acid and it is derived from myristic acid (14:0). N-myristylation is a lipid modification and it is also a post co-translational modification. This process plays an important role in protein-protein interaction, lipid-protein interactions, signal transduction pathway, etc. This enzyme has specific functions when it attaches the fatty acid group to specific proteins and NMT is necessary for survival of parasites like *L. donovani, T. brucie,* and *P. falciparum*. So this enzyme has the ability to become a potential drug target to develop and treat various diseases like visceral leishmaniasis and cutaneous leishmaniasis. There is 42% sequence similarity between LdNMT and human NMT (HsNMT); it has provided the opportunity to identify novel inhibitors as therapeutic agents to combat the diseases [60]. Literatures have mentioned that non-hydrolyzable myristoyl-CoA analogue S-(2-oxo) pentadecyl-CoA (NHM) can inhibit visceral leishmaniasis infection. The targeted gene deletion reveals that the single copy of NMT gene from *L. major* is necessary for promastigote viability. As like this, one copy of the NMT gene from *L. donovani* is essential for the requirement of propagation of parasites [61].

6.4.9 Phosphofructokinase

Phosphofructokinase (Pfk) is the enzyme which is involved in the glycolytic pathway and located in glycosomes. The enzymes which are involved in glycolytic pathway are mostly conserved, but Pfk is not that much conserved and it shows the variability in different species. There are two types of Pfks: first one is ATP-dependent while another is PPi-dependent Pfk. PPi dependent undergoes modification, binds with different ligands, and then it provides the chance to know the difference between humans and parasitic enzymes; it gives the information to design a drug based on structure.

Pfk of *L. donovani* and *L. braziliensis* is allosteric in nature, and when it binds with the substrate, it requires AMP for the activation. The inhibition process will be reversed by the activator, AMP [62]. ATP Pfk contains glycine residue at the position of 124 where as in kinetoplastids contains lysine at that position. The substitution of lysine residue by glycine did not show any different effect in enzyme activity in *L. donovani* Pfk, but it shows the effect in *T.brucie*, so they mentioned that the mutation creates effect on the behavior of the enzyme Pfk [63].

6.4.10 cdc2-Related Protein Kinase (CRK3)

The regulation of the leishmania cell cycle occurs by the participation of two molecules which is related to cyclin-dependent kinase (CDK) family. These two molecules belong to cdc2-related serine–threonine protein kinases namely CRK1 and CRK3. CDC-2 and CDC-28 are key regulators of cell cycle regulation and these are ubiquitous in nature. CDK2 and CDK1 are useful to control the progression of G1/S and G2/M, respectively. This process is active in metacyclic promastigotes but not in amastigotes. With IC_{50} value of 100 nM flavopiridol compound is useful to inhibit histidine-tagged CRK3 of *L. maxicana* in vitro. Few inhibitors are not only specific to CDK but they also inhibit other types of kinases. *L. maxicana* CRK3 gene has shown 99% similarity with *L. major* gene. ATP-binding site shows the differences between HsCDK2 and Gln85 and is replaced by alanine in Leishmania CRK3, and His 84 with glutamate [64].

6.4.11 Glyceraldehyde 3-Phosphate Dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an enzyme which is involved in the sixth reaction of the glycolytic pathway. This enzyme is involved in activation of transcription, initiation of apoptosis, vesicle shutting from ER to Golgi, and axoplasmic transport. GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1, 3-bisphosphate. This reaction is in two steps; within these, the first step is favorable and another is unfavorable reaction. The gene encoding this enzyme is considered as housekeeping gene because of its constitutive high expression levels. The inhibitors of this enzyme selectively block

the trypanosomal GAPDH but do not block human GAPDH. In one study, the author revealed that the Golgi GAPDH enzymes consist of sequences with targeting glycosomal signals and this enzyme of Golgi and cytoplasm show 55% identity. The difference between the cytosolic GAPDH enzyme in between *L. donovani* and *L. major* plays a crucial role in survival of *L. donovani* in visceral organs. The deletion of cytosolic GAPDH eventually led to depletion of uptake of glucose. The inhibitors should have the capacity to inhibit both cytosolic and glycosolic enzymes. Then it may be an ideal inhibitor. So this enzyme provides the chance to treat as a drug target. In another study, the authors gave the information that adenosine analogs as tight binding inhibitor with the pocket of enzyme NADPH and the co-substrate id NAD^+ even though adenosine is a very poor inhibitor with IC_{50} value of 50 Mm. When tested against *T. brucei* and *T. cruzi* N^6-(1-naphthalenemethyl)-2′-(3-chlorobenzamido) adenosine inhibited the growth in micro molar range [65].

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