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Molecular targets and pathways for the treatment of visceral leishmaniasis

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Visceral leishmaniasis (VL) represents the most severe form of the tropical disease, leishmaniasis. Treatment of VL is complicated because of the few clinically approved antileishmanial drugs available; emerging resistance to first-line drugs; need for a temperature-controlled 'cold' supply chain; serious toxicity concerns over drugs such as amphotericin B; high cost of medication; and unavailability of clinically approved antileishmanial vaccines. Attacking potential molecular targets, specific to the parasite, is a vital step in the treatment of this and other infectious diseases. As we discuss here, comprehensive investigation of these targets could provide a promising strategy for the treatment of visceral leishmaniasis.

Introduction

Leishmaniasis is a debilitating disease caused by various species of protozoan parasite belonging to the genus *Leishmania*. It is particularly prevalent in underdeveloped and developing countries. VL represents the most severe form of leishmaniasis, and can be fatal if left untreated. *Leishmania infantum* (also known as *Leishmania chagasi* or *Leishmania infantum chagasi*) and *Leishmania donovani* are the causative parasites of zoonotic and anthroponotic visceral leishmaniasis. The life cycle of *Leishmania* parasites has two stages: (i) a flagellate promastigote stage, which propagates extracellularly in the gut of sand flies; and (ii) a flagellate amastigote stage, which proliferates in phagocytes within the phagolysosomes of mammalian hosts [1–6].

VL is included in the WHO's list of the most-neglected diseases and affects various organs of the body, including the liver and spleen. The disease is characterized by extreme parasitic loads in the liver, spleen, and bone marrow. The large number of causative species and manifestation of disease in different clinical forms complicates the treatment strategies. The high cost of medication, long treatment schedule, nonavailability of an effective vaccine, emergence of resistance, need for a temperature-controlled 'cold' supply chain, severe toxicity, and lack of drug specificity further complicate the treatment of this tropical disease [1,2,4,5,7,8].

There is urgent need to investigate new targets for the chemotherapy of leishmaniasis because of the unavailability of vaccines and the poor availability of the few antileishmanial drugs that have been developed, which also have serious adverse effects. The presence of unique biological features (specific to either the parasite as a whole or to pathways and/or molecules that could be targeted specifically without inhibiting the corresponding pathways and/or molecules in mammals), including glycosomes, acidocalcisomes, enzymes involved in metabolism, such as trypanothione reductase (TR) and ornithine decarboxylase (ODC), and in DNA replication and repair mechanisms, translation, and unique features of the cell cycle, provide opportunities for investigating new target molecules for the treatment of VL. Various molecular targets and pathways are being investigated to develop new drug molecules and vaccines for the treatment and prevention of infectious diseases, including VL. Here, we review recent studies investigating new targets for the design of novel chemotherapeutic agents for the treatment of VL.

Mechanisms of resistance

One of the main issues relating to the use of existing antileishmanial drugs is the emergence of resistance. Ghosh *et al.* [9] observed that, in *L. donovani*, metabolic reconfiguration is important in the

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development of resistance to antileishmanial drugs, including sodium stibogluconate, amphotericin-B and miltefosine, and could be explored as important target for the development of novel antileishmanial drugs as well as to address emerging resistance [9]. Thiol metabolism was also found to be linked with resistance to pentavalent antimonial drugs. In resistant strains. thiol levels are elevated, accompanied by an increase in the expression level of enzymes including TR, ODC, mitochondrial tryparedoxin peroxidase, and mitochondrial tryparedoxin, which are involved in thiol metabolism [9].

Leishmania parasites have been found to be resistant to the nitric oxide (NO) defence mechanism of phagocytes, and NO-resistant strains of *L. chagasi* were observed to be more infective than NO-sensitive strains [3]. Relapse cases of *L. infantum* have shown resistance to antimonial drugs, which reinforce the killing mechanisms of macrophages. Furthermore, these parasites were also resistant to NO and macrophage-killing mechanisms, showing both increased infectivity and survival within host macrophages [2].

Miltefosine was the first antileishmanial drug found to be effective orally, although high tolerance has been observed for this drug. The emergence of resistance in *L. donovani* towards miltefosine was observed to be associated with mitochondrial heat shock protein HSP70 (or HSPA9B) in the promastigote stage, affording the parasite protection from high temperatures and low pH [8]. The miltefosine transporter (LiMT)/LiRos3 (β-subunit of the miltefosine transporter) complex also has a role in the development of resistance towards miltefosine [10].

In recent years, next-generation sequencing (NGS) has become more available via the development of NGS platforms such as 454 Sequencing (Roche), Illumina; PacBio (Pacific Biosciences); Ion Torrent (PGM/Proton; Life Technologies); and SOLiD (Applied BioSystems). The power of NGS enabled scientists to rapidly and specifically identify biomarkers and determinants of drug resistance in *Leishmania* spp., including gene dosage alterations, modulations of gene expression, and single nucleotide polymorphisms (SNPs) [11]. Laffitte and co-workers recently reported that the plastic genome of *Leishmania* enables gene copy number variation in response to drug pressure, which the authors suggest as a possible drug target [12].

In summary, resistance of *Leishmania* spp. to antileishmanial drugs is related to some extent to metabolic pathways, including glucose and thiol metabolism, the overexpression of certain enzymes, and increased membrane fluidity, resulting in altered interactions with antileishmanial drugs. These biochemical and biophysical changes must be taken into consideration while investigating potential novel therapeutic strategies for the treatment of VL.

Molecular targets and pathways for the treatment of leishmaniasis

Targeting molecules specific to *Leishmania* parasites provides an attractive strategy to develop an optimum therapeutic device for the treatment of VL. Important molecular targets and metabolic pathways of *Leishmania* parasites are depicted in Fig. 1 and summarized in Table 1.

Glycosome: compartmentalized pathways and enzymes

Kinetoplastids contain a unique cell organelle known as the glycosome, which compartmentalizes various crucial metabolic

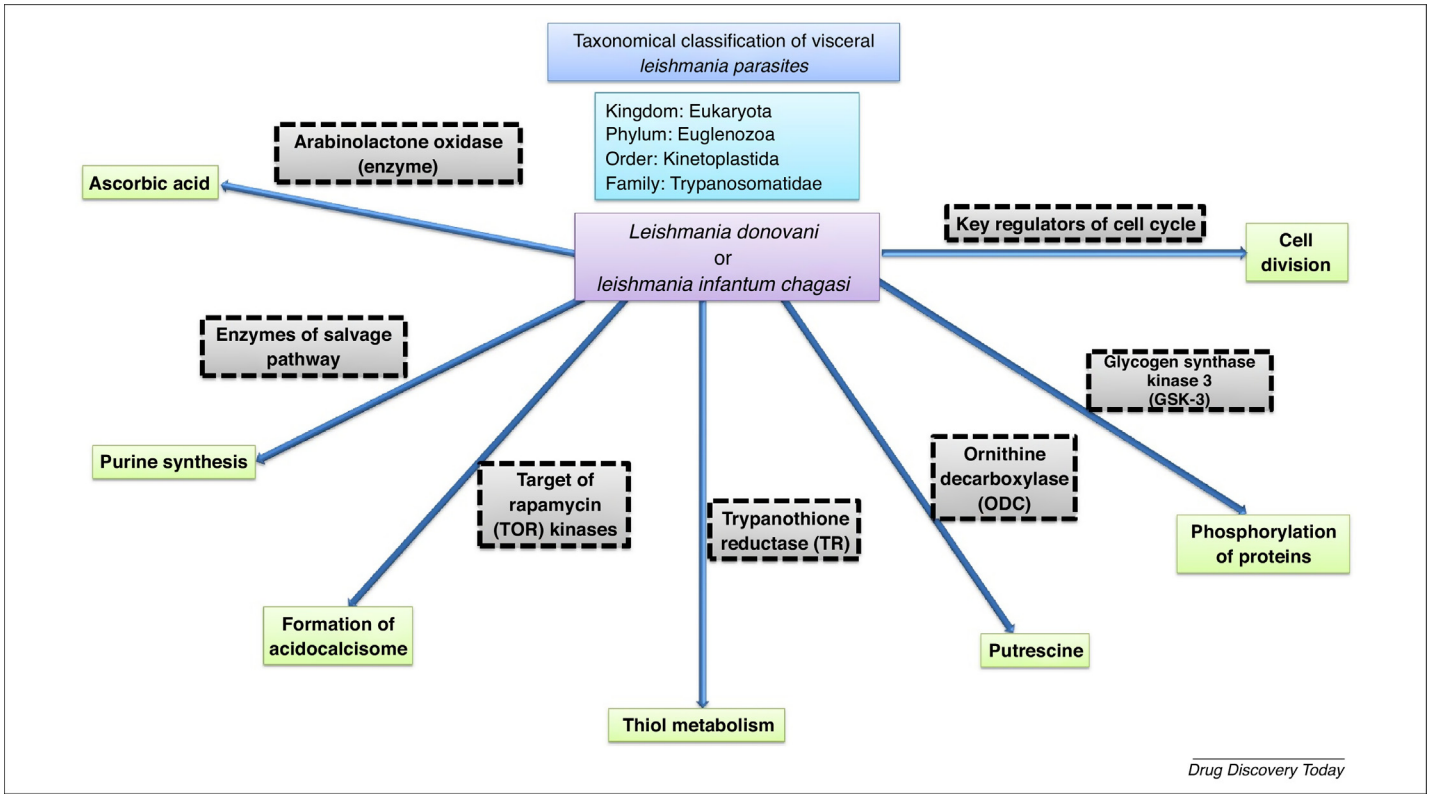

FIGURE 1

New molecular targets for antileishmanial therapy (broken boxes indicate anticipated molecular targets).

TABLE 1

Molecular targets for antileishmanial therapy

Target	Description
Arabinolactone oxidase	Essential for ascorbate synthesis in *Leishmania* spp.
Targets of rapamycin kinases	Essential for trypanosomatid virulence; its deficiency leads to glucose starvation
Purine *de novo* synthesis pathways	*L. donovani* uses this pathway to maintains its supply of purines
TR	Enzyme involved in trypanosomatid thiol metabolism; also has a role in the formation of DNA precursors
ODC	Enzyme that catalyzes formation of putrescine from ornithine via decarboxylation
GSK-3	A serine/threonine protein kinase that is investigated extensively as a molecular target for many diseases, including infections caused by trypanosomatids
Leishmania cell cycle	Fundamental regulators of the leishmanial cell cycle could be exploited as targets for therapy

pathways, including purine salvage, glycolysis, and β-fatty acid oxidation. *Leishmania* parasites are unable to synthesize purines *de novo*. The parasites obtain purines from their host via the salvage synthesis of three different degradation products of purines: inosine-5′-monophosphate (IMP), guanine, and xanthine (Fig. 2). Thus, the purine salvage pathway is vital for the viability and growth of *Leishmania* parasites [13–15]. The parasites are able to adapt to purine-scarce environments by surveying the intracellular purine nucleotide pools, which induces metabolic changes that enable them to survive in purine-deficient environments [15].

Guanosine monophosphate reductase (GMPR) and IMP dehydrogenase (IMPDH) are compartmentalized into the leishmanial glycosome mediated by COOH-terminal peroxisomal targeting signals. In contrast to IMPDH-null mutants of *L. donovani*, GMPR- or GMPR- and IMPDH-null mutants show highly restricted growth phenotypes. GMPR-null mutants were unable to grow in the presence of xanthine, guanine, or corresponding nucleosides, highlighting the importance of GMPR in the purine salvage pathway of these parasites [16]. Bastos *et al.* [17] investigated the role of two nucleoside triphosphate diphosphohydrolases (NTPDases) from *L. infantum chagasi* (LicNTPDase1 and -2) in the purine salvage pathway by expressing these nucleosides in bacterial (*Escherichia coli*) and mammalian cell (COS-7) systems, respectively. The authors observed that the activity of rLicNTPDase2 was increased in *E. coli*, as did that of LicNTPDase1 in COS-7 cells [17].

Thus, enzymes in the purine salvage pathway could be promising targets for the development of new antileishmanial treatments.

Polyamine biosynthesis, thiol metabolism, and the role of trypanothione

Polyamines are low-molecular-weight aliphatic polycations of *Leishmania* that are attractive molecular targets for the treatment

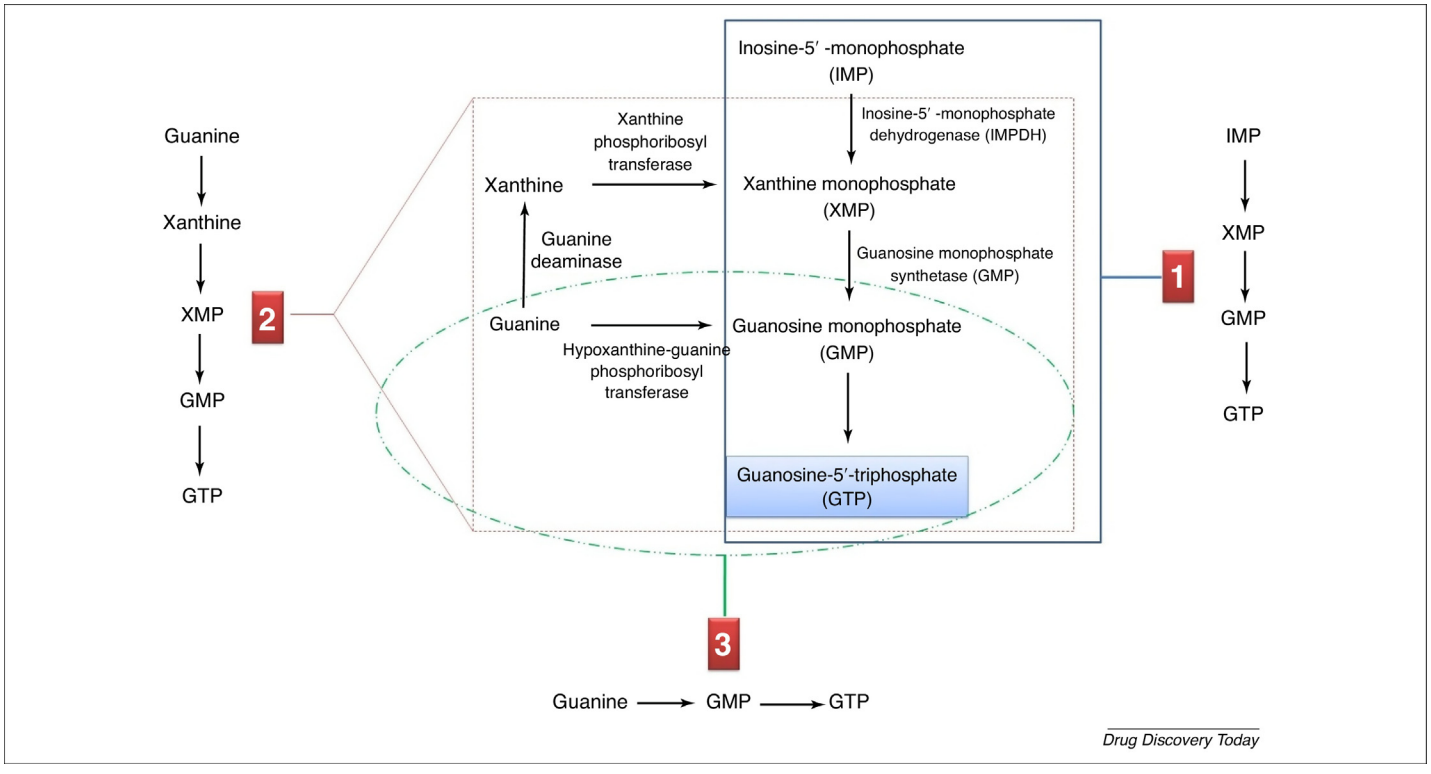

FIGURE 2

Purine salvage pathways in *Leishmania*.

of VL because of their roles in the growth, development, virulence, and survival these parasites. The polyamine biosynthetic pathway and enzymes involved in spermidine biosynthesis in *L. donovani*, including arginase, ODC, and spermidine synthase, have been investigated as drug targets in the search for novel chemotherapeutic agents for the treatment of VL [18–20].

Trypanothione is synthesized by *Leishmania* to combat oxidative stress. Structurally, it comprises two glutathione units linked by the polyamine, spermidine. Trypanothione provides electrons to the tryparedoxin-tryparedoxin peroxidase couple to reduce reactive oxygen species (ROS) produced by macrophages. TR maintains trypanothione in the reduced stage, which enables the parasite to survive even in conditions of oxidative stress created by macrophages (Fig. 3) [19–21].

Arginase and spermidine synthase

Arginase is involved in polyamine biosynthesis that has been investigated as a drug target in trypanosomatid diseases including VL, African sleeping sickness and Chagas' disease. The absence of extracellular arginase can lead to cell death in *Leishmania* via caspase-independent apoptosis mediated by ROS [22,23]. Da Silva and co-workers [22] reported the inhibition of this enzyme by the flavonols quercetin, quercitrin, and isoquercitrin. Glucoside flavonoids obtained from ethanolic extracts of the plant *Cecropia pachystachya* were found to reduce the growth of *Leishmania amazonensis* promastigotes by inhibiting the activity of arginase, an enzyme that is vital to the polyamine biosynthesis of this parasite, and resulting in the alteration of mitochondrial kinetoplastid DNA assembly [24].

Spermidine synthase catalyzes the conversion of putrescine to spermidine (Fig. 3). The second enzyme in the polyamine biosynthesis pathway of *Leishmania*, spermidine synthase, has also been investigated as a possible drug target. Using a homology modeling approach, Grover *et al.* [25] investigated the tertiary structure of spermidine synthase from *L. donovani* to distinguish its active site from that of human spermidine synthase. The authors also used molecular dynamics simulations to model the structure of leishmanial spermidine synthase and identified its active sites, which differ from those of the human enzyme. On this basis, they also screened two selective inhibitors of *L. donovani* spermidine synthase that did not affect the human form of the enzyme [25].

Although previous studies suggest that arginase and spermidine synthase could serve as important drug targets, a recent study suggested that arginine is essential for the survival of promastigotes but not of amastigotes, which reside within the phagolysosome inside macrophages. It was also reported that amastigotes can readily salvage arginine and ornithine but not spermidine. Boitz *et al.* [18] created arginase-null mutants of *L. donovani* and found that the promastigotes were unable to grow in the absence

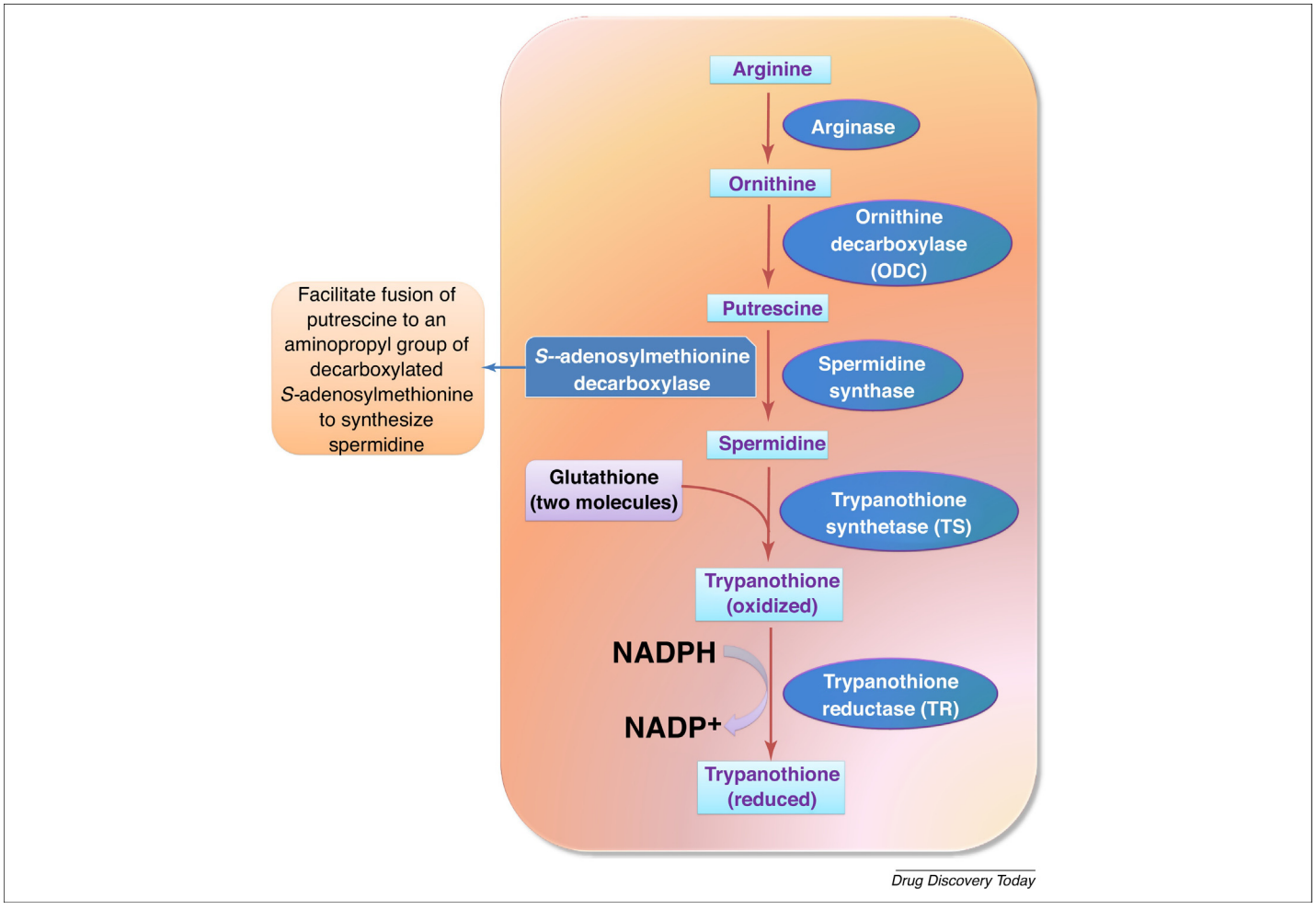

FIGURE 3

Polyamine biosynthesis and its role in the formation of trypanothione and reduction of oxidative stress.

of polyamine supplementation with ornithine, putrescine, or glycosomal or cytosolic arginase, showing that arginase is essential for promastigote growth. However, arginase was not found to be essential for the growth of intracellular amastigotes, given that *L. donovani* amastigotes readily salvage ornithine and achieve polyamine biosynthesis by having access to spermidine pools in the host. Furthermore, it is possible that putrescine cannot be salvaged from the host by the parasite [18]. However, further systematic studies are required of both arginase and spermidine synthase to confirm their role as drug targets for VL.

Ornithine decarboxylase

In polyamine biosynthesis, ODC forms putrescine by catalyzing the decarboxylation of ornithine (a rate-limiting enzyme in polyamine biosynthesis). It also has an important role in redox metabolism by controlling the synthesis of trypanothione and in the pathogenesis of *L. donovani* by modulating the immune response. Given the significant differences between human and *L. donovani* ODC, as well as the significant increase in oxidative stress and ROS levels resulting from ODC inhibition following by trypanothione depletion in the parasite, research has focused on the development of novel specific inhibitors of ODC [19,20,26]. ODC deficiency caused by α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, reduced the virulence of *Leishmania*, which was restored by supplying putrescine. Given that DFMO inhibits the promastigote stage of the parasite but is not effective against the infectious amastigote stage, the discovery and development of new ODC inhibitors could lead to promising drug candidates [27].

Yadav *et al.* [20] examined the role of ODC in the pathogenesis and immunological responses of VL. The authors purified a recombinant ODC from *L. donovani* (r-LdODC) and investigated its effects on the immunological responses of peripheral blood mononuclear cells from patients with VL as well as from patients who had been successfully treated. The growth of both the promastigote and axenic amastigote stages was found to be directly linked to r-LdODC. Furthermore, there was increased production of interleukin (IL)-10 without any effect on interferon (IFN)-γ production by $CD4^+$ T cells, and reduced IL-12, NO, and ROS levels, highlighting the importance of ODC in modulating the immune response and supporting parasite growth in susceptible hosts. In addition, the cultures of promastigotes and amastigotes, and blood samples from patients showed decreased growth with reduced parasite numbers and increased levels of IFN-γ, IL-12, and ROS in the presence of DFMO, which is an ODC inhibitor [20].

Kumar Pandey *et al.* [26] modeled the dimensional structure of ODC using molecular modeling and virtual screening approaches to design ODC-inhibiting drug for the treatment of VL. The authors revealed ZINC67909154 (Fig. 4) to be a potent inhibitor of ODC and suggested that it could be a suitable VL drug candidate [26].

Thus, ODC has important roles in the survival, metabolism and immune responses of *Leishmania*. The development of molecules acting on this molecular target could provide important leads in the design of novel antileishmanial drugs.

Trypanothione synthetase and trypanothione reductase

Following the synthesis of spermidine, trypanothione synthetase (TS) catalyzes the conjugation of spermidine with two glutathione units to produce trypanothione. TR is involved in the redox metabolism of trypanosomatid parasites by converting the oxidized form of trypanothione into its reduced form by connecting the thiol- and NADPH-based redox systems. TR performs the same function as glutathione reductase in the insect and mammalian host of trypanosomatids. TR also assists in retaining the intracellular reducing environment by detoxifying hydroperoxides in trypanosomatids. It also takes an active role in the formation of DNA precursors using NADPH as a co-substrate and FAD as a flavin co-factor. Given these important roles of TS and TR in *Leishmania* and their absence in humans, they have been explored as validated molecular targets for VL treatment. However, studies with TR have shown that *Leishmania* can survive at very low concentrations of TR and reduced trypanothione (10% and 5%, respectively). Thus, it will be necessary to identify effective inhibitors of TR that are active in the nanomolar range or are irreversible inhibitors [21,28–32]. For a review of thiol metabolism in these parasites, and its role in redox reactions and potential as a drug target, as well as a list of the various inhibitors of TS and TR, see Ref. [31].

Zahir *et al.* [33] synthesized silver and titanium dioxide nanoparticles (NPs) by green synthesis. In studies against *L. donovani* intracellular amastigotes and promastigotes, silver NPs were found to be most effective, with IC_{50} values of 3.89 and 14.94 μg/ml, respectively. The mechanism of action of silver NPs was found to be due to inhibition of the trypanothione/TR system in *L. donovani*. The authors speculated that these silver NPs could be explored as safe and cost-effective alternatives for the treatment of VL [33].

Pandey and co-workers [34] evaluated nitroimidazole analogs as inhibitors of TR of *L. donovani* and compared their TR inhibitory activity with that of a positive inhibitor of TR, clomipramine [34]. Iman *et al.* [29] investigated the antileishmanial activity and interaction of selenocyanate and diselenide derivatives against TR from *L. infantum* via molecular dynamic simulation and docking experiments, and observed that the binding energies of the aryl rings of the most potent compounds were higher than the reference drugs miltefosine or edelfosine [29]. Baquedano *et al.* [28] also investigated heteroaryl selenocyanate and diselenide derivatives bearing different bioactive scaffolds, such as quinoline, quinoxaline, acridine, chromane, and furane, as TR inhibitors and as prospective antileishmanial agents (Fig. 4). Of these derivatives, two compounds [3,5-dimethyl-4-isoxazolyl selenocyanate and 3,3′-(diselenodiyldimethanediyl) bis(2-bromothiophene)] showed significant inhibition of TR with corresponding antileishmanial activity [28].

Ramu *et al.* [35] examined hybrids of β-carboline-quinazolinone with different stereochemical orientations as inhibitors of *L. donovani* TR, based on the observation that drug–protein binding is affected by the chirality of drug molecule. One isomer among the different stereoisomers under investigation showed significant antileishmanial activity and binding affinity towards *L. donovani* TR in promastigotes, intracellular amastigotes, and *in silico* docking studies [35]. Polyamine derivatives of hydroxybenzotriazole have also shown TR inhibition. Among different synthesized derivatives, N2-spermidine-benzotriazole derivatives have shown activity against intramacrophagic amastigotes of *L. donovani*, with IC_{50} values of 3 MM [36]. Plumbagin, a naphthoquinone metabolite obtained from *Plumbago indica*, shows both TR inhibitory activity and activity against leishmanial promastigotes and amastigotes. It

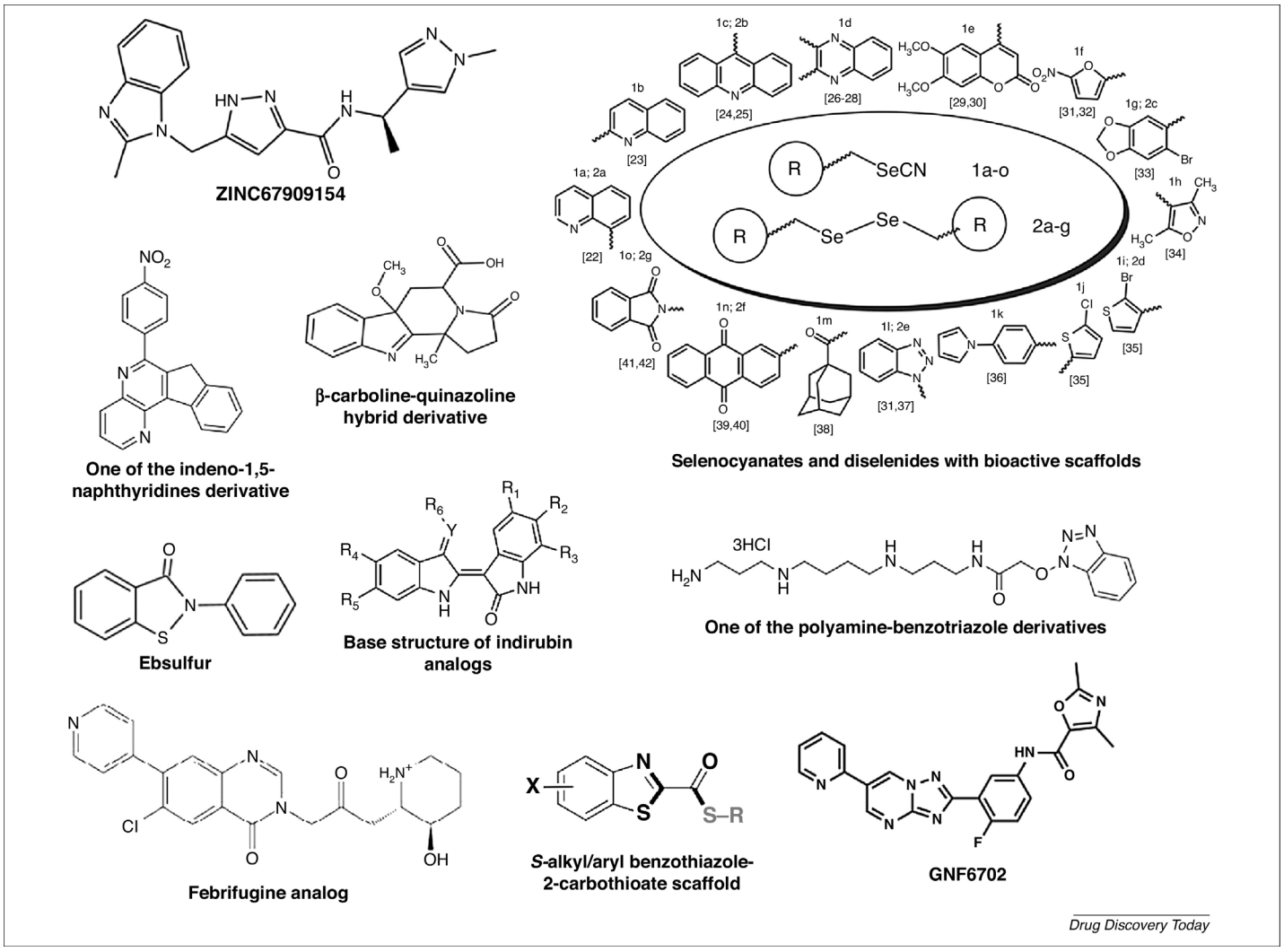

FIGURE 4

Structures of novel antileishmanial chemotherapeutic molecules acting on molecular targets and pathways currently under preclinical investigation.

was reported that plumbagin induced apoptosis-like death in response to an increase in oxidative stress [37].

In a molecular dynamics simulation study, Pandey *et al.* [38] investigated the antileishmanial activity of benzoxaborole-based analogs. The authors reported the interaction of these analogs with TR and suggested that benzoxaborole analogs could be promising novel antileishmanial drug candidates [38]. In a docking study, one febrifugine analog, 6-chloro-3-[3-(3-hydroxy-2-piperidyl)-2-oxo-propyl]-7-(4-pyridyl) quinazolin-4-one, showed potential inhibition of both TR and *L. donovani* [39]. Benzothiazole derivatives also showed antileishmanial activity against *L. donovani*, possibly as a result of TR inhibition, without any toxicity towards RAW 264.7 macrophages [40].

Tricyclic antidepressants have shown leishmanicidal action that was speculated to be via TR inhibition. For example, cyclobenzaprine (CBP) showed therapeutic potential against experimental VL by inhibition of TR, immunomodulation and induction of oxidative stress evinced by enhanced release of ROS, and decreased release of IL-6 in promastigotes and infected macrophages [30].

TS inhibitors have also been designed by scientists including conessine, cynaropicrin, phenyl-thiazole, phenyl-tetrazole, phenyl-indazole, and oxabicyclo[3.3.1]nonanone [31]. Thus, TR and TS could be further explored as druggable biomolecular targets for the development of antileishmanial therapeutic agents.

DNA replication and the cell cycle

Attacking the replication and repair mechanisms of DNA or the cell cycle or of key regulators of the cell cycle in *Leishmania* and other trypanosomatid parasites is another approach used to develop novel treatment strategies or novel biomolecular targets for VL treatment [7,41,42].

Balaña-Fouce *et al.* [42] reviewed trypanosomatid DNA topoisomerases in terms of their structure and their role in the physiology and virulence of trypanosomatids, including *L. donovani*. DNA topoisomerases relax the torsional tensions during DNA replication and transcription. This ubiquitous family of proteins also maintains the stability of the genome during DNA replication. Given that inhibition of these enzymes can induce cell death, DNA topoisomerases are also being investigated for the development of antiparasitic drugs [42]. *Leishmania* topoisomerases differ structurally from mammalian topoisomerases (Fig. 5) and, thus, have been investigated as druggable targets in VL [42,43]. Tejería *et al.* [43]

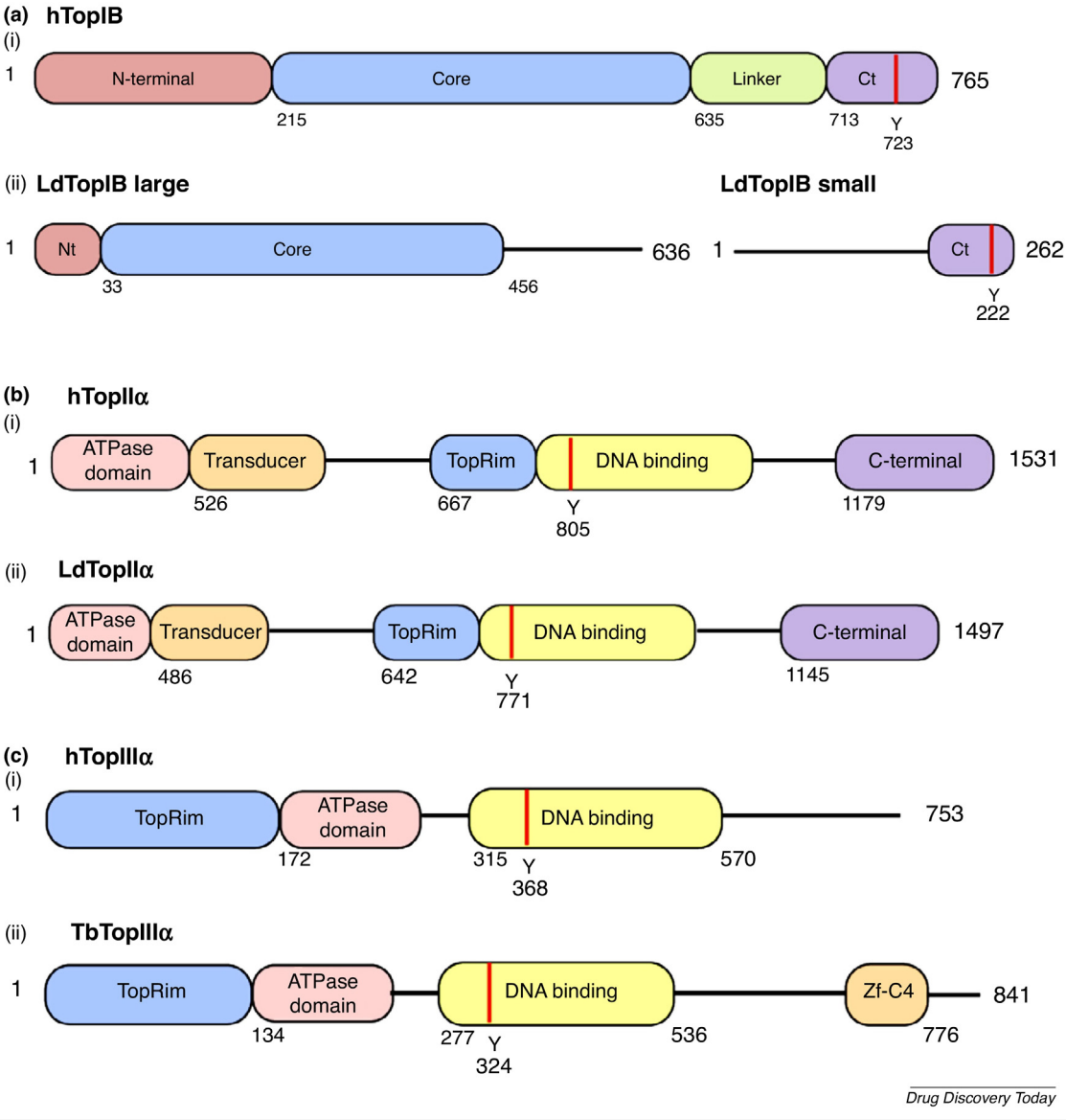

FIGURE 5

Graphical comparison of topoisomerases of trypanosomatids with their human counterparts. **(a)** Human (i) and bi-subunit *Leishmania donovani* (ii) topoisomerase IB (TopIB) structural domains: nonconserved hydrophilic N-terminal domain (Nt); central 'core' domain with the representative active-site amino acids ('tetrad'); nonconserved 'linker' subdomain that connects the 'core', C-terminal domain (Ct), and Ct containing the DNA-cleaving Tyr (red strip). **(b)** Human (i) and *L. donovani* topoisomerase II (TopII) (ii) structural domains: N-terminal domain containing the ATPase and transducer domains; the Mg^{2+}-binding site of the TopRim motif; a DNA-binding domain that contains the DNA-cleaving Tyr (red strip); and the C-terminal domain. **(c)** Human (i) and *Trypanosoma brucei* topoisomerase III (TopIII) (ii) structural domains: N-terminal domain containing the Mg^{2+}-binding site at the TopRim motif; the ATPase and C-terminal domains; DNA-binding site that contains the DNA-cleaving Tyr (red strip); and Znf-C4 (the zinc-finger domain). Not to scale. Adapted, with permission, from Ref. [41].

synthesized indeno-1,5-naphthyridines polycyclic heterocyclic compounds and evaluated their ability to inhibit both *Leishmania* as well as human topoisomerases (Fig. 4). The antileishmanial activity of these compounds was also tested against promastigotes and amastigote-infected splenocytes and compared with that of amphotericin B. Synthesized indeno-1,5-naphthyridines inhibited *Leishmania* topoisomerases without affecting human topoisomerases, with a high selectivity index towards *L. Infantum* amastigotes. Furthermore, tetrahydro indeno-1,5-naphthyridines derivatives showed equivalent or even higher antileishmanial activity than amphotericin B towards *L. Infantum* amastigotes [43].

A volatile compound from plants, pentadecane, has been shown to inhibit the growth of *L. donovani* by more than 85%, with IC_{50} values of 65.3 and 60.5 μM towards promastigotes and amastigotes, respectively. A study using propidium iodide (PI) showed that pentadecane arrested the sub-G0/G1 and G1 phases of the *L. donovani* cell cycle. Furthermore, pentadecane was found to be negligibly cytotoxic towards primary epithelial cells of Cercopiteco and the immortal cell lines U937 and DH82 [41]. Kumar and Saha [44] observed that removal of histone acetyl transferase 3 (HAT3) from *L. donovani* led to a decrease in cell viability attributed to defects in histone deposition and aberrant cell progression patterns [44].

Kinases

Kinases have important roles in the survival, growth, and virulence of parasites [45–47]. Glycogen synthase kinase 3 (GSK-3), a protein kinase, has been investigated as a putative target for the treatment of trypanosomatid infections [48]. Analogs of indirubin, a bis-indole that occurs naturally in mollusks and plants, including 6-bromoindirubin-3′-oxime (6BIO) and 6-substituted analogs, target leishmanial cyclin-dependent kinase-1 (LCDK1) and leishmanial GSK-3 (LGSK-3), with more potent effects on LCDK1. Efstathiou *et al.* [48] synthesized seven new indirubin analogs with 3′-bulky amino substitutions, including piperazine or pyrrolidine rings, as selective inhibitors of LGSK-3 with GI_{50} values <1.5 μM[48].

Using whole-cell phenotypic assays and high-throughput screening, Peña *et al.* [49] investigated a new set of compounds with activity against *L. donovani*, *Trypanosoma cruzi*, and *Trypanosoma brucei*. The prospective mechanisms of these compounds were found to be their activity against kinetoplastid kinases, proteases, cytochromes, and potential host–pathogen targets [49]. *L. donovani* MAPK1 (LdMAPK1) has been suggested to be a promising target because it decreases the P-glycoprotein efflux of antimonial drugs; in addition, the downregulation of LdMAPK1 has also been observed in antimony-resistant isolates of *L. donovani* [50]. The mitotic kinase Aurora kinase, has important roles in eukaryotic cell division. The *L. donovani* homolog of Aurora kinase has been identified to be important in cell cycle progression, cell division (in mitosis and cytokinesis) and the viability of the parasite [45]. Since kinases play important role in cell cycle and propagation of parasite as well as in modifying the immune response of host hence they could also be explored to devise new strategy for treatment of infectious diseases including leishmaniasis.

Arabinolactone oxidase

Arabino-1,4-lactone oxidase (ALO) is an enzyme in the ascorbate biosynthesis pathway in *L. donovani* [51,52]. Ascorbic acid has an important role in physiological processes of *L. donovani*, and ALO-null mutants (ΔALO) of *L. donovani* showed reduced infectivity in mice and decreased survival within macrophages. Furthermore, these mutants also showed induction of IFN-γ, IL-12, and tumor necrosis factor (TNF)-α, with increased susceptibility towards oxidative stress [52]. Anand and Madhubala [51] observed that genetically engineered ascorbic acid-deficient live attenuated ΔALO parasites served as safe immunogens and induced long-lasting immunity against VL in Balb/c mice [51].

Thus, ALO could be an additional molecular target in the treatment strategy for VL, given that ALO-null mutants showed decreased survival rates and a reduction in virulence.

Future directions

Proteins and other enzymes that are important for the survival and virulence of *L. donovani* are currently under investigation as druggable biomolecular targets for the design of novel antileishmanial drug molecules or potential antileishmanial vaccines. In addition to the molecular strategies discussed above, other molecules that could be pursued as drug targets include serine proteases, which are highly expressed in *L. donovani* and important for its virulence, and stress-specific eIF2α kinases, which have important roles in the survival of parasites under stress conditions, such as acidic pH and high temperatures, by inducing the phosphorylation of eIF2α [53,54].

Based on the fact that kinetoplastid parasites, such as *Leishmania* and *Trypanosoma* spp., are similar in terms of their genomic sequences and biology, Khare *et al.* [55] investigated GNF6702, a noncompetitive and selective inhibitor of the kinetoplastid proteasome as a prospective broad-spectrum drug. This research also paved the way for a new direction in the investigation of treatment strategies for diseases caused by kinetoplastids, because GNF6702 was found to be reasonably safe in mice and selective towards the kinetoplastid proteasome without affecting mammalian cells or the mammalian proteasome. The structure of GNF6702 as well as that of other compounds acting on leishmanial molecular targets and pathways are shown in Fig. 4.

Nanotechnology approaches are also being investigated for the delivery of conventional and novel antileishmanial drug molecules [56–60]. Clinical trials are also ongoing to study the efficacy of nanotechnology-based formulations, and combinations of antileishmanial drugs, antileishmanial vaccines, and novel drugs, such as fexinidazole (a nitroheterocyclic compound), which is a prodrug converted into cytotoxic species by nitroreductase. These cytotoxic species then exert their antileishmanial action by damaging DNA, lipids and proteins [61–64].

Concluding remarks

The development of an optimum treatment strategy for VL appears challenging because of the emergence of resistance, cost, and toxicity. However, the important roles of enzymes, such as ODC, TR, and G6PDH, of pathways, such as thiol metabolism, carbon metabolism, cell cycle, and DNA replication, as well as other proteins and biomolecules in the survival and virulence of *Leishmania* spp. provide additional targets for the design of new molecules for the development of new antileishmanial drugs and vaccines.

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