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REVIEW ARTICLE

Polyamine metabolism in *Leishmania*: from arginine to trypanothione

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Abstract Polyamines (PAs) are essential metabolites in eukaryotes, participating in a variety of proliferative processes, and in trypanosomatid protozoa play an additional role in the synthesis of the critical thiol trypanothione. The PAs are synthesized by a metabolic process which involves arginase (ARG), which catalyzes the enzymatic hydrolysis of L-arginine (L-Arg) to L-ornithine and urea, and ornithine decarboxylase (ODC), which catalyzes the enzymatic decarboxylation of L-ornithine in putrescine. The *S*-adenosylmethionine decarboxylase (AdoMetDC) catalyzes the irreversible decarboxylation of *S*-adenosylmethionine (AdoMet), generating the decarboxylated *S*-adenosylmethionine (dAdoMet), which is a substrate, together with putrescine, for spermidine synthase (SpdS). *Leishmania* parasites and all the other members of the trypanosomatid family depend on spermidine for growth and survival. They can synthesize PAs and polyamine precursors, and also scavenge them from the microenvironment, using specific transporters. In addition, Trypanosomatids have a unique thiol-based metabolism, in which trypanothione (N1-N8-bis(glutathionyl)spermidine, $T(SH)_2$) and trypanothione reductase (TR) replace many of the antioxidant and metabolic functions of the glutathione/glutathione reductase (GR) and thioredoxin/thioredoxin reductase (TrxR) systems present in the host. Trypanothione synthetase (TryS) and TR are necessary for the protozoa survival. Consequently, enzymes involved in spermidine synthesis and its utilization, i.e. ARG, ODC, AdoMetDC, SpdS and, in particular, TryS and TR, are promising targets for drug development.

Keywords Polyamines · *Leishmania* · Arginine · Trypanothione · Redox metabolism · Drug targets

Abbreviations

AdoMet	*S*-Adenosylmethionine
AdoMetDC	*S*-Adenosylmethionine decarboxylase
APA	3-Aminooxy-1-aminopropane
APC	Amino acid-polyamine-organocation
APX	Ascorbate peroxidase
ARG	Arginase
CAT	Cationic amino acid transporter
dAdoMet	Decarboxylated *S*-adenosylmethionine
DFMO	α-Difluoromethylornithine
eEF1B	Eukaryotic translation elongation factor 1B
GR	Glutathione reductase
GspS	Glutathionyl-spermidine synthetase
L-Arg	L-Arginine
MCF	Mitochondrial carrier family
NO	Nitric oxide
NOS	Nitric oxide synthase
nsGPX	Non selenium glutathione peroxidase-like enzyme
ODC	Ornithine decarboxylase
PA	Polyamine
PAO	Polyamine oxidase
PLP	Pyridoxal 5′-phosphate
PV	Parasitophorous vacuole
ROS	Reactive oxygen species

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RNOS	Reactive nitric oxide species
RR	Ribonucleotide reductase
Spd	Spermidine
SpdS	Spermidine synthase
Spm	Spermine
TDPX	Tryparedoxin peroxidase
TR	Trypanothione reductase
TrxR	Thioredoxin reductase
TryS	Trypanothione synthetase
$T(SH)_2$	Trypanothione, or N1, N8-bis(glutathionyl)spermidine
TXN	Tryparedoxin

Introduction

Leishmaniasis is a disease characterized by high morbidity, which is deeply linked to malnutrition, humanitarian emergencies and environmental changes that affect vector biology. It causes an estimated 70,000 deaths annually, a rate surpassed among parasitic diseases only by malaria (Alvar et al. 2006). Usually, the parasite primarily infects a feral or domestic mammalian host. Most forms of the disease are zoonotic, i.e. transmissible only among animals, but human leishmaniasis is increasingly spreading throughout the world, with a sharp increase in the number of recorded cases over the last 10 years.

Indeed, 2 million new cases are considered to occur annually, with an estimated 12 million people presently infected worldwide. Leishmaniasis comprises two major clinical forms, visceral leishmaniasis, caused by *L. donovani* and *L. infantum*, which is invariably fatal, if untreated, and the cutaneous form, which can heal spontaneously but leaves disfiguring scars.

The *Leishmania* parasites exist in two different forms; the promastigotes (flagellates) are transmitted into mammalian host by the bite of sand flies of the genus *Lutzomyia* in the New World and of the genus *Phlebotomus* in the Old World. Once inside host cells, promastigotes transform into aflagellated amastigotes, which can multiply inside parasitophorous vacuoles (PVs) of the infected cell. Many cell types can be infected by the parasites including dendritic cells, fibroblasts and neutrophils, but the major target of the parasites are the macrophages. The fate of intracellular *Leishmania* parasites is determined by the activation status of macrophages. Fully activated macrophages can produce leishmaniacidal molecules, such as nitric oxide (NO) and oxidative mediators, and kill parasites effectively, whereas "suboptimally" and "alternatively" activated cells preferentially turn on the Arginase (ARG) pathway to produce polyamines (PAs) and enhance parasite replication/persistence (Martinez et al. 2009). Although these two distinct types of macrophage activation can lead to divergent outcomes of infection, L-arginine (L-Arg) is a common substrate for their enzymatic activities. Therefore the L-Arg is positioned at a crossroads between life and death of the intracellular parasites, and its metabolism is a key determinant for *Leishmania* infection.

L-Arg, as mentioned above, is the precursor of PAs, simple aliphatic compounds found in all tissues and microorganisms. Indeed, the PAs biosynthetic pathway starts with the synthesis of the polyamine precursor L-ornithine, catalyzed by ARG. L-ornithine is then decarboxylated by ornithine decarboxylase (ODC), which produces the putrescine molecule. Putrescine is used as the substrate for the constitutive spermidine synthase (SpdS) that adds the aminopropyl group donated from the decarboxylated *S*-adenosylmethionine (dAdoMet) provided by the *S*-adenosylmethionine decarboxylase (AdoMetDC). Finally, spermine synthase performs a similar function on spermidine (Spd), producing spermine (Spm) by the addition of another aminopropyl group.

Under physiological conditions, PAs are totally protonated and behave as natural polycations, capable of interacting with both DNA and RNA, thereby inducing conformational transitions and affecting nucleic acid function (Wallace 2003; Ruiz-Chica et al. 2003; Ouameur and Tajmir-Riahi 2004; N'soukpo-Kossi et al. 2008). It has been shown that biogenic PAs cause DNA condensation in both isolated DNA (Patel and Anchoroquy 2006) and chromatin (Todd et al. 2008). However, the exact nature of the polyamine–DNA interaction is not clearly established even if is well known that putrescine, Spd and Spm bind to DNA through the minor and major grooves of the double strand, interacting electrostatically with the phosphate backbone (Ouameur and Tajmir-Riahi 2004). On account of absolute PA requirement for cell growth, interference with PA biosynthesis can be a rather promising therapeutic approach against a number of diseases (Wallace 2007; Casero and Marton 2007). PAs are essential for eukaryotic cell growth and differentiation, and are known to be implicated in a number of cellular roles as well as in maintaining the native structure of several biological macromolecules while affecting the activity of others through tightly regulated concentration-dependent processes. Moreover, several findings suggest that the deregulation of PA metabolism may induce apoptosis (Seiler and Raul 2005).

Intracellular PAs concentrations are maintained at a cell type-specific level by a highly regulated metabolic pathway, an energy-dependent transport system, and an export system that leads to the efflux of both modified and unmodified PAs. Highly regulated rate-limiting enzymes facilitate the fine control of both biosynthesis and catabolism.

Apart from being of vital importance for the propagation and viability of most cells, the natural PAs Spm and Spd are also the source of cytotoxic metabolites. In most eucaryotes, oxidative deamination of Spm by amine oxidase generates hydrogen peroxide and aldehyde(s), whereas in bacteria polyamine oxidase (PAO) converts Spd and Spm into 4-aminobutyraldehyde and 3-(aminopropyl)-4-aminobutyraldehyde, respectively, in addition to 1,3-diaminopropane, with production of hydrogen peroxide. The products of the PA catabolism may induce both apoptotic and non-apoptotic cell death (Agostinelli et al. 2007, 2009).

The last few years have seen a steady increase in studies on the role of the intracellular PAs in the onset of several human diseases, and many enzymes of PAs metabolism are now considered as potential targets for chemotherapy and chemoprevention.

In *Leishmania*, Spd together with glutathione is the substrate for the trypanothione synthetase (TryS), which synthesizes the trypanothione ($T(SH)_2$). This molecule is kept reduced by the trypanothione reductase (TR). The $T(SH)_2$/TR system replaces many of the antioxidant and metabolic functions of the glutathione/glutathione reductase (GR) and thioredoxin/thioredoxin reductase (TrxR) systems present in other organism and therefore is necessary for the parasite survival. The $T(SH)_2$ pathway is crucial for the parasite but is absent in the host and for this reason may provide clues for rational drug design. Fairlamb and coworkers have shown that pentavalent antimonials in vivo interfere with the $T(SH)_2$ metabolism by inducing rapid efflux of intracellular $T(SH)_2$ and by inhibiting TR in intact cells (Cunningham and Fairlamb 1995). Moreover, Baiocco and coworkers solved the structure of TR in complex with Sb(III), disclosing the molecular basis of the interaction of antimonials with TR and of its inhibition (Baiocco et al. 2009).

In this review, the main features of the PA metabolic pathways of *Leishmania* spp. are described with a special emphasis on the enzymes that can be considered as main drug targets against Leishmaniasis.

L-arginine metabolism in the host macrophage

A complex interplay between parasite and host metabolism exists. Infection of the mammalian host is initiated by flagellated promastigotes, which are deposited into the host skin during a sandfly bloodmeal. Promastigotes are phagocytized by macrophages, either directly or after infection of neutrophils initially recruited to the sandfly bite (van Zandbergen et al. 2004). Promastigotes are targeted to vacuolar compartments in the macrophage that have the characteristics of mature phagolysosomes, where they differentiate to the smaller aflagellated amastigotes.

Leishmania parasites are auxotrophic for many amino acids, which must be scavenged from the phagolysosome (McConville et al. 2007; Naderer and McConville 2008).

In mammals, L-Arg is a semi-essential amino acid (Yeramian et al. 2006; Morris 2007). It can be synthesized from proline or glutamate via ornithine and citrulline. However, mammalian cells must also import L-Arg since its endogenous synthesis is not sufficient to feed pathways that use it as a precursor molecule. In particular, L-Arg is essential for the efficient activation and function of T cells; depletion of this amino acid in the extracellular microenvironment induces profound T-cell hyporesponsiveness (Rodriguez et al. 2007; Munder 2009).

In the macrophages, L-Arg can be catabolized in two different ways, depending on the expression of two inducible enzymes, NO synthase 2 (NOS2) and ARG, who are competitively controlled by Th1 and Th2 cytokines (Fig. 1). The way L-Arg is used by these enzymes may determine the outcome of parasite infection, since macrophages may either kill the parasites or promote their growth (Munder et al. 1998, 1999; Gordon 2003; Martinez et al. 2009).

Activation of macrophages with Th1 cytokines such as interferon-γ induces tumor necrosis factor α, which triggers the activation of L-Arg metabolic pathways needed for production of NO (Green et al. 1990; Liew et al. 1991).

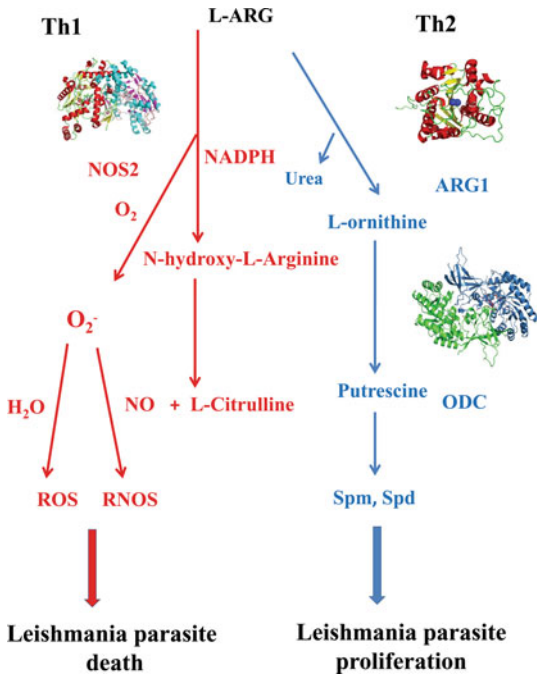

Fig. 1 L-Arg metabolism in the host macrophage. L-Arg can be catabolized in two different ways, since it is a common substrate for NOS2 (*red*, *left*) and ARG (*blue*, *right*), who are competitively controlled by Th1 and Th2 cytokines, thus determining the outcome of parasite infection: macrophages may either kill the parasites or promote their growth

Th1 cytokines induce NOS2 that oxidizes L-Arg to L-citrulline and NO, a metabolite responsible for parasite killing (MacMicking et al. 1997; Bogdan 2001). Moreover, when L-Arg concentration is limiting, electrons are transferred by NOS2 to the cosubstrate O_2, generating O_2^-. This species combines either with NO to generate $ONOO^-$ and other reactive nitric oxide species (RNOS) or with water to produce hydrogen peroxide and reactive oxygen species (ROS) (Fig. 1).

NO production is facilitated by increased L-Arg uptake and by the inhibition of host enzymes that lead L-Arg to L-ornithine and PAs biosynthesis (Gordon 2003). *Leishmania* spp. are dependent on exogenous sources of L-Arg for protein synthesis and for PAs and $T(SH)_2$ biosynthesis (Roberts et al. 2004). Depletion of host arginine/ornithine levels may inhibit amastigote growth and also increase their vulnerability to oxidative stress.

Th2 cytokines determine the induction of host ARG, which converts L-Arg to urea and L-ornithine, yielding in a tissue-specific and stimulus-dependent catabolism urea, proline, glutamate, creatine, agmatine and PAs (Iniesta et al. 2002; Barksdale et al. 2004; Morris 2004). In particular, PAs are essential for parasite growth (Kropf et al. 2005). ARG activity deprives NOS2 of substrate, decreasing NO synthesis, and provides PAs, which can increase parasite proliferation (Gordon 2003) (Fig. 1).

The Th2-type response likely promotes amastigote growth, at least in part, by increasing availability of essential nutrients (Sacks and Anderson 2004). Increase in parasite load in both healing and non-healing strains of mice are associated with induction of ARG-1, while resolution of infection in healing mice is associated with reduced host ARG activity (Iniesta et al. 2005; Kropf et al. 2005). In addition, inhibitors of ARG-1 reduce the parasite load in *Leishmania*-infected BALB/c mice (Iniesta et al. 2001; Kropf et al. 2005), while provision of exogenous L-ornithine reverses the effects of these inhibitors and prevents parasite killing in interferon-γ- and lipopolysaccharide-activated macrophages (Iniesta et al. 2001).

This dual role for L-Arg can be exemplified by immune cells, which typically synthesize NOS or ARG, but not both, depending on their role in host defense and pathology (Modolell et al. 1995; Peranzoni et al. 2007; Bronte and Zanovello 2005).

L-Arg metabolism is therefore a key determinant for infection outcome (Wanasen and Soong 2008). Further, different species of *Leishmania* modulate macrophage signalling pathways and metabolism to different extents (Qi et al. 2004; Wanasen et al. 2007).

Mammals have two ARG isoforms (ARG-1 and ARG-2), which are structurally and kinetically similar, but differ for subcellular localization, tissue distribution and regulation of expression. ARG-1 is cytosolic and functions mainly in the urea cycle; ARG-2 is mitochondrial and acts primarily in proline, glutamate and PAs biosynthesis (Ash 2004; Mori and Gotoh 2004). The human type 1 and type 2 ARGs have 58% sequence identity, are trimeric and contain an active site with two essential Mn(II) ions separated by about 3.3 Å and bridged by oxygens derived from two aspartate residues and a solvent-derived hydroxide (Fig. 2), which is the proposed nucleophile that attacks the guanidinium carbon of substrate L-Arg (Pace et al. 1980; Reczkowski 1992; Kanyo et al. 1996; Scolnick et al. 1997).

Compounds such as *N*-hydroxy-guanidinium or boronic acid derivatives such as *N*(G)-hydroxy-L-arginine, an intermediate product of the inducible NOS pathway, *N*ω-hydroxy-nor-L-arginine, 2(*S*)-amino-6-boronohexanoic acid and *S*-(2-boronoethyl)-L-cysteine, which can bridge the binuclear manganese cluster of ARG, are considered highly effective and specific inhibitors of the enzyme, which is a potential drug target for the treatment of several pathological conditions (Christianson 2005; Morris 2009; Huynh et al. 2009; Jung et al. 2010).

L-Arg has to be imported from the host cytosol into the phagolysosome, and therefrom to *Leishmania* cells. L-Arg shares the same transport systems with other cationic

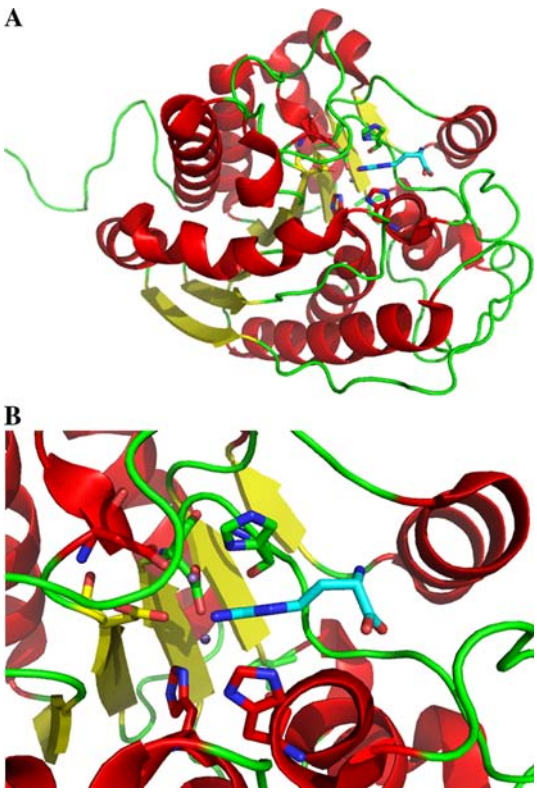

Fig. 2 Human ARG-1 (PDB code 3KV2) in complex with its inhibitor *N*ω-hydroxy-nor-L-arginine. **a** Overall view. **b** Blow up of the catalytic site. The inhibitor and residues important for the catalysis are depicted in *sticks*, whereas the Mn(II) ions are depicted as *spheres*. The picture has been generated in PyMol (DeLano 2008)

amino acids such as lysine and ornithine, i.e. several Na^+-independent transport systems and one Na^+-dependent system (White 1985; Van Winkle et al. 1988; Devés et al. 1992; Devés and Boyd 1998; Closs et al. 2004). Transporters in the $y+$ system include those of the cationic amino acid transporter (CAT) family, which form a subfamily of the solute carrier family 7. To date, six members in the CAT family have been identified, among which four (CAT-1, CAT-2A, CAT-2B and CAT-3) are known to transport cationic amino acids such as L-Arg, L-ornithine, and L-lysine; the function of CAT-4 and CAT-14 is unclear (Closs et al. 2006). The most relevant CAT members involved in macrophage functions are CAT-2A and CAT-2B, which are two splice variants of the same gene.

Leishmania expresses its own L-Arg transporter and the enzymes for L-Arg metabolism.

L-arginine metabolism in Leishmania

L-Arg is an essential amino acid for *Leishmania* growth, since promastigotes can not be maintained in L-Arg-free media (Krassner and Flory 1971; Steiger and Steiger 1977).

L. donovani can transport L-Arg through the LdAAP3 transporter, which has been localized to the surface membrane of promastigotes and contains 480 amino acids, with 11 predicted transmembrane domains. Unlike macrophage CAT-2B, that binds with high affinity several basic amino acids (e.g., L-Arg, L-lysine, and L-ornithine), LdAAP3 specifically binds with high affinity L-Arg (Shaked-Mishan et al. 2006). Moreover, while CAT-2B shows decreased activity at low pH, LdAAP3 is highly active at pH 5.5, the physiological pH of *Leishmania*-containing vacuoles of the infected cells (Closs et al. 1997). LdAAP3 belongs to a closely related family of amino acids permeases that is exclusive to organisms of the Trypanosomatidae family. LdAAP3 belongs to the amino acid-polyamine-organocation (APC) superfamily (Busch and Saier 2003) and has only 19% sequence identity with respect to mammalian arginine transporters such as CAT1-3. LdAAP3 appears in two copies that are adjacent in chromosome 31.

Recently, Darlyuk et al. (2009) established that LdAAP3 expression and activity depends on L-Arg availability and hypothesized that *L. donovani* is able to sense cellular concentrations of the cationic amino acid and to adapt LdAAP3 expression and activity.

Leishmania also expresses other enzymes in the L-Arg metabolic pathway, such as its own ARG which, like host arginases, catalyses the hydrolysis of L-Arg to L-ornithine and urea. It is a trimeric metalloenzyme with a binuclear manganese center, essential for enzyme activity, where the two manganese atoms, Mn(II)A and Mn(II)B, responsible for the activation of a water molecule that is used to attack the L-arginine substrate, form a metal bridge. Its sequence identity with human arginases is 42% with respect to ARG-1 and 38% with respect to ARG-2.

A model of its structure, based on the amino acid sequence deduced from the *arg* gene, indicates His212 and His213 as candidate residues for binding Mn(II) in the coordinated binuclear center in the active site. ARG is expressed in glycosomes from a single-copy gene and is essential for the parasite survival; its K_M and V_{max} values are 23.9 mM and 192.3 mol/min mg protein, respectively (Roberts et al. 2004; da Silva et al. 2008). *Δarg* knockout mutants revealed that ARG is essential for promastigote viability and that the lethality of the null mutation could be conditionally bypassed by either low concentrations of putrescine, high concentrations of ornithine or spermidine, or episomal complementation (Roberts et al. 2004).

As a therapeutic approach for the control of *Leishmania* infection, the physiological inhibitor of ARG, *Nω*-hydroxy-L-arginine, has been used to interfere with ARG activity (Iniesta et al. 2001).

Polyamine biosynthesis

The first and rate-limiting step in the PAs biosynthetic pathway is the conversion of the ARG product, L-ornithine, to putrescine (Fig. 3). This reaction is catalyzed by ODC, a pyridoxal 5′-phosphate (PLP)-dependent enzyme, known to form obligate dimers in eukaryotes.

Studies carried out in *L. donovani* revealed that, as found with ARG, ODC is expressed from a single-copy gene vital for parasite growth. Both *Δodc L. donovani* promastigotes and amastigotes require putrescine or Spd supplementation; *L. donovani* cells require ODC activity and are not capable of scavenging PAs from the host milieu

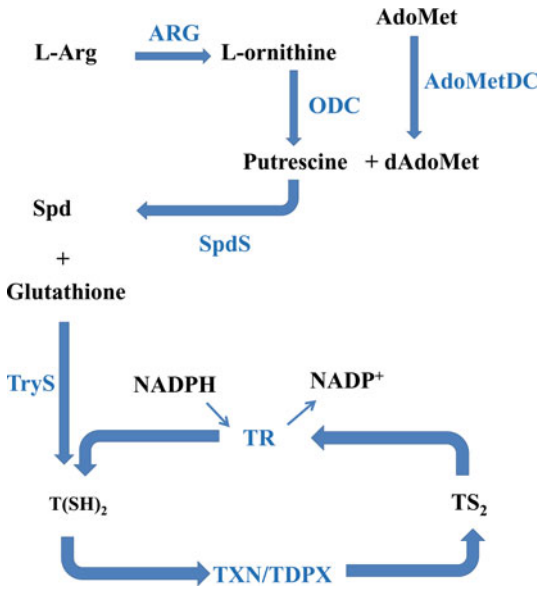

Fig. 3 Polyamine metabolism in Leishmania

in amounts sufficient to sustain an infection (Jiang et al. 1999; Boitz et al. 2009). In addition, ODC from *Leishmania* is much more stable than that of the mammalian host, which is a therapeutic advantage when attempting to achieve a long-lasting inhibition of the enzyme (Hanson et al. 1992; Roberts et al. 2002). These findings indicate that ODC represents a rational therapeutic target for the treatment of leishmaniasis.

The sequences of *Leishmania* spp. ODCs are quite similar, being at least 90% identical for *L. major*, *L. donovani* and *L. infantum*, while *L. brasiliensis* ODC is 70% identical to *L. major* ODC. *Leishmania* ODCs are about 700 residues long and comprise a long N-terminal domain, which contains about 250 amino acids and is absent in mouse, *T. brucei* and human ODCs, and a ~450 residues long C-terminal domain, the Type III PLP-Dependent Enzyme ODC domain, whose sequence is 30–40% identical to the whole ODC in mammals or in *T. brucei*. In mammals, the inducible antizyme binds two PEST regions of mammal ODC (absent in *Leishmania* and *Trypanosoma* ODCs), thus inactivating the enzyme and targeting it for 26 S proteasome-dependent degradation (Kahana 2007).

The X-ray structures of ODCs from mouse, *T. brucei* and human have been solved (Kern et al. 1999; Grishin et al. 1999; Almrud et al. 2000). They show a dimeric enzyme in which each N-terminal region is a TIM-like α/β-barrel and each C-terminal domain forms a β-sheet. The two active sites are found at the dimer interface, between the N-terminal domain of one monomer and the C-terminal domain of the other, with a Lys residue and a Cys residue from two-fold symmetry related monomers contributing to each active site.

The ODC enzyme-activated irreversible inhibitor α-difluoromethylornithine (DFMO) is used for the treatment of African sleeping sickness (Bacchi et al. 1994). DFMO and several other fluorinated ornithine analogs are growth inhibitory and cytotoxic not only to *T. brucei* but also to *L. donovani* (Kaur et al. 1986) and *L. infantum* (Reguera et al. 1995). Another ODC inhibitor, 3-aminooxy-1-aminopropane (APA), an isosteric analog of putrescine, binds to the catalytic site of ODC as shown by the X-ray structure of the complex (Fig. 4) and is considerably more effective than DFMO against *Leishmania* promastigotes and amastigotes multiplying in macrophages (Khomutov 2002; Singh et al. 2007). APA binds to the substrate-binding pocket of ODC, in close proximity to PLP; DFMO binds to the active sites of ODC, forming a Schiff base with the PLP on one monomer and a covalent bond to the Cys residue of the other monomer (Grishin et al. 1999; Dufe et al. 2007).

A high affinity *S*-adenosylmethionine (AdoMet) transporter, AdoMetT1, has been recently identified in

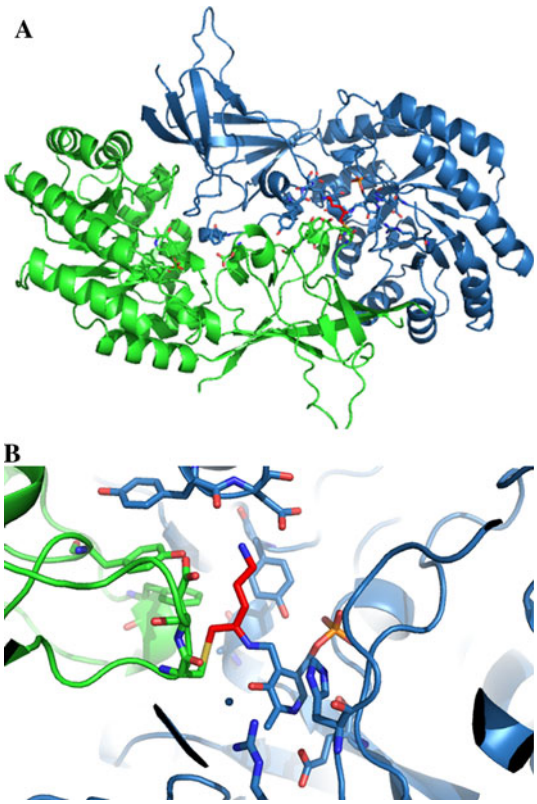

Fig. 4 *Leishmania donovani* ODC in complex with 3-aminooxy-1-amino propane (PDB code 2NO3). **a** Overall structure. **b** Blowup of the active site. The inhibitor, the residues important for the catalysis and the PLP cofactor are depicted as *sticks*. The picture has been generated in PyMol (DeLano 2008)

Leishmania major. AdoMetT1 is a plasma membrane protein belonging to the FBT family, a novel class of membrane proteins, which is part of the major facilitator superfamily (Dridi et al. 2010).

The AdoMetDC catalyzes the irreversible decarboxylation of AdoMet, generating the *S*-adenosyl-5′-(3-methylthiopropylamine) (dAdoMet) substrate for spermidine synthase (SpdS). Decarboxylation reactions in enzymes frequently depend on PLP or thiamine pyrophosphate as a cofactor for catalysis. AdoMetDC belongs to a small class of enzymes that depend on a pyruvoyl cofactor for the decarboxylation process (Pegg et al. 1998). AdoMetDCs exhibit high homology among the trypanosomatid species, with identity values ranging between 62 and 97%, while the identity with respect to the mammalian enzymes is about 30% (Persson 2007).

In mammals and in trypanosomatidae, AdoMetDC is expressed as an inactive proenzyme (~380 amino acids) which contains a highly conserved region around an ESS (Glu-Ser-Ser) sequence and that undergoes autoprocessing to yield the active enzyme: the cleavage of the proenzyme into the two subunits occurs between the glutamic acid and

the serine residues of the ESS, and also transforms the serine into the covalently bound pyruvate. The decarboxylation reaction begins with the formation of a Schiff base between the pyruvoyl cofactor and the α-amino group of the substrate, AdoMet. Upon removal of the α-carboxylate group from the substrate, this leaving group is then replaced by a proton, and the Schiff base is hydrolyzed to yield the decarboxylated product.

Putrescine regulates AdoMetDC activity; the putrescine-binding site is distant from the active site, suggesting a mechanism of allosteric regulation (Beswick et al. 2006). Almost all of the residues that were identified by structural and genetic analyses to be critical for catalysis, ligand binding, and proenzyme processing in human AdoMetDC are conserved in the *L. donovani* enzyme (Roberts et al. 2002). Based on human AdoMetDC crystal structure (Ekstrom et al. 1999), *Leishmania* AdoMetDC is an $(\alpha\beta)_2$ dimer, where α and β represent the products of the proenzyme self-cleavage reaction. The architecture of each $(\alpha\beta)$ monomer is a four-layer α/β-sandwich fold, comprised of two antiparallel eight-stranded β sheets flanked by several α and 3_{10} helices.

The *Δadometdc* strains are incapable of growth in media without PAs; auxotrophy can be rescued by Spd but not by putrescine, Spm, or methylthioadenosine. Incubation of *Δadometdc* parasites in media lacking PAs results in a marked increase of putrescine and glutathione levels and in a decrease in the amounts of Spd and of the Spd-containing thiol $T(SH)_2$ (Roberts et al. 2002). A specific irreversible inhibitor of AdoMetDC is 5′-{[(Z)-4-amino-2-butenyl] methylamino}-5′-deoxyadenosine (MDL 73811; AbeAdo), a structural analog of dAdoMet (Bitonti et al. 1990; Roberts et al. 2007); CGP 40215A, a competitive inhibitor of AdoMetDC, has strong leishmanicidal activity (Mukhopadhyay et al. 1996).

SpdS catalyzes the transfer of an aminopropyl group from dAdoMet to putrescine, thus generating Spd and 5′-deoxy-5'-methylthioadenosine. The *L. donovani* SpdS gene encodes a polypeptide of 300 amino acids that exhibits 56% amino acid identity with the human counterpart. SpdS is an essential enzyme for *L. donovani* amastigotes: Δ*spds* parasites can not convert putrescine to Spd and are auxotrophic for PAs. The PA auxotrophy can be circumvented by exogenous Spd but not by putrescine (1,4-diaminobutane), cadaverine (1,5-diaminopentane), 1,3-diaminopropane, or Spm (Roberts et al. 2002).

SpdS may represent a valid target for chemotherapy; pentamidine decreases ODC activity and inhibits in a non-competitive fashion SpdS (Bachrach et al. 1979, Basselin et al. 1997).

There are no spermine synthase enzymes encoded in the leishmanial genomes, and the parasites lack Spm (Jiang et al. 1999), a major PA in the mammalian host.

Polyamine transport in Leishmania

Leishmania parasites are also able to import PAs from the environment.

According to Basselin and coworkers, at least two PA transporters are expressed in *Leishmania*, one specific for putrescine and one for Spd. The aromatic diamidine pentamidine, one of the drugs of choice for the treatment of antimonial-resistant cases of leishmaniasis, inhibits both putrescine and Spd transport non-competitively. Promastigote and amastigote transporters function optimally at different pH: the amastigote transporters operate optimally at pH 5.5 (close to the pH of the PV in which they reside), while the promastigote transporters operate optimally at pH 7.4 (Basselin et al. 2000).

The PA transporter LmPOT1, with high affinity for putrescine and Spd, has been identified in the membrane fraction of *Leishmania major* (Hasne and Ullman 2005), and highly homologous genes have been found in the genomes of *L. infantum* and *L. brasiliensis* (Ivens et al. 2005; Peacock et al. 2007). LmPOT1 is 803 amino acids long, is a member of APC superfamily, contains an amino acid permease domain and 10 probable transmembrane helices.

Trypanothione synthesis

Mammals use the glutathione (L-γ-Glu-Cys-Gly)/glutathione reductase (GR) system, together with glutathione peroxidase, to regulate the intracellular thiol levels and the redox metabolism. Trypanosomatids lack genes for GR, selenocysteine-containing glutathione peroxidases and thioredoxin reductase (TrxR) as well as catalase. While in most eukaryotic organisms the glutathione/GR and thioredoxin/TrxR systems maintain the intracellular thiol redox homeostasis, trypanosomatids possess a redox metabolism that is based on the low molecular mass dithiol trypanothione ($T(SH)_2$) and TR, which maintains it in the reduced form.

The absence of the $T(SH)_2$ system in mammals, the lack of a functional redundancy within the parasite thiol system together with the sensitivity of Trypanosomatidae against oxidative stress render the components of this metabolism attractive drug target molecules.

In *Leishmania* and *Trypanosoma* species, the bifunctional trypanothione synthetase-amidase (TryS) catalyzes biosynthesis and hydrolysis of the glutathione-Spd adduct $T(SH)_2$. The structure of *Leishmania major* TryS, determined in three crystal forms, reveals two catalytic domains (Fyfe et al. 2008). The N-terminal domain, a cysteine, histidine-dependent amidohydrolase/peptidase amidase, is a papain-like cysteine protease, and the C-terminal synthetase domain displays an ATP-grasp family fold common

to C:N ligases. The C-terminal domain catalyzes $T(SH)_2$ biosynthesis by the stepwise addition of two molecules of glutathione onto Spd with hydrolysis of two ATP molecules. The biosynthesis of $T(SH)_2$ starts with the formation of a glutathionylspermidine intermediate, which then reacts with a second glutathione molecule to form $T(SH)_2$. The N-terminal domain of TryS is capable of hydrolyzing $T(SH)_2$ and/or glutathionylspermidine back to Spd and glutathione and is classified as a cysteine-histidine-dependent amidohydrolase/peptidase amidase. TryS is important in *T. brucei* as shown by RNA interference experiments, which indicate a loss of proliferation and viability with increased sensitivity to oxidative stress. TryS is unlikely to have resistance or toxicity issues because it is a single copy gene in *Trypanosoma* and *Leishmania* species, there is no alternative bypass mechanism, and there is no equivalent enzyme in humans.

Such data are consistent with studies on *Crithidia fasciculata*, where glutathione-like tripeptides, inhibitors of Glutathionyl-spermidine synthetase (GspS) (De Craecker et al. 1997; Amssoms et al. 2002a, b) displayed potent trypanocidal effects. In particular the phosphinate analogue of glutathionylspermidine, previously shown to be a potent inhibitor of GspS from *Escherichia coli*, also inhibits with high affinity recombinant TryS from *C. fasciculata*, *L. major*, *T. cruzi* and *T. brucei*, and is the most potent TryS inhibitor identified to date (Oza et al. 2008). A recent work by Fairlamb and coworkers shows that potent TryS inhibitor compounds have trypanosomicidal effects and decrease intracellular trypanothione levels down to 10% of wild type (Torrie et al. 2009). All these observations strongly suggest that the enzyme is an important potential drug target.

Trypanothione metabolism

$T(SH)_2$ is a low-molecular-mass dithiol consisting of two glutathione molecules covalently linked to Spd. It was identified 25 years ago in trypanosomatids, where it replaces glutathione in the majority of thiol–disulfide exchange reactions of the cell (Fairlamb et al. 1985). $T(SH)_2$ is more reactive than glutathione, a property explained by the p*K*a of its cysteines, coincident with the intracellular pH, and its dithiol nature, which favors the formation of an intramolecular disulfide bridge after one or two electron oxidations and prevents the formation of sulfinyl radicals (RSO·), which are able to propagate oxidation to other molecules. In *Leishmania* species, $T(SH)_2$ levels are maintained between 1.0 and 2.0 mM (Krauth-Siegel and Comini 2008). The concentration of $T(SH)_2$ does not change significantly during the different logarithmic growth phases but decreases when the cells enter the stationary phase. $T(SH)_2$ is necessary for the parasite survival since it is used by several enzymes to neutralize the ROS produced by the macrophages during the infection.

The $T(SH)_2$ molecule is kept reduced by TR (Fig. 5). The $T(SH)_2$/TR system substitutes the glutathione/GR and thioredoxin/TrxR systems present in other organisms and, for this reason, it is necessary for the protozoa survival. TRs are members of the large and well-characterized family of FAD-dependent NAD(P)H oxidoreductases. These are dimeric flavoenzymes that catalyze the transfer of electrons between pyridine nucleotides and disulfide/dithiol compounds and promote catalysis via FAD and a redox active disulfide, such as GR, lipoamide dehydrogenase, and mercuric ion reductase.

The only *Leishmania* solved TR structure is that of *L. infantum* (Baiocco et al. 2009). It closely resembles that of the other TRs solved so far (TR from *C. fasciculata* and from *T. cruzi*). Each monomer is formed by three different domains: the FAD binding domain (residues 1–160 and 289–360, *L. infantum* TR numbering), the NADPH binding domain (residues 161–289) and the interface domain (residues 361–488).

TRs resemble GRs in structure and mechanism of action. Substrate specificity is largely determined by five amino acids at the substrate binding site, making the active site pocket of TR wider, more hydrophobic and negatively charged than that of GR.

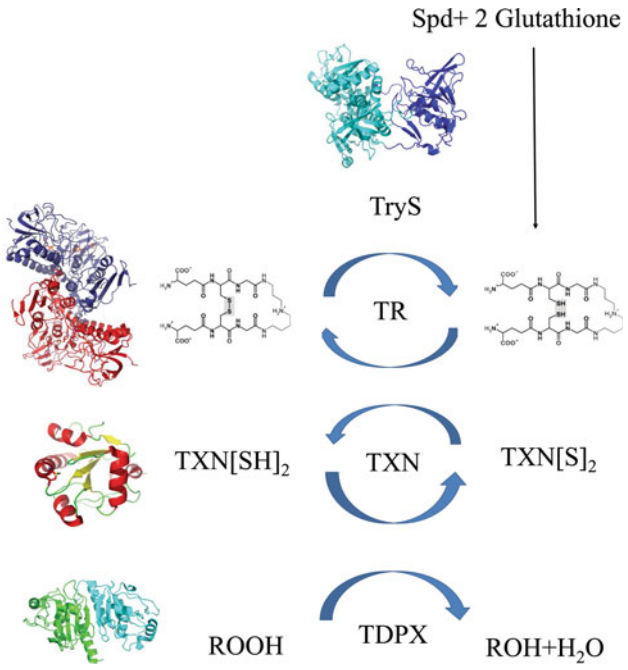

Fig. 5 Trypanothione biosynthesis and metabolism. X-ray structures of trypanothione synthetase, trypanothione reductase, tryparedoxin and tryparedoxin peroxidase are shown

The reaction begins with the binding of NADPH, which transiently reduces the flavin. Reduction of the cystine disulfide in the active site by the reduced flavin follows, by formation of a short-lived covalent intermediate with Cys57, and the subsequent formation of a stable charge-transfer complex between the flavin and the Cys57 thiolate. After formation of the charge-transfer complex, $NADP^+$ dissociates and is replaced by another NADPH. One of the protein cysteines, Cys52, which is activated similarly to serine and cysteine proteases by the His461′-Glu466′ pair (belonging to the second subunit of the dimer), can then react with TS_2 to produce a mixed disulfide followed by attack of the second protein cysteine (Cys57) on Cys52 (Fig. 6).

Fairlamb and coworkers showed that the antimonials, the main used drugs against Leishmaniasis, interfere with the trypanothione metabolism (Cunningham and Fairlamb 1995). They also demonstrated that the antimonials bind to TR inhibiting its enzymatic activity. Baiocco and coworkers solved the structure of the reduced *Leishmania* TR in complex with Sb(III) and explained the molecular basis of the antimonial activity in *Leishmania* parasites. This trivalent ion binds to the catalytic pocket of the enzyme engaging the residues involved in the catalytic mechanism in the formation of a complex. Sb(III) binds to Cys52, to Cys57, to His461' of the two-fold symmetry related subunit and to Thr335 (Baiocco et al. 2009) (Fig. 6).

Also arsenical drugs, (terpyridine)platinum(II) complexes, nitrosoureas and carmustin are irreversible inhibitors of TR activity targeting TR cysteines. PAs analogues

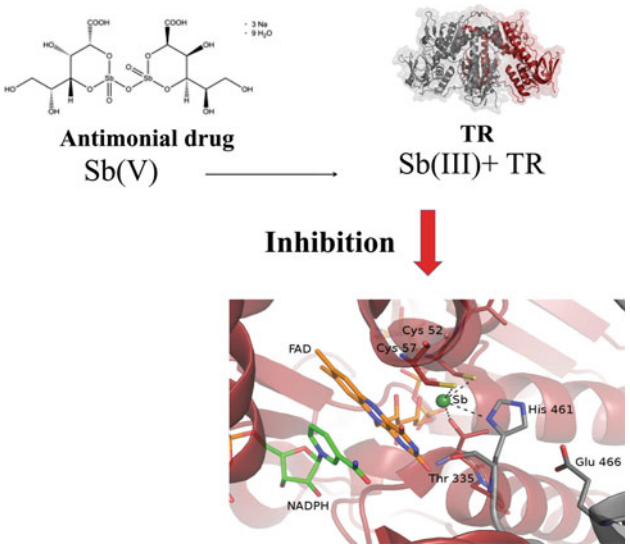

Fig. 6 Molecular basis of antimony treatment in leishmaniasis. Sb(V)-based antimonials are prodrugs: Sb(III) binds to the active site of *Leishmania* TR and inhibits the enzyme. Sb(III) is depicted as a *sphere*. The residues which bind Sb(III) are depicted as *sticks*

and tricyclic compounds, like chlorpromazine and mepacrine, are interesting examples of competitive inhibitors of TR (Liñares et al. 2006).

Reverse genetic approaches in *L. donovani* have shown that TR is essential for parasite viability thus validating it as a good drug target. In the case of another member of the Trypanosomatidae family, *T. brucei*, conditional gene deletion studies have revealed that parasites can survive with only 10% of their normal TR activity (Krieger et al. 2000), indicating that low levels of TR are sufficient to maintain $T(SH)_2$ in its reduced state under optimal growth conditions (Tovar et al. 1998). However, TR-depleted parasites grown in a thiol-free medium could not efficiently detoxify hydrogen peroxide (H_2O_2) and showed a reduced capacity to infect mice when compared to wild type parasites.

Role of trypanothione in the redox metabolism of Leishmania

Although $T(SH)_2$ biosynthesis is likely to take place in the cytosol of the parasite cell (Oza et al. 2005), the presence of $T(SH)_2$ in other cellular compartments has been suggested based on the occurrence of enzymes that use it as electron donor.

$T(SH)_2$ is used as electron donor in several metabolic pathways where it delivers reducing equivalents to intermediary molecules, such as oxidized glutathione, dehydroascorbate and the dithiol protein tryparedoxin (TXN) which, in turn, reduces different enzymes.

Leishmania parasites lack both catalases and classical seleno-containing glutathione peroxidases, the two major enzymes that in eukaryotes eliminate hydrogen peroxide. Instead, the hydroperoxide metabolism depends indirectly on $T(SH)_2$. The enzymatic system linking $T(SH)_2$ to the hydroperoxide reduction consists of two proteins, TXN and tryparedoxin-dependent peroxidase (TDPX), which are analogues of thioredoxin and thioredoxin peroxidase, respectively (Holmgren 1995; Saitoh et al. 1998; Zhang et al. 1997). This unique $T(SH)_2$-dependent system is common to all *Leishmania* species and is important for the parasite survival since it neutralizes the effects of the ROS.

TXN represents a distinct molecular clade within the thioredoxin superfamily of oxidoreductases; TXNs are characterized by a WCPPC motif at their catalytic center replacing the WCG/APG motifs found in thioredoxins. Two genes coding for TXNs have been identified in *L. infantum*, *LiTXN1* and *LiTXN2*, which share the same genetic *locus*. These genes are both single copy and code for two active TXN enzymes, *Li*TXN1 and *Li*TXN2, with different biochemical and biological features. *Li*TXN1 is located in the cytosol and is upregulated in the infectious forms of the parasite, strongly suggesting that it might play

an important role during infection. *Li*TXN2 is the first mitochondrial tryparedoxin described in Kinetoplastida (Castro et al. 2004). *Li*TXN1 is essential to the mammalian stage of *L. infantum*, and therefore can be regarded as a potential candidate for drug targeting (Romao et al. 2009), although specific TXN inactivation could be challenging, since there are many similarities between TXNs and thioredoxins, and the active site of TXNs is located on the surface of the protein, thus preventing tight binding of inhibitors to the enzyme (Comini et al. 2007).

The structures of *Leishmania* spp. TXNs have not been solved yet, but the structures of TXNs of different members of the Trypanosomatidae family, homologous to the *Leishmania* TXNs, have been well analyzed. The three-dimensional structures of *C. fasciculata* TXN-I and of *T. brucei* TXN-I have been solved as well as the three-dimensional structure of *C. fasciculata* TXN-II. TXNs fold as thioredoxins in a twisted five stranded central β-sheet with two helices on either side. In addition, the TXNs contain a β-hairpin at the protein N-terminus. The active site WCPPC motif is located between strand β3 and the N terminus of helix α1 (Alphey et al. 1999, 2003). The reaction of *T. cruzi* TXN-I with $T(SH)_2$ has a K_M around 40 μM and an apparent second-order rate constant of 8×10^3 M^{-1} s^{-1} (Wilkinson et al. 2002).

TXN is used as source of reducing electrons during removal of peroxides by TDPXs (Fig. 5). TDPXs catalyze the reduction of H_2O_2 and organic hydroperoxides to water or alcohols, thereby detoxifying the *Leishmania* parasites. The TDPXs include members of the peroxiredoxin and of the non-selenium glutathione peroxidase families.

The peroxiredoxins represent an ubiquitous family of redox enzymes which reduce hydroperoxide and peroxynitrite. These enzymes, which lack prosthetic groups, use active cysteines to reduce their substrates. TDPXs of Trypanosomatidae are typical 2-Cys peroxidoxins and are localized both in the cytosol and in the mitochondria. *Leishmania* 2-Cys peroxidoxins act preferentially on hydrogen peroxide and peroxynitrite, whereas are rapidly inactivated by complex lipid hydroperoxide (Trujillo et al. 2008).

The *Leishmania* spp. possess more than one TDPXs (8 in *L. major*, 3 in *L. infantum* and *L. brasiliensis*). X-ray structures of *Leishmania* TDPXs have not been solved so far, but these proteins display an high degree of homology with the 2 Cys-peroxidases of the other members of the Trypanosomatidae family. In particular the TDPXs from *L. infantum* display identities up to 69% with the TDPX from *T. cruzi* and to 72% with the *C. fasciculata* TDPX, whose crystal structures have been solved (Alphey et al. 1999; Piñeyro et al. 2005).

Also, the structure of TDPX proteins closely resemble those of the members of the thioredoxin superfamily. The 2-Cys peroxidoxins are homodimers of about 22 KDa containing two identical catalytic centers. Basically each TDPX monomer is formed by a seven stranded β-sheet with parallel and anti-parallel alignments surrounded by four α-helices of varying length (Alphey et al. 2000).

According to the generally accepted mechanism for these enzymes, they participate in two distinct reactions. Oxidized TDPX first binds a redox partner which reduces the intersubunit disulfide bridge (Cys52–Cys173′ in *C. fasciculata*); then the reduced enzyme has to react with and process hydroperoxides. In the first reaction, the dimeric enzyme reacts with TXN, which carries two cysteine residues at the active site, which cycle between sulfhydryl groups and a disulfide (Alphey et al. 1999). The first cysteine residue of TXN is reduced by $T(SH)_2$ and subsequently reduces the TDPX disulfide bond, releasing reduced Cys52 in *C. fasciculata* TDPX (Cys39 in *T. cruzi* TDPX), and forming a new disulfide with Cys173′ in *C. fasciculata* TDPX (Cys87′ in *T. cruzi* TDPX). This inter-protein disulfide bond subsequently undergoes nucleophilic attack by the second Cys of TXN to leave TDPX Cys173′ (Cys87′ in *T. cruzi* TDPX) as a thiol or thiolate. TXN is returned to the oxidized form to be recharged by $T(SH)_2$. In the second reaction, the Cys of one enzyme subunit reduces the peroxide substrate, being oxidized to a cysteine sulfenic acid. This residue subsequently undergoes a nucleophilic attack by the second cysteine of the two-fold symmetry related subunit to form a stable disulfide bond.

The two cysteines in the active site of TDPX are placed at long distance from each other. This means that TDPXs are predicted to undergo significant conformational changes between their respective reduced and oxidized structures (Piñeyro et al. 2005), as observed for Glutathione peroxidase from *Populus trichocarpaxdeltoides* (Alphey et al. 2008). Alphey and coworkers have built a model of oxidized *Tc*TDPX, suggesting that α2 must completely unravel, causing loop β4–α2 to bulge outwards and allowing Cys87′ of one subunit to form a disulphide bond with Cys39 of the other subunit. It can be hypothesized that the trigger for this conformational change must reside in conversion of the Cys39 thiolate group into a sulphenate group. The TDPXs could be used as drug targets since these enzymes have a key role in the detoxification pathway of the Trypanosomatidae. The reduced form of TDPX2 displays a defined pocket near the Cys87' that could be exploited for drug design (Alphey et al. 2008).

In Trypanosomatidae, the detoxifying action from hydroperoxides is exerted also by another class of enzymes, the "non selenium glutathione peroxidase-like enzymes" (nsGPXs). In this class of enzymes, the catalytic C-terminal selenocysteine is replaced by a simple cysteine. In *Leishmania* two separate genomic loci contain two

genes which code for two different nsGPX enzymes, classified as A and B (Castro and Thomas 2008). Both nsGPX-As and nsGPX-Bs have a weak affinity for glutathione, since all the residues involved in glutathione binding are either mutated or deleted.

The two nsGPX families share only 30% identity and display a different catalytic behavior. The nsGPX-As use TXN instead of glutathione as main reducing agent in the cell and are likely to accommodate in the catalytic pocket complex hydroperoxides. nsGPX-Bs are also able to oxidize fatty acids or phospholipids but do not accept TXN as reductant.

An additional class of enzymes, the ascorbate peroxidases (APX), is able to reduce hydrogen peroxide in *Leishmania* parasites. These 30 kDa heme-containing enzymes utilize ascorbate, which is synthesized directly by the parasites as reducing agent to reduce hydrogen peroxide to water. The catalytic mechanism of trypanosomatidal APXs likely conforms to that of plant APXs. The two monodehydroascorbates radicals resulting from the reaction are directly reduced by $T(SH)_2$ (Adak and Datta 2005).

In Trypanosomatids, a trypanothione *S*-transferase activity catalyzes the nucleophilic attack of $T(SH)_2$ on a wide variety of hydrophobic substrates. In *L. major* this activity is associated to the eukaryotic translation elongation factor 1B (eEF1B). The *L. major* eEF1B displays $T(SH)_2$-dependent peroxidase activity, reacting preferentially with linoleic acid hydroperoxide, but not with H_2O_2 (Vickers et al. 2004a). This, added to the fact that eEF1B localizes to the endoplasmic reticulum, has led to the suggestion that this molecule may be involved in the elimination of oxidized lipids within this organelle.

$T(SH)_2$ also plays a role in DNA synthesis in the members of Trypanosomatidae family. $T(SH)_2$ acts as electron donor in the reaction catalyzed by the class I ribonucleotide reductase (RR) to reduce the 2′-OH group of ribonucleotide diphosphates to the corresponding deoxynucleotides. In fact, it has been shown that, in contrast to glutathione, $T(SH)_2$ is a direct reductant of *T. brucei* RR with a K_M value of 2 mM, being the first example of a natural low molecular mass thiol directly delivering reducing equivalents for ribonucleotide reduction (Dormeyer et al. 2001). Since in the presence of TXN the electron flux is more efficient, probably in vivo the dithiol protein is the reductant of RR. *Leishmania* spp. possess RRs which are highly similar to that of *T. brucei*.

Lastly, $T(SH)_2$ can react with a variety of electrophiles and as such has been implicated in detoxification of ketoaldehydes, heavy metals, and xenobiotics. The metalloenzymes glyoxalase I (lactoylglutathione lyase) and glyoxalase II (hydroxyacylglutathione lyase) catalyse the step-wise dismutation of 2-oxoaldehydes into the corresponding 2-hydroxyacids, using glutathione as a cofactor (Greig et al. 2006). The principal role of the glyoxalase system is thought to be the detoxification of methylglyoxal (2-oxopropanal), a highly toxic α-oxoaldehyde produced as a by-product of glycolysis (Vickers et al. 2004b, Padmanabhan et al. 2005). The inherent toxicity of this molecule stems from its propensity to react with the nucleophilic centres of DNA, RNA and proteins. In particular, the oxoaldehyde reacts with the side chains of arginine, lysine and cysteine, and with the base guanine. In the majority of the organisms glutathione is used as cofactor but in *Leishmania* species $T(SH)_2$ carries out this role. $T(SH)_2$ reacts directly with the ketoaldehyde and at the end of the catalytic cycle is recovered in reduced form (Ariza et al. 2006; Greig et al. 2009).

$T(SH)_2$ has also been implicated in the detoxification of heavy metals and drugs. Most evidence comes from the analysis of *Leishmania* species resistant to arsenite- or antimony-containing drugs. In vitro selected drug-resistant parasites have higher levels of $T(SH)_2$ than susceptible ones. Additionally, some resistant parasites display an associated amplification of the PGPA (p-glycoprotein-like protein A) gene. This gene codes for an intracellular ATP-binding cassette (ABC) transporter of the multidrug resistance proteins subfamily, known to facilitate the efflux of metal-thiol conjugates (Légaré et al. 1997, 2001; Haimeur et al. 2000).

Recently, these two features were also described in field isolates of *L. donovani* naturally resistant to antimony. Moreover, it has been shown that $T(SH)_2$ can form adducts with arsenite in vitro and in vivo. Thus, resistance could be associated with the formation of $T(SH)_2$-drug conjugates, followed by their extrusion or sequestration. In this scenario, the existence of a $T(SH)_2$-*S*-transferase activity had been proposed as another feature that could contribute to parasite resistance, even if a recent study has shown that at least in *L. tarentolae*, $T(SH)_2$-*S*-transferase activity is not increased in antimony-resistant parasites.

Polyamine transport and redox function in mitochondria

Kinetoplastids have a single mitochondrion with a kinetoplast, a disk-shaped mass of circular DNA containing many copies of the mitochondrial DNA, associated with the flagellar bases.

Like mitochondria from higher eukaryotes, the mitochondrion of *Leishmania* spp. is thought to be impermeable to several metabolites, implying the presence of specific membrane-bound transporters. Such transporters are required for the maintenance of the cellular redox balance, and most of the known or postulated *Leishmania* mitochondrial pathways require the transport of metabolites between the mitochondrion and other cellular compartments, i.e. the glycosome and the cytosol. Colasante and

coworkers identified 24 different genes codifying for the Mitochondrial Carrier Family (MCF) proteins in *T. brucei* (Colasante et al. 2009). In particular, a gene orthologous to yeast or human ornithine MCF (Tb.11.03.0870) and a gene orthologous to human or yeast AdoMet MCF (Tb10.61.2510) have been identified in *T. brucei*. Similar genes (LmjF25.0210 and LmjF32.0110, respectively) were identified also in *Leishmania major*, indicating that PAs may be transported inside *Leishmania* mitochondrion.

PAs strongly affect the mitochondrial metabolism. PA analogues inhibit leishmanial proliferation and cause mitochondrial potential changes and modifications compatible with apoptosis (Tavares et al. 2005); pentamidine accumulates in the *Leishmania* mitochondrion and the development of the resistance phenotype in *Leishmania* involves drug extrusion from the mitochondrion (Basselin et al. 2002). In addition, the putrescine analogue 1,4-diamino-2-butanone impairs *L. amazonensis* promastigote proliferation dose-dependently and reduces parasite putrescine concentration by nearly 50%. This analogue markedly inhibits both ODC activity and putrescine uptake by promastigotes and causes mitochondrial damage. Putrescine may be involved in leishmanial survival, possibly by maintaining the parasite's mitochondrial function, since PAs may protect mitochondrial DNA from oxidative species (Khan et al. 1992; Vannier-Santos et al. 2008).

Mitochondria are well-known sites for the intracellular production of ROS (H_2O_2 and alkylhydroperoxide). Parasite survival depends on the presence of an efficient mitochondrial detoxification pathway from ROS. In the mitochondrion of the *Leishmania* spp., hydroperoxide reduction is supported by enzymes belonging to the families of the nsGPX and of the peroxiredoxins. Biochemical assays show that both classes of enzymes use electrons provided by TXNs, to reduce their hydroperoxide substrates (Castro and Tomàs 2008). In *L. infantum* one of the two TXNs is mitochondrial and reduces mitochondrial TDPXs. The mechanism of reduction of the mitochondrial TXN is still unclear: since TR and TryS are not detectable in this organelle, it remains to be elucidated if the parasite possesses a redox shuttle mechanism [$T(SH)_2/TS_2$ transporter] between cytosol and mitochondria (Krauth-Siegel and Comini 2008). Castro and coworkers have shown that the system $TR/T(SH)_2$ is not active inside the mitochondrion and that other redox systems may reduce TXN inside the organelle (Castro et al. 2008).

In *Leishmania*, mitochondrial TXN reduces the UMSBP (universal minicircle sequence-binding protein) involved in the initial step of replication of the kinetoplast, the trypanosomatid mitochondrial DNA and the monothiol glutaredoxin (1-C-Grx1) which is probably involved in iron metabolism and/or in the biogenesis of iron sulfur clusters (Motika et al. 2006; Comini et al. 2008). Apart from the hydroperoxide-detoxifying enzymes, two superoxide dismutase (SOD) isoforms, SODA and SODC, are localized in Trypanosomatidae mitochondrion, and are able to metabolize the ion superoxide which is mainly produced in this organelle (Dufernez et al. 2006).

Conclusions

In this review we describe the metabolism of PAs in *Leishmania* parasites, that displays many features which are absent in the host cells. Therefore, the unique pathway of PA metabolism furnishes excellent targets for rational drug design.

First-line treatments against leishmaniasis rely mainly on the pentavalent antimonials meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), compounds discovered more than 50 years ago, which present severe, undesirable side effects. For this reason, they have to be administered in low doses, and hence, drug resistance has appeared so rapidly that in some regions of India, for example, the compounds can no longer be used. The second-line drugs are pentamidine and amphotericine B, which suffer from similar toxicity concerns (Maltezou 2008; Kedzierski et al. 2009). To overcome nephrotoxicity of this latter antifungal compound, different colloidal and lipid formulations have been successfully prepared, and in particular amphotericine B liposomal formulation has revealed a milestone in leishmaniasis treatment, due to its safety and the high administrable dosage. However, despite the drastic price reduction in May 2007, the cost of US $300 for an average course is still prohibitive for visceral leishmaniasis-endemic countries. Recently, miltefosine, an alkylphosphocholine derivative developed as an anticancer drug, has been registered in India for oral treatment of visceral leishmaniasis. However, it is contraindicated in women of childbearing age and shows severe gastrointestinal side effects. Liposomal amphotericin B in combination with oral miltefosine has displayed 90% cure rates for the visceral disease form. All these drugs have many limitation including high toxicity and the emergence of drug resistance. In this scenario, there is an urgent need to develop new, less toxic and more affordable drugs (Maltezou 2010).

In this paper we show how the enzymes involved in the PA metabolism are necessary for the parasites survival and/or virulence and therefore may represent targets for developing new drugs. In this perspective, we have described how the infection is directly related to a complex interplay between host and parasite involving L-Arg, which is the precursor of the PAs and of the PA derivatives. In fact, the fate of intracellular *Leishmania*

parasites is determined by the activation status of macrophages. Fully activated macrophages can produce leishmanicidal molecules, such as NO and oxidative mediators, and kill parasites effectively, whereas alternatively activated cells preferentially induce the ARG pathway to produce PAs and enhance parasite replication/persistence. Although two distinct types of macrophages activation can lead to divergent outcomes of infection, these two pathways use L-Arg as a common substrate for their enzymatic activities. On the other hand, L-Arg is essential for *Leishmania* growth, since it is the precursor of the PAs, and promastigotes can not be maintained in L-Arg-free media.

The PAs biosynthetic pathway starts with the synthesis of the PA precursor ornithine, catalyzed by ARG. Ornithine is then decarboxylated by ODC, which produces the putrescine molecule. Putrescine is used as the substrate for the SpdS, that adds the aminopropyl group donated from the dAdoMet, which is provided by AdoMetDC. All these enzymes are in principle good drug targets, since their inhibition may cause the death of *Leishmania* parasites.

In *Leishmania*, the PA spermidine is used to synthesize $T(SH)_2$, which is kept reduced by the TR: the $T(SH)_2$/TR system replaces the glutathione/GR system used in mammals. $T(SH)_2$ is utilized by a number of enzyme involved in a number of metabolic pathways. In particular, it is used by the TPX\TDPX system, that replaces the catalase and the classical Glutathione peroxidases (GPX) in the host, to reduce hydrogen hydroperoxides, thereby neutralizing the effects of ROS produced by macrophages during the infection.

The inhibition of the enzymes involved in the $T(SH)_2$ metabolism reduces the vitality of the parasites. The enzymes of the $T(SH)_2$ pathway are also target for the most used drugs. In this respect, the crystal structures of oxidized TR from *L. infantum* and of the complex of reduced TR with NADPH and Sb(III) (Baiocco et al. 2009) disclose for the first time the TR-mediated mechanism of action of antimonial drugs against the parasite: Sb(III) binds to the active site of the enzyme, thereby inhibiting the reduction of $T(SH)_2$. The $T(SH)_2$-mediated hydroperoxide metabolism represents the Achilles' heel of *Leishmania* and the other members of Trypanosomatidae family. The enzymes involved in this metabolism are not present in the mammalian host and therefore are targets for developing new less toxic and more affordable drugs.

In conclusion, the metabolism of PAs is necessary for parasite survival. The structural and functional studies are helpful for the identification of the differences between the parasite enzymes and the human enzymes with similar function. The authors strongly believe that these studies will bring to the identification of inhibitors that can be used as lead compound for rational drug design.

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