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Targeting the polyamine biosynthetic enzymes: a promising approach to therapy of African sleeping sickness, Chagas' disease, and leishmaniasis

Review Article

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Summary. Trypanosomatids depend on spermidine for growth and survival. Consequently, enzymes involved in spermidine synthesis and utilization, i.e. arginase, ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, trypanothione synthetase (TryS), and trypanothione reductase (TryR), are promising targets for drug development. The ODC inhibitor α-difluoromethylornithine (DFMO) is about to become a first-line drug against human late-stage gambiense sleeping sickness. Another ODC inhibitor, 3-aminooxy-1-aminopropane (APA), is considerably more effective than DFMO against *Leishmania* promastigotes and amastigotes multiplying in macrophages. AdoMetDC inhibitors can cure animals infected with isolates from patients with rhodesiense sleeping sickness and leishmaniasis, but have not been tested on humans. The antiparasitic effects of inhibitors of polyamine and trypanothione formation, reviewed here, emphasize the relevance of these enzymes as drug targets. By taking advantage of the differences in enzyme structure between parasite and host, it should be possible to design new drugs that can selectively kill the parasites.

Keywords: African sleeping sickness – Chagas' disease – Leishmaniasis – Polyamines

Abbreviations: AbeAdo, 5′-{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine, (MDL 73811); AdoDATO, *S*-adenosyl-1,8-diamino-3-thiooctane; AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; APA, 3-aminooxy-1-aminopropane; CGP 40215A, (bis{[3-(aminoiminomethyl)phenyl]methylene}carbonimidic dihydrazide trihydrochloride); CNS, central nervous system; dcAdoMet, decarboxylated *S*-adenosylmethionine; DFMO, DL-α-difluoromethylornithine; LmPOT1, *Leishmania major* polyamine transporter 1; MGBG, methylglyoxal-bis(guanylhydrazone); MTA, 5′-deoxy-5′-methylthioadenosine; ODC, ornithine decarboxylase; SpdS, spermidine synthase; SpmS, Spermine synthase; TryR, trypanothione reductase; TryS, trypanothione synthetase

Introduction

Protozoan pathogens cause disease and death in millions of humans, primarily in developing countries in tropical and subtropical regions. In this brief review, we will focus on the pathogens causing African sleeping sickness, Chagas' disease, and leishmaniasis. Attempts to develop vaccines for parasitic diseases have been futile, and current therapies rely on a very small number of drugs, most of which are inadequate because of severe host toxicity. In addition, the emergence of drug resistance has become a serious problem.

A whole new approach to treatment of parasitic diseases was taken by Bacchi et al. (1980). By employing inhibitors of the enzymes in the polyamine biosynthetic pathway (Fig. 1) they made remarkable progress. Initially, they found that treatment with DL-α-difluoromethylornithine (DFMO), a specific, irreversible inhibitor of ornithine decarboxylase (ODC), cures mice infected with a virulent rodent-passaged strain of *Trypanosoma brucei brucei* (Bacchi et al., 1980). This parasite is closely related to the trypanosomes that cause African sleeping sickness in humans. DFMO treatment was subsequently evaluated in the Southern Sudan in an open clinical field trial in patients with gambiense sleeping sickness, i.e. infected with *T. b. gambiense* (Van Nieuwenhove et al., 1985). The patients were in late-stage disease with central nervous

system (CNS) involvement, and were refractory to arsenical treatment. Monotherapy with oral DFMO resulted in disappearance of parasites from cerebrospinal fluid and reversal of clinical symptoms, and was curative in several patients (Van Nieuwenhove et al., 1985). The International Scientific Council for Trypanosomiasis Research

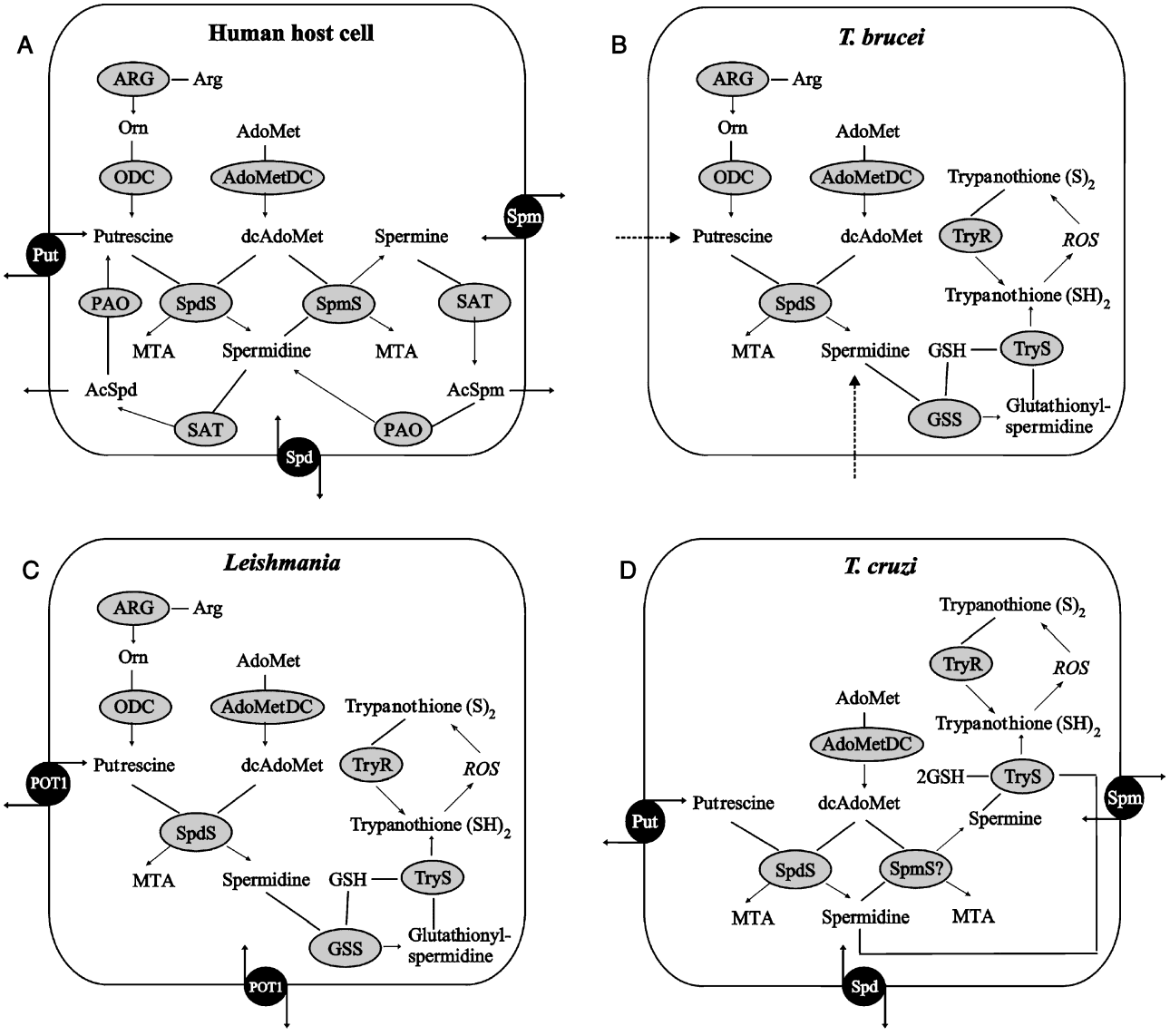

Fig. 1. Transport, biosynthesis, interconversion and utilization of polyamines in parasitic protozoa and human host cells. Arginase is essential for production of the polyamine precursor ornithine in most types of cells, but not in *T. cruzi*. **A** In human cells, the decarboxylases (ODC and AdoMetDC) are highly regulated, and have very short half-lives, whereas the synthases (SpdS and SpmS) are constitutively expressed. SAT and PAO provide a pathway for back-conversion from spermine to spermidine to putrescine via acetylated intermediates (AcSpm and AcSpd). Polyamine transporters (black circles) can regulate the intracellular polyamine levels. **B** In *T. brucei*, ODC and AdoMetDC have long half-lives, and SpmS is lacking. These organisms conjugate spermidine and glutathione (GSH), using two enzymes (GSS and TryS) to form trypanothione, which is involved in defence against chemical and oxidant stress. **C** In *L. donovani* the properties of the enzymes that catalyze the synthesis of polyamines and trypanothione are similar to those of *T. brucei*, but *Leishmania* parasites have a more efficient polyamine transporter (POT1). **D** *T. cruzi* parasites have no ODC, but AdoMetDC and two aminopropyltransferases are present. One may be a SpmS because these parasites have been shown to contain spermine, which is conjugated with GSH by TryS. TryS is a single enzyme with wide polyamine substrate specificity, catalyzing the formation of trypanothione, and various trypanothione analogs. *T. cruzi* parasites depend on efficient putrescine uptake and have a high-affinity transporter. *AcSpd* acetylated spermidine; *AcSpm* acetylated spermine, *AdoMet* S-adenosylmethionine; *AdoMetDC* S-adenosylmethionine decarboxylase; *ARG* arginase; *dcAdoMet* decarboxylated S-adenosylmethionine; *GSH* glutathione; *GSS* glutathionylspermidine synthase; *MTA* 5′-deoxy-5′-methylthioadenosine; *ODC* ornithine decarboxylase; *PAO* polyamine oxidase; *Put* putrescine; *ROS* reactive oxygen species; *SAT* spermidine/spermine N^1-acetyltransferase; *Spd* spermidine; *Spm* spermine; *TryR* trypanothione reductase; *TryS* trypanothione synthetase

and Control is presently considering to recommend DFMO (eflornithine) for use as first-line drug to treat the late stages of gambiense sleeping sickness.

Follow-up studies, in which the polyamine biosynthetic enzymes were either specifically inhibited by substrate or product analogs or eliminated by double targeted gene replacement, have clearly demonstrated that other protozoan parasites also depend on polyamines for growth and survival (Müller et al., 2001; Heby et al., 2003).

Since polyamine biosynthetic enzymes exhibit structural features that differ significantly between the parasite and the human host, and also because some enzymes that use polyamines as substrates are unique to the parasites, these enzymes are promising targets in drug design against tropical parasitic diseases.

Specific irreversible inhibitors of the polyamine biosynthetic enzymes that have been successfully used against protozoan pathogens

ODC, which catalyzes the decarboxylation of ornithine to putrescine (Fig. 1), can be irreversibly inactivated by substrate and product analogs (Seiler, 2003).

Among the substrate analogs, DFMO is by far the most widely used. It is a so-called ''suicide inhibitor'', implying that its mechanism of action is based on activation by the target enzyme, which ensures high specificity, and irreversible binding to the active site.

S-adenosylmethionine decarboxylase (AdoMetDC) catalyzes the decarboxylation of *S*-adenosylmethionine (AdoMet) to decarboxylated AdoMet (dcAdoMet) (Fig. 1), which serves as the aminopropyl group donor in spermidine and spermine synthesis. A very potent and specific irreversible inhibitor of AdoMetDC is 5′-{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (MDL 73811; AbeAdo), a structural analog of dcAdoMet (Bitonti et al., 1990).

Spermidine synthase (SpdS) catalyzes the transfer of an aminopropyl group from dcAdoMet to putrescine, thus generating spermidine and 5′-deoxy-5′-methylthioadenosine (MTA) (Fig. 1). A very potent and selective inhibitor of SpdS is *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO), a multisubstrate adduct inhibitor designed and synthesized by Coward and Pegg (1987).

Spermine synthase (SpmS) catalyzes the transfer of an aminopropyl group from dcAdoMet to spermidine, thus generating spermine and MTA (Fig. 1). This enzyme is present in the human host, but not in the protozoan parasites (Fig. 1), with the possible exception of *T. cruzi*, in which two candidate aminopropyltransferase genes have been found (Berriman et al., 2005). Nevertheless, some parasites have been reported to contain spermine, apparently because of uptake from their host, or because SpdS may use spermidine as a substrate, thus forming spermine to a small extent under certain conditions (Ikeguchi et al., 2006). For obvious reasons, SpmS inhibitors are not candidates in the development of antiparasitic agents.

Alkyl-substituted polyamine analogs, which may interfere with the functions of the polyamines rather than their biosynthesis, also possess antiparasitic activity (Zou et al., 2001), but are beyond the scope for this brief review.

A spermidine-bis(glutathionyl) conjugate called trypanothione is unique to trypanosomes and *Leishmania* (Fig. 1) (Fairlamb, 2003). It is involved in defending the parasite against chemical and oxidant stress. As a result of spermidine depletion in DFMO-treated parasites trypanothione cannot be formed, and a situation arises that may be detrimental to the parasite. Enzymes involved in trypanothione metabolism, notably trypanothione synthetase and trypanothione reductase (Fig. 1), are current drug targets of choice, but will not be discussed at length in this review.

African sleeping sickness (African trypanosomiasis)

Trypanosomes transmitted to humans and livestock by the bite of tsetse flies represent a severe constraint to health and development in sub-Saharan Africa. The human-infective forms that give rise to African sleeping sickness are *T. b. rhodesiense* and *T. b. gambiense*.

Infection with *T. b. rhodesiense*, which occurs in eastern and southern Africa, usually leads to an acute disease, whereas infection with *T. b. gambiense*, which occurs in western and central Africa, usually leads to a chronic infection. The incidence may approach 500,000 cases per year. Both forms are invariably fatal if untreated, usually due to meningoencephalitis. The infective trypomastigotes, formed from epimastigotes in the salivary glands of the tsetse fly, enter the blood, lymph, and eventually the CNS.

DFMO has proven very effective against both early- and late-stage gambiense sleeping sickness (Van Nieuwenhove et al., 1985) and is now licensed for use in many countries. The effectiveness of DFMO against late-stage disease is based on the fact that it can pass the blood-brain barrier. Accordingly, patients with late-stage gambiense sleeping sickness who had treatment failure after oral DFMO, had lower concentrations of DFMO in their cerebral spinal fluid than those who had successful treatment (Na-Bangchang et al., 2004). The reason why DFMO treatment is not effective against rhodesiense sleeping

sickness has not been established, but may be a consequence of the more rapid progression of this disease.

The high efficacy of DFMO against *T. brucei gambiense* infections may have multiple causes: (i) the parasite ODC is stable, because it lacks the C-terminal degradation signal (Phillips et al., 1987) which makes the human enzyme turn over rapidly; implying that rapid synthesis of active ODC molecules in the host, but not in the parasite, allows the host cells to transiently escape from being inhibited by DFMO; (ii) the depletion of putrescine and spermidine in the parasite cannot be prevented by utilization of the low levels of polyamines present in the blood of the host, because the parasite exhibits negligible putrescine uptake capability (Hasne and Ullman, 2005); (iii) the depletion of spermidine causes a reduction in trypanothione, a conjugate of spermidine and glutathione (Fig. 1B), thus seriously compromising the antioxidant defences of the parasite (Fairlamb, 2003); (iv) in the absence of putrescine and spermidine, there is a marked decrease in the synthesis of macromolecules, especially of variant surface glycoproteins (Bitonti et al., 1988), which are essential for evasion of the host immune response; (v) the depletion of putrescine causes a marked accumulation of AdoMet and dcAdoMet that may lead to aberrant methylation in the parasite (Yarlett and Bacchi, 1988; Byers et al., 1991); and (vi) polyamine depletion causes irreversible differentiation to nondividing short stumpy forms with a limited lifespan in the host (Giffin et al., 1986).

ODC gene knockout *T. b. brucei* cell lines have been constructed by homologous recombination and disruption of the two alleles of the ODC gene (Li et al., 1996). These Δodc null mutants require exogenous putrescine for proliferation. When injected into mice, a bloodstream form of the Δodc mutant was unable to multiply and was quickly cleared from the blood (Li et al., 1996), presumably because the polyamine transport system of the parasite is very inefficient and thus cannot retrieve the small amounts of polyamines present in the blood (Hasne and Ullman, 2005).

Irreversible inhibition of AdoMetDC by treatment with AbeAdo was shown to cure *T. b. brucei* infections in rodents (Byers et al., 1991). However, since the parasites disappeared from the bloodstream before their spermidine content was depleted, the antitrypanosomal effect of AbeAdo had to be attributed to other changes. Dramatic increases in AdoMet and dcAdoMet were found to correlate with the decrease in parasitemia (Byers et al., 1991). The accumulation of AdoMet, the major biological methyl donor, may cause aberrant methylation, which in turn may interfere with growth and survival of the parasite. Being the second most widely used enzyme substrate after ATP, AdoMet is involved in many essential biochemical processes (Fontecave et al., 2004). A role for AdoMet in the trypanocidal action of ODC and AdoMetDC inhibitors is also suggested by the finding that clinical isolates of *T. b. rhodesiense* accumulated much larger amounts of AdoMet and dcAdoMet if they were sensitive to DFMO than if they were refractory (Bacchi et al., 1993).

AbeAdo was much more potent than DFMO, and combinations of the two drugs were synergistic, even curing mice infected with clinical isolates of *T. b. rhodesiense* (Bacchi et al., 1992). These data indicate that by inhibiting both ODC and AdoMetDC, it may be possible to achieve effective therapy against rhodesiense sleeping sickness, possibly even drug-refractory forms.

Another AdoMetDC inhibitor that exhibits trypanocidal activity is CGP 40215A (bis{[3-(aminoiminomethyl)phenyl]methylene}carbonimidic dihydrazide trihydrochloride). It resembles the trypanocidal diamidines berenil and pentamidine, and was developed on the basis of the structure of methylglyoxal-bis(guanylhydrazone) (MGBG). CGP 40215A proved to be highly active in vitro against *T. b. rhodesiense* and multidrug-resistant *T. b. brucei* (Brun et al., 1996), and in vivo against clinical isolates of *T. b. rhodesiense* and *T. b. gambiense* (Bacchi et al., 1996). It was considerably more effective, and less toxic, than the mother compound. Interestingly, African green monkeys infected with *T. b. rhodesiense* were cured by treatment with CGP 40215A, but since the drug did not pass the blood-brain barrier, a prerequisite for cure of CNS infection, further development of the compound was stopped (Brun et al., 2001). To be considered, however, is the possibility of using a combination of DFMO and CGP 40215A, which was shown to cure a model CNS system infection (Bacchi et al., 1996).

AdoDATO was shown to be a potent inhibitor of *T. brucei* SpdS, as was cyclohexylamine and dicyclohexylamine (Bitonti et al., 1984), two competitive inhibitors with respect to putrescine. Since dicyclohexylamine treatment did not increase the survival time of mice infected with *T. b. brucei* the other SpdS inhibitors were not tested in vivo.

Chagas' disease (American trypanosomiasis)

T. cruzi is the causative agent of Chagas' disease. It is estimated that 16–18 million people are infected, primarily in rural zones of Central and South America. In the acute phase the disease can be lethal, but it usually evolves into a chronic stage accompanied by severe debilitation and untimely death. The parasite is transmitted by reduviid bugs, blood transfusion or organ transplantation

from an infected donor, or by congenital transmission from an infected mother. The infective metacyclic trypomastigotes are present in the vector feces, which are deposited near the bite wound. Once in the dermal layers or conjunctival mucosa, trypomastigotes invade host cells, mainly macrophages. There they transform into amastigotes, which represent the infective replicative stage. After several cycles of binary fission, transformation to trypomastigotes with host cell disruption occurs, releasing the infective forms into the bloodstream from where they invade other cells.

DFMO treatment is ineffective against *T. cruzi* (Kierszenbaum et al., 1987; Carillo et al., 2000) for an obvious reason – this parasite lacks ODC (Carrillo et al., 1999) and therefore cannot synthesize putrescine de novo (Fig. 1D). Instead *T. cruzi* is dependent on putrescine (or cadaverine) or spermidine uptake for growth and survival (Hunter et al., 1994; Carrillo et al., 1999). Although the amastigotes reside intracellularly, where the di- and polyamine levels are significantly higher than in the blood (where the trypomastigotes reside) it is not known to what extent these polyamines are available to the parasite. It has been estimated that only 5% of the spermine and 15% of the spermidine present in lymphocytes and liver cells are freely available, i.e. not bound to DNA, RNA, phospholipids or ATP (Watanabe et al., 1991).

Putrescine uptake by *T. cruzi* epimastigotes is 10–50-fold higher than in *Leishmania mexicana* and *Crithidia fasciculata* (González et al., 1992). A high-affinity polyamine transporter for both putrescine and spermidine (LmPOT1) has been identified and characterized from *L. major* (Fig. 1C), and a putative homolog has been found in *T. cruzi* (Fig. 1D) (Hasne and Ullman, 2005). A homologous *T. cruzi* high-affinity spermidine transporter, with 55% amino acid identity to LmPOT1, was recently cloned and characterized (Carrillo et al., 2006). It was also found to transport putrescine, but at a lower rate.

Expression of a foreign ODC gene in *T. cruzi* overcomes the requirement of exogenous putrescine or spermidine for proliferation (Carrillo et al., 1999), and DFMO treatment causes growth arrest of *T. cruzi* that express the foreign ODC provided that the growth medium is free of polyamines (Carrillo et al., 2000).

Although the gene for ODC, the initial enzyme in the polyamine biosynthetic pathway, is lacking in *T. cruzi*, an AdoMetDC gene encoding a low-activity AdoMetDC has been identified and characterized (Persson et al., 1998; Kinch et al., 1999). AbeAdo was shown to affect *T. cruzi* parasites adversely in their ability to infect and to replicate within heart myoblasts (Yakubu et al., 1993). As in AbeAdo-treated *T. brucei* (Byers et al., 1991), it seems that the mechanism of action does not involve a decrease in production of polyamines caused by inhibition of AdoMetDC, because the addition of spermidine or spermine did not reverse the inhibition of infectivity caused by treatment with AbeAdo. Instead, accumulation of AdoMet is a more plausible explanation in view of the fact that exogenous AdoMet significantly inhibited the infectivity of the parasites (Yakubu et al., 1993).

Although CGP 40215A exhibited trypanocidal effects against *T. b. brucei* and *T. b. rhodesiense*, it was inactive against *T. cruzi* amastigotes in murine macrophages in vitro (Brun et al., 1996).

Two candidate aminopropyltransferase genes have been found in *T. cruzi*, in contrast to one in *L. major* and in *T. brucei* (Fig. 1B–D) (Berriman et al., 2005). One of these may be a SpmS, which would explain the presence of spermine and the spermine-containing analog of trypanothione in *T. cruzi* (Fig. 1D) (Ariyanayagam et al., 2003). To our knowledge it has not been determined whether SpdS or SpmS inhibitors exert any effect on *T. cruzi* growth and survival.

Considering the efficiency of the polyamine transporter in *T. cruzi* (Le Quesne and Fairlamb, 1996) it seems unlikely that the intracellular amastigote form of the parasite can be killed by using inhibitors of AdoMetDC or spermidine synthase. The host cells constitute a rich source of polyamines (depending on how hard the polyamines are bound), and the polyamine transporter of the parasite probably has to be blocked in order markedly reduce the putrescine and spermidine content of the parasite. It remains to be determined whether inhibitors of the polyamine transporter(s) will be capable of arresting growth of *T. cruzi*.

In *T. cruzi* a single enzyme catalyzes the formation of trypanothione from spermidine and glutathione (Fig. 1D) (Oza et al., 2002). Since it may be difficult to deplete spermidine to an extent that will significantly reduce the production of trypanothione, development of inhibitors that inactivate the enzymes involved in trypanothione metabolism should be a much better approach.

Leishmaniasis

Infection with pathogenic *Leishmania* results in a broad spectrum of human diseases in Central and South America, Asia, Africa, and across the Mediterranean. An estimated 12 million people are suffering from leishmaniasis worldwide. *Leishmania* parasites are transmitted to the human host by sandflies, small blood-sucking insects in the subfamily Phlebotominae. Metacyclic promastigotes are in-

jected when the sandfly takes a blood meal, but can also gain entry when infected sandflies are crushed into the skin or mucous membrane. The motile metacyclics are engulfed by macrophages, and in the phagolysosome they transform into round, nonmotile amastigotes. These proliferate and eventually lyse the macrophage, permitting spreading of the parasite within the host. The outcome of infection is determined by the infecting species, and there are over 17 species of *Leishmania* known to be infective to humans. *L. major* and *L. mexicana* cause cutaneous ulcers, whereas *L. infantum* and *L. donovani* are responsible for visceral leishmaniasis, or kala azar, a potentially fatal disease.

Unlike human cells, *L. mexicana* promastigotes express only one arginase enzyme, whose sole vital function is to provide a polyamine precursor (ornithine) for the parasite (Fig. 1C) (Roberts et al., 2004). Δ*arg* null mutants that were created by double-targeted gene replacement were shown to lack ornithine, and were auxotrophic for ornithine or polyamines (Roberts et al., 2004). The ability of the Δ*arg* null mutants to proliferate was restored by addition of low putrescine, or high ornithine or spermidine concentrations, or by complementation with an *arginase* episome. Since arginase is essential for parasite viability it is a potential drug target. In fact, *n*-hydroxyarginine, an arginase inhibitor that is produced by macrophages as an intermediate during the conversion of arginine to citrulline and nitric oxide, has been shown to reduce polyamine levels in *L. major* and *L. infantum*, and to inhibit their growth (Iniesta et al., 2001).

The importance of the polyamine biosynthetic enzymes for *L. donovani* promastigote growth and survival was investigated by creating null mutants of the genes encoding these enzymes. The Δ*odc* knockout strain lacking both *ODC* alleles was incapable of growth in a polyamine-deficient medium (Jiang et al., 1999). The transfer of Δ*odc* cells to a polyamine-deficient medium caused a rapid depletion of putrescine and trypanothione. The fact that the cellular spermidine content was relatively unaffected, may indicate that the trypanothione turnover replenished the spermidine pools. The auxotrophy for polyamines was overcome by addition of putrescine or spermidine, but not spermine. Addition of putrescine restored the intracellular pools of putrescine, spermidine, and trypanothione. The study showed that *L. donovani* lacks the polyamine back-conversion pathway (Fig. 1C) (Jiang et al., 1999) that is present in the human host (Fig. 1A).

Δ*adometdc* and Δ*spds* mutants of *L. donovani* were incapable of growth in the absence of exogenous spermidine (Roberts et al., 2001, 2002). Their auxotrophy was overcome by addition of spermidine, but not by putrescine or spermine. AdoMetDC or SpdS overexpression, resulting from transfection and amplification of an episomal *AdoMetDC* or *SpdS* construct, abrogated spermidine auxotrophy in the Δ*adometdc* and Δ*spds* parasites (Roberts et al., 2002). The stability of *L. donovani* AdoMetDC and the lability of human AdoMetDC suggests that irreversible inhibitors of AdoMetDC may be able to eradicate leishmanial infections. Since SpdS is a stable enzyme not only in *L. donovani* but also in human cells (Roberts et al., 2001), SpdS inhibitors are likely to affect parasite and host cells similarly, unless structural differences can be exploited to selectively target the parasite enzyme.

DFMO and several other fluorinated ornithine analogs are growth inhibitory and cytotoxic not only to *T. brucei* subspecies but also to *L. donovani* (Kaur et al., 1986) and *L. infantum* (Reguera et al., 1995). Like *T. brucei* ODC, leishmanial ODC exhibits a long half-life (Hanson et al., 1992), which is a therapeutic advantage when attempting to achieve a long-lasting inhibition of the enzyme.

3-Aminooxy-1-aminopropane (APA) is an isosteric analog of putrescine that binds strongly, but reversibly to ODC (Khomutov, 2002). Its aminooxy group may form an oxime with the pyridoxal phosphate cofactor, and the positively charged amino group may play a role in anchoring APA in the active site of the enzyme. APA proved to be a potent inhibitor of *L. donovani* promastigote growth with an IC_{50} value of 42 μM (Singh et al., 2007). APA treatment reduced the putrescine, spermidine and trypanothione levels, and upon addition of putrescine or spermidine the antiproliferative effect of APA was abolished. Importantly, APA was even more effective against amastigotes in the macrophage model, with an IC_{50} value of 5 μM. APA also proved to be 10-fold more effective than DFMO against amastigotes in the macrophage assay (Singh et al., 2007). Parasites that overexpressed ODC, as a consequence of transfection and amplification of an episomal construct encoding ODC, exhibited resistance to APA, indicating that the effect of APA is primarily a result of its inhibition of ODC activity.

AbeAdo was shown to inhibit the growth of wild type cultures of *L. donovani* promastigotes with an EC_{50} value of 40 μM (Roberts et al., 2002). The fact that AdoMetDC overexpression, due to amplification of an episomal construct encoding AdoMetDC, alleviated the antiproliferative effect of AbeAdo, implies that AdoMetDC is the actual target of AbeAdo (Roberts et al., 2002). This approach has been used to establish whether antiparasitic agents specifically target enzymes in the polyamine biosynthetic pathway (Roberts et al., 2007). Thus, strains of *L. donovani* that overproduce ODC, AdoMetDC or SpdS were found to exhibit high levels of resistance to DFMO, AbeAdo and

n-butylamine, respectively, confirming the target specificity of each of these agents (Roberts et al., 2007).

The fact that ODC, AdoMetDC or SpdS overproduction did not affect the sensitivity of *L. donovani* to pentamidine, berenil, and MGBG, drugs that have been postulated to target the polyamine biosynthetic enzymes, implies alternative and/or additional targets for these agents (Roberts et al., 2007).

In another recent study it was observed that *L. donovani* ODC overexpressors exhibited significant resistance to sodium stibogluconate (Pentostam), a pentavalent antimony (Sb^V) complex and presently a first-choice drug against visceral leishmaniasis (Singh et al., 2007).

Moreover, clinical *L. donovani* isolates exhibiting Pentostam resistance were found to overexpress ODC, and to contain elevated putrescine and spermidine levels. As a consequence, they exhibited significant resistance to APA, the ODC inhibitor (Singh et al., 2007). Despite the stimulation of polyamine synthesis, the Pentostam-resistant clinical isolates exhibited no accumulation of trypanothione, suggesting that the resistance to Pentostam is polyamine-related. These findings indicate that APA may not be a suitable drug, despite its potent antileishmanial effect, in regions (especially in Bihar, India) where resistance to pentavalent antimonials is a major problem.

The *L. donovani* overexpressor strains (Roberts et al., 2007) have not yet been used to test the specificity of CGP 40215A, which is an aromatic analog of MGBG (Brun et al., 1996). However, the fact that the antiproliferative effect of CGP 40215A against *L. donovani* promastigotes was reversed by the addition of spermidine or spermine (Mukhopadhyay et al., 1996), is consistent with inhibition of AdoMetDC activity.

Conclusion and outlook

Gene deletion experiments, targeting the genes encoding ODC, AdoMetDC and SpdS, clearly demonstrate that *T. b. brucei* and *L. donovani* parasites cannot survive unless they can access sufficient amounts of exogenous putrescine or spermidine. Accordingly, the naturally ODC-deficient *T. cruzi* parasites, also depend on exogenous putrescine or spermidine for their survival. Potent trypanocidal activities of specific inhibitors of the polyamine biosynthetic enzymes further underscore the relevance of these enzymes as therapeutic targets.

Comparative modeling and site-directed mutagenesis studies indicate that sufficient differences exist in the structures of the polyamine biosynthetic enzymes such that development of selective inhibitors of the parasite enzymes will be feasible. As the three-dimensional structures for these enzymes become solved, it may be possible to design structure-based inhibitors that will selectively kill the parasites while exerting minimal or at least tolerable effects on the parasite-infected individual.

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References

Ariyanayagam MR, Oza SL, Mehlert A, Fairlamb AH (2003) Bis(glutathionyl)spermine and other novel trypanothione analogues in *Trypanosoma cruzi*. J Biol Chem 278: 27612–27619

Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A (1980) Polyamine metabolism: a potential therapeutic target in trypanosomes. Science 210: 332–334

Bacchi CJ, Nathan HC, Yarlett N, Goldberg B, McCann PP, Bitonti AJ, Sjoerdsma A (1992) Cure of murine *Trypanosoma brucei rhodesiense* infections with an S-adenosylmethionine decarboxylase inhibitor. Antimicrob Agents Chemother 36: 2736–2740

Bacchi CJ, Garofalo J, Ciminelli M, Rattendi D, Goldberg B, McCann PP, Yarlett N (1993) Resistance to DL-α-difluoromethylornithine by clinical isolates of *Trypanosoma brucei rhodesiense*. Role of S-adenosylmethionine. Biochem Pharmacol 46: 471–481

Bacchi CJ, Brun R, Croft SL, Alicea K, Bühler Y (1996) In vivo trypanocidal activities of new S-adenosylmethionine decarboxylase inhibitors. Antimicrob Agents Chemother 40: 1448–1453

Berriman M et al. (2005) The genome of the African trypanosome *Trypanosoma brucei*. Science 309: 416–435

Bitonti AJ, Kelly SE, McCann PP (1984) Characterization of spermidine synthase from *Trypanosoma brucei brucei*. Mol Biochem Parasitol 13: 21–28

Bitonti AJ, Cross-Doersen DE, McCann PP (1988) Effects of α-difluoromethylornithine on protein synthesis and synthesis of the variant-specific glycoprotein (VSG) in *Trypanosoma brucei brucei*. Biochem J 250: 295–298

Bitonti AJ, Byers TL, Bush TL, Casara PJ, Bacchi CJ, Clarkson AB Jr, McCann PP, Sjoerdsma A (1990) Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of S-adenosylmethionine decarboxylase. Antimicrob Agents Chemother 34: 1485–1490

Brun R, Bühler Y, Sandmeier U, Kaminsky R, Bacchi CJ, Rattendi D, Lane S, Croft SL, Snowdon D, Yardley V, Caravatti G, Frei J, Stanek J, Mett H (1996) In vitro trypanocidal activities of new S-adenosylmethionine decarboxylase inhibitors. Antimicrob Agents Chemother 40: 1442–1447

Brun R, Burri C, Gichuki CW (2001) The story of CGP 40215: studies on its efficacy and pharmacokinetics in African green monkey infected with *Trypanosoma brucei rhodesiense*. Trop Med Int Health 6: 362–368

Byers TL, Bush TL, McCann PP, Bitonti AJ (1991) Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in S-adenosyl-L-methionine. Biochem J 274: 527–533

Carrillo C, Cejas S, González NS, Algranati ID (1999) *Trypanosoma cruzi* epimastigotes lack ornithine decarboxylase but can express a foreign gene encoding this enzyme. FEBS Lett 454: 192–196

Carrillo C, Cejas S, Cortes M, Ceriani C, Huber A, González NS, Algranati ID (2000) Sensitivity of trypanosomatid protozoa to DFMO

and metabolic turnover of ornithine decarboxylase. Biochem Biophys Res Commun 279: 663–668

Carrillo C, Canepa GE, Algranati ID, Pereira CA (2006) Molecular and functional characterization of a spermidine transporter (*Tc*PAT12) from *Trypanosoma cruzi*. Biochem Biophys Res Commun 344: 936–940

Coward JK, Pegg AE (1987) Specific multisubstrate adduct inhibitors of aminopropyltransferases and their effect on polyamine biosynthesis in cultured cells. Adv Enzyme Regul 26: 107–113

Fairlamb AH (2003) Chemotherapy of human African trypanosomiasis: current and future prospects. Trends Parasitol 19: 488–494

Fontecave M, Atta M, Mulliez E (2004) S-adenosylmethionine: nothing goes to waste. Trends Biochem Sci 29: 243–249

Giffin BF, McCann PP, Bitonti AJ, Bacchi CJ (1986) Polyamine depletion following exposure to DL-α-difluoromethylornithine both in vivo and in vitro initiates morphological alterations and mitochondrial activation in a monomorphic strain of *Trypanosoma brucei brucei*. J Protozool 33: 238–243

González NS, Ceriani C, Algranati ID (1992) Differential regulation of putrescine uptake in *Trypanosoma cruzi* and other trypanosomatids. Biochem Biophys Res Commun 188: 120–128

Hanson S, Adelman J, Ullman B (1992) Amplification and molecular cloning of the ornithine decarboxylase gene of *Leishmania donovani*. J Biol Chem 267: 2350–2359

Hasne M-P, Ullman B (2005) Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. J Biol Chem 280: 15188–15194

Heby O, Roberts SC, Ullman B (2003) Polyamine biosynthetic enzymes as drug targets in parasitic protozoa. Biochem Soc Trans 31: 415–419

Hunter KJ, Le Quesne SA, Fairlamb AH (1994) Identification and biosynthesis of N^1, N^9-bis(glutathionyl)aminopropylcadaverine (homotrypanothione) in *Trypanosoma cruzi*. Eur J Biochem 226: 1019–1027

Ikeguchi Y, Bewley MC, Pegg AE (2006) Aminopropyltransferases: function, structure and genetics. J Biochem 139: 1–9

Iniesta V, Gómez-Nieto LC, Corraliza I (2001) The inhibition of arginase by N^1-hydroxy-L-arginine controls the growth of *Leishmania* inside macrophages. J Exp Med 193: 777–783

Jiang Y, Roberts SC, Jardim A, Carter NS, Shih S, Ariyanayagam M, Fairlamb AH, Ullman B (1999) Ornithine decarboxylase gene deletion mutants of *Leishmania donovani*. J Biol Chem 274: 3781–3788

Kaur K, Emmett K, McCann PP, Sjoerdsma A, Ullman B (1986) Effects of DL-α-difluoromethylornithine on *Leishmania donovani* promastigotes. J Protozool 33: 518–521

Khomutov AR (2002) Inhibition of enzymes of polyamine biosynthesis by substrate-like O-substituted hydroxylamines. Biochemistry (Moscow) 67: 1159–1167

Kierszenbaum F, Wirth JJ, McCann PP, Sjoerdsma A (1987) Arginine decarboxylase inhibitors reduce the capacity of *Trypanosoma cruzi* to infect and multiply in mammalian host cells. Proc Natl Acad Sci USA 84: 4278–4282

Kinch LN, Scott JR, Ullman B, Phillips MA (1999) Cloning and kinetic characterization of the *Trypanosoma cruzi* S-adenosylmethionine decarboxylase. Mol Biochem Parasitol 101: 1–11

Le Quesne SA, Fairlamb AH (1996) Regulation of a high-affinity diamine transport system in *Trypanosoma cruzi* epimastigotes. Biochem J 316: 481–486

Li F, Hua SB, Wang CC, Gottesdiener KM (1996) Procyclic *Trypanosoma brucei* cell lines deficient in ornithine decarboxylase activity. Mol Biochem Parasitol 78: 227–236

Mukhopadhyay R, Kapoor P, Madhubala R (1996) Antileishmanial effect of a potent S-adenosylmethionine decarboxylase inhibitor: CGP 40215A. Pharmacol Res 33: 67–70

Müller S, Coombs GH, Walter RD (2001) Targeting polyamines of parasitic protozoa in chemotherapy. Trends Parasitol 17: 242–249

Na-Bangchang K, Doua F, Konsil J, Hanpitakpong W, Kamanikom B, Kuzoe F (2004) The pharmacokinetics of eflornithine (α-difluoromethylornithine) in patients with late-stage *T. b. gambiense* sleeping sickness. Eur J Clin Pharmacol 60: 269–278

Oza SL, Tetaud E, Ariyanayagam MR, Warnon SS, Fairlamb AH (2002) A single enzyme catalyses formation of trypanothione from glutathione and spermidine in *Trypanosoma cruzi*. J Biol Chem 277: 35853–35861

Persson K, Åslund L, Grahn B, Hanke J, Heby O (1998) *Trypanosoma cruzi* has not lost its S-adenosylmethionine decarboxylase: characterization of the gene and the encoded enzyme. Biochem J 333: 527–537

Phillips MA, Coffino P, Wang CC (1987) Cloning and sequencing of the ornithine decarboxylase gene from *Trypanosoma brucei*. Implications for enzyme turnover and selective difluoromethylornithine inhibition. J Biol Chem 262: 8721–8727

Reguera RM, Fouce RB, Cubria JC, Bujidos ML, Ordonez D (1995) Fluorinated analogues of L-ornithine are powerful inhibitors of ornithine decarboxylase and cell growth of *Leishmania infantum* promastigotes. Life Sci 56: 223–230

Roberts SC, Jiang Y, Jardim A, Carter NS, Heby O, Ullman B (2001) Genetic analysis of spermidine synthase from *Leishmania donovani*. Mol Biochem Parasitol 115: 217–226

Roberts SC, Scott J, Gasteier JE, Jiang Y, Brooks B, Jardim A, Carter NS, Heby O, Ullman B (2002) S-adenosylmethionine decarboxylase from *Leishmania donovani*. Molecular, genetic, and biochemical characterization of null mutants and overproducers. J Biol Chem 277: 5902–5909

Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B (2004) Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants. J Biol Chem 279: 23668–23678

Roberts SC, Jiang Y, Gasteier J, Frydman B, Marton LJ, Heby O, Ullman B (2007) *Leishmania donovani* polyamine biosynthetic enzyme overproducers as tools to investigate the mode of action of cytotoxic polyamine analogs. Antimicrob Agents Chemother 51: 438–445

Seiler N (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. Curr Drug Targets 4: 537–564

Singh S, Mukherjee A, Khomutov AR, Persson L, Heby O, Chatterjee M, Madhubala R (2007) Antileishmanial effect of 3-aminooxy-1-aminopropane is due to polyamine depletion. Antimicrob Agents Chemother 51: 528–534

Van Nieuwenhove S, Schechter PJ, Declercq J, Boné G, Burke J, Sjoerdsma A (1985) Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-a-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. Trans Roy Soc Trop Med Hyg 79: 692–698

Watanabe S, Kusama-Eguchi K, Kobayashi H, Igarashi K (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. J Biol Chem 266: 20803–20809

Yakubu MA, Majumder S, Kierszenbaum F (1993) Inhibition of S-adenosy-L-methionine (AdoMet) decarboxylase by the decarboxylated AdoMet analog 5′-{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (MDL 73811) decreases the capacities of *Trypanosoma cruzi* to infect and multiply within a mammalian host cell. J Parasitol 79: 525–532

Yarlett N, Bacchi CJ (1988) Effect of DL-a-difluoromethylornithine on methionine cycle intermediates in *Trypanosoma brucei brucei*. Mol Biochem Parasitol 27: 1–10

Zou Y, Wu Z, Sirisoma N, Woster PM, Casero RA Jr, Weiss LM, Rattendi D, Lane S, Bacchi CJ (2001) Novel alkylpolyamine analogues that possess both antitrypanosomal and antimicrosporidial activity. Bioorg Med Chem Lett 11: 1613–1617

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