Polyamine Metabolism as Chemotherapeutic Target in Protozoan Parasites

C.J. Bacchi*[1,2] and N. Yarlett[1,3]

[1]*Haskins Laboratories and Departments of Biology*[2] *and Chemistry*[3]*, Pace University, 41 Park Row, New York, NY 10038, USA*

Abstract: Polyamines are essential cell constituents for all organisms. The present review highlights important differences in the synthesis, degradation, and interconversion of polyamines between the protozoan parasites (*Trypanosoma brucei, Trypanosoma cruzi, Cryptosporidium parvum and Trichomonas vaginalis*) and their mammalian hosts. Approaches include development of mono- and di-substituted polyamine analogs targeting polyamine interconversion, as well as more traditional targeting of synthetic enzymes and related pathways.

Polyamines are naturally-occurring cations which are found universally in cells. The most common polyamines are putrescine (diaminobutane), spermidine (N^1-aminopropyl-putrescine) and spermine (N^1,N^4-*bis*(aminopropyl)putrescine). Since they are cations at physiological pH, and because they are conformationally flexible and reversibly bind to negatively-charged molecules, polyamines function in a wide range of biochemical roles including: as cofactors for synthesis of macromolecules, cell division and differentiation, and as conformational stabilizers for nucleic acids [1]. In mammalian cells, polyamine content correlates with the rate of cell division. Polyamines are unique as cations since their intracellular content is carefully regulated through synthesis, uptake, interconversion and excretion [1]. Because of the essential nature of polyamines to cell division and other processes, intervention of this pathway "Fig. (**1**)" has been the focus of significant antitumor and antiparasite studies.

Synthesis of polyamines in mammalian cells "Fig. (**1**)" is initiated through ornithine decarboxylase (ODC) which decarboxylates ornithine to form putrescine. In mammals this enzyme is inducible, with a $t_{1/2}$ of ~ 10 min. Aminopropyl groups are added from decarboxylated S-adenosylmethionine (dAdoMet) via specific spermidine and spermine aminopropyl transferases. dAdoMet is supplied by AdoMet decarboxylase, a putrescine-activated inducible enzyme with a short half-life [2]. Aminopropyl groups are donated from dAdoMet by the synthase, and methylthioadenosine (MTA) is formed as a byproduct. This is normally recycled to methionine and adenine in a 5-step pathway [3].

Mammalian cells take up spermine and spermidine, interconverting them to spermidine and putrescine, respectively "Fig. (**1**)". The enzymes responsible are spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO) [4]. Since SSAT is inducible and the intermediates in the backconversion pathway, N^1-acetylspermine and N^1-acetylspermidine, are excretable, the backconversion pathway serves to regulate intracellular polyamine levels by preventing over-synthesis or uptake. Acetaminoproprionaldehyde (APA) and H_2O_2 are byproducts of the interconversion reactions [4].

POLYAMINE METABOLISM IN PROTOZOAN PARASITES

A number of considerations set protozoan parasites apart from their mammalian hosts and give reason to hope that parasite polyamine metabolism may be selectively different from that of the host, so that differential sensitivity to various agents may be present and exploitable chemotherapeutically.

1. African Trypanosomes

African trypanosomes make spermidine but not spermine "Fig. (**2A**)"; Table (**1**). Instead, spermidine is combined with 2 molecules of glutathione to form *bis*(glutathionyl) spermidine or trypanothione [5]. Glutathione reductase has not been detected, but trypanothione reductase, which uses only trypanothione as substrate, is present instead. Thus African trypanosomes as well as *Leishmania* spp and *Trypanosoma cruzi*, have a unique mechanism for handling oxidative stress [5]. African trypanosomes do not easily assimilate exogenous polyamines, and do not have the spermine to spermidine interconversion pathway [6]. Hence they rely on *de novo* synthesis for polyamine supplies.

A major difference between polyamine metabolism in African trypanosomes and mammals lies in the half-lives of the major synthetic enzymes ODC and AdoMet decarboxylase. In mammalian cells, these have very short half-lives (10−20 min) [2] while in trypanosomes, both have extended half-lives [7, 8]. The idiosyncratic non-regulatory nature of parasite polyamine metabolism also extends to AdoMet metabolism: AdoMet synthetase, which is heavily regulated in mammalian cells by its product, remains active in African trypanosomes even in the presence of high levels of AdoMet and dAdoMet [9]. Thus inhibition of polyamine synthesis leads to accumulation of AdoMet. MTA, the

*Address correspondence to this author at the Haskins Laboratories and Departments of Biology, Pace University, 41 Park Row, New York, NY 10038, USA; Tel: (212) 346-1246; Fax: (21)346-1586; E-mail: cbacchi@pace.edu

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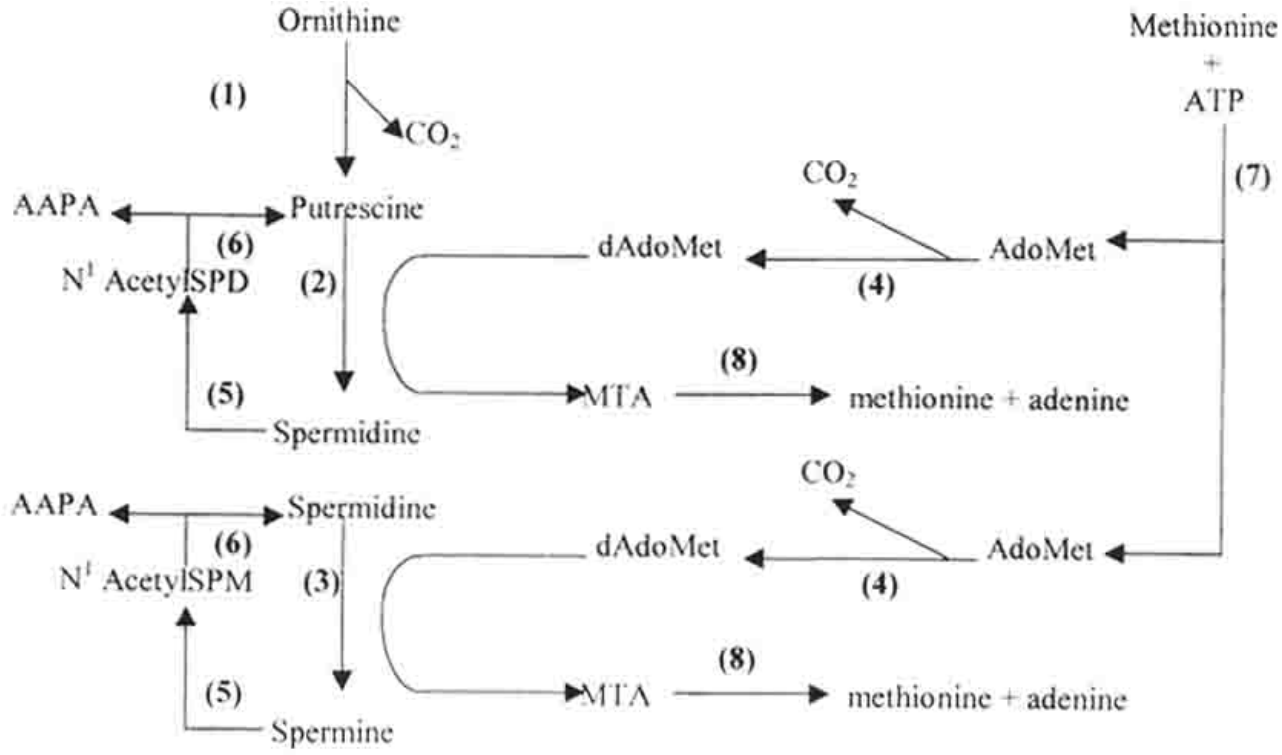

Fig. (1). Polyamine Metabolism in Mammalian Cells. AdoMet, S-adenosylmethionine; MTA, methylthioadeonsine; AAPA, acetaminoproprionaldehyde. Enzymes: (**1**), ornithine decarboxylase; (**2**), spermidine synthase; (**3**), spermine synthase; (**4**), AdoMet decarboxylase; (**5**), Spermidine/spermine N^1 acetyltransferase; (**6**), polyamine oxidase; (**7**), AdoMet synthase; (**8**), MTA phosphorylase.

byproduct of aminopropyl group donation from dAdoMet, is rapidly broken down in most mammalian cells and some parasites to adenine and methylthioribose-1-PO_4 by MTA phosphorylase (MTA-Pase) [3, 10]. The latter ribose derivative is recycled in a 5-step pathway to methionine. In African trypanosomes, MTA-Pase has a broad substrate specificity, allowing substrate analogs to be cleaved as well [11, 12]. African trypanosomes depend on exogenous purines to satisfy their purine requirement, and utilize a variety of nucleoside transporters for this purpose [13, 14]. Recent studies, however, have demonstrated the presence of a purine nucleoside transporter specific for AdoMet [15, 16]. This site is unique in being independent of inosine, adenosine and related uptake sites, but able to take up analogs of MTA and AdoMet [17].

2. *Trypanosoma Cruzi*

T. cruzi, the agent of Chagas' disease, presents a different pattern of polyamine metabolism from that of African

Table 1. Free Polyamine Content of Selected Parasites as Nmols Per 10^6 Cells

Organism	Putrescine	Spermidine	Spermine	Reference
T. b. brucei:				
Blood Trypomastigotes[a]	0.03	0.12	< 0.004	[28]
Procyclics[b]	0.17	0.22	0	[28]
T. cruzi:				
Epimastigotes[c]	0.008	0.0002	0.0018	[28]
C. parvum[d]	0.002	0.048	0.020	[29]
T. vaginalis[e]	5.40	0.49	0.18	[90]

[a] Trypomastigotes from rat blood
[b] Late log phase cells in culture
[c] Late log phase cells cultured with 10% fetal calf serum
[d] Sporozoites
[e] Log phase cells.

trypanosomes "Fig. (**2B**)". As a rapidly dividing parasite, it too is dependent on a constant supply of polyamines. Despite this, it does not appear to have ODC. Instead, a number of studies had indicated that *T. cruzi* epimastigotes have arginine decarboxylase (ADC) activity which converts arginine to agmatine, with the latter subsequently being converted to putrescine, a pathway usually found in plants. ADC was therefore thought to be the lead enzyme in the polyamine pathway of *T. cruzi* [18− 22]. Other studies, however, have failed to detect ADC and ODC activity [23, 24], while others found that neither intact epimastigotes, trypomastigotes nor amastigotes synthesize polyamines *de novo* from arginine or ornithine [25−27]. In addition, agmatine is not converted to putrescine in *T. cruzi* epimastigotes, indicating that agmatine iminohydrolase is absent and a further indication that the ADC pathway is not functional [28]. Thus it appears from the latter studies that uptake and interconversion are the main routes of diamine and polyamine acquisition in *T. cruzi*.

Lifecycle stages of *T. cruzi* (epimastigotes, trypomastigotes, amastigotes) avidly assimilate exogenous spermidine and the diamines cadaverine and putrescine. Putrescine is converted to spermidine and trypanothione, and spermidine to putrescine [25, 27]. Some conversion of cadaverine to the spermidine analog aminopropyl cadaverine and the related trypanothione analog was also found [27]. Putrescine transport in *T. cruzi* epimastigotes varies according to the stage of growth, and can be down regulated 20-fold in going from exponential to stationary phase of growth. The half-life of this transporter is much longer than that in mammalian cells (18 h vs. 30 min) [26]. Thus in light of apparent polyamine and diamine auxotrophy in *T. cruzi*, targeting of polyamine metabolism might well focus on the high affinity diamine/polyamine transport system demonstrated in epimastigotes [26], and more recently for trypomastigotes and intracellular amastigotes [25].

3. *Cryptosporidium Parvum*

The water-borne opportunistic parasite, *C. parvum* has a plant-like polyamine biosynthetic pathway "Fig. (**2C**)" which utilizes arginine to form agmatine via ADC [29]. The agmatine is further metabolized to form putrescine via the action of agmatine ureahydrolase (AUH). The remainder of the forward pathway from putrescine to spermine appears to be similar to other organisms thus far examined "Fig (**2**)", and the enzymes AdoMet decarboxylase and spermine and spermidine synthase have been reported in the organism [29]. The parasite also has a very active retro-conversion pathway from spermine to spermidine, and to a lesser extent to putrescine [29; Yarlett *et al.*, submitted). The activity of the enzymes involved in polyamine retro-conversion are 20-fold greater than the lead enzyme of the forward pathway. This information coupled with the fact that the mammalian gut has a high content of spermine [30, 31] leads one to believe that *in situ* the synthesis of spermidine via the retroconversion pathway is more significant to parasite survival than the forward directed pathway.

4. *Trichomonas Vaginalis*

The urogenital parasite *T. vaginalis* differs significantly from other eukaryotes in several aspects of its polyamine metabolism. The parasite produces and excretes large amounts of putrescine via an energy generating arginine dihydrolase pathway [32, 33]. The putrescine excreted is used to drive the uptake of spermine via a putrescine: spermine antiporter that selectively transports 1 mol of spermine into the cell while exporting 2 mols of putrescine, effectively balancing the counterion charge [34]. This parasite therefore relies upon exogenously supplied polyamines to satisfy its needs for these molecules. The spermine taken up by the parasite is retro-converted to spermidine by SSAT and PAO coupled enzyme activity [35]. The SSAT catalyses the transfer of an acetyl group from acetyl-CoA to the terminal aminopropyl nitrogen of spermine forming N^1-acetylspermine which in turn is metabolized to spermidine and APA by the action of PAO "Fig (**2D**)". In mammalian cells these enzymes are constitutively present at low levels, but are highly inducible by certain agents such as thioacetamide and certain polyamine analogues such as *bis*(ethyl)spermidine and *bis*(ethyl)*nor*spermine (BENSpm) [36, 37]. In contrast the parasite enzymes are constitutively made at high levels [35]. The *T. vaginalis* ODC has been partially purified and was found to be a pyridoxal phosphate dependent enzyme that was also capable of decarboxylating lysine and arginine at 1.0% and 0.1%, respectively of the activity with ornithine [38]. In common with other ODC's examined, the *T. vaginalis* enzyme was sensitive to inhibition by DFMO, with a K_i of 27 μM [39]. The trichomonad enzyme had a native *Mr* of 210,000 (gel-filtration) and a subunit *Mr* of 55,000 (SDS-PAGE), suggesting that the trichomonad enzyme is a tetramer. Based upon the subunit *Mr* and binding ratio of DFMO, it was calculated that there was 137 μg of ODC per mg of *T. vaginalis* protein (0.013%), which supports the hypothesis that this pathway plays a significant role in parasite metabolism and survival *in situ*.

TARGETING POLYAMINE METABOLISM IN PARASITES

1. African Trypanosomes

African sleeping sickness has experienced a resurgence within the last several years, with estimated annual cases ranging to 350−500,000 [40]. Since surveillance, vector control, and medical care have been significantly affected by the ongoing civil unrest on the Continent, the escalation in cases is not unexpected. Conventional agents for sleeping sickness include pentamidine and suramin for early stage disease, and melarsoprol (Mel B, Arsobal®) for late-stage central nervous system (CNS) disease.

Reports of melarsoprol treatment failures not attributable to drug levels and pharmacokinetics have increased [41, 42]. This apparent resistance, coupled with existing reactive encephalopathy associated with 5% of melarsoprol-treated patients and deaths in 50% of these cases, make it imperative that new therapy be developed for African sleeping sickness [43].

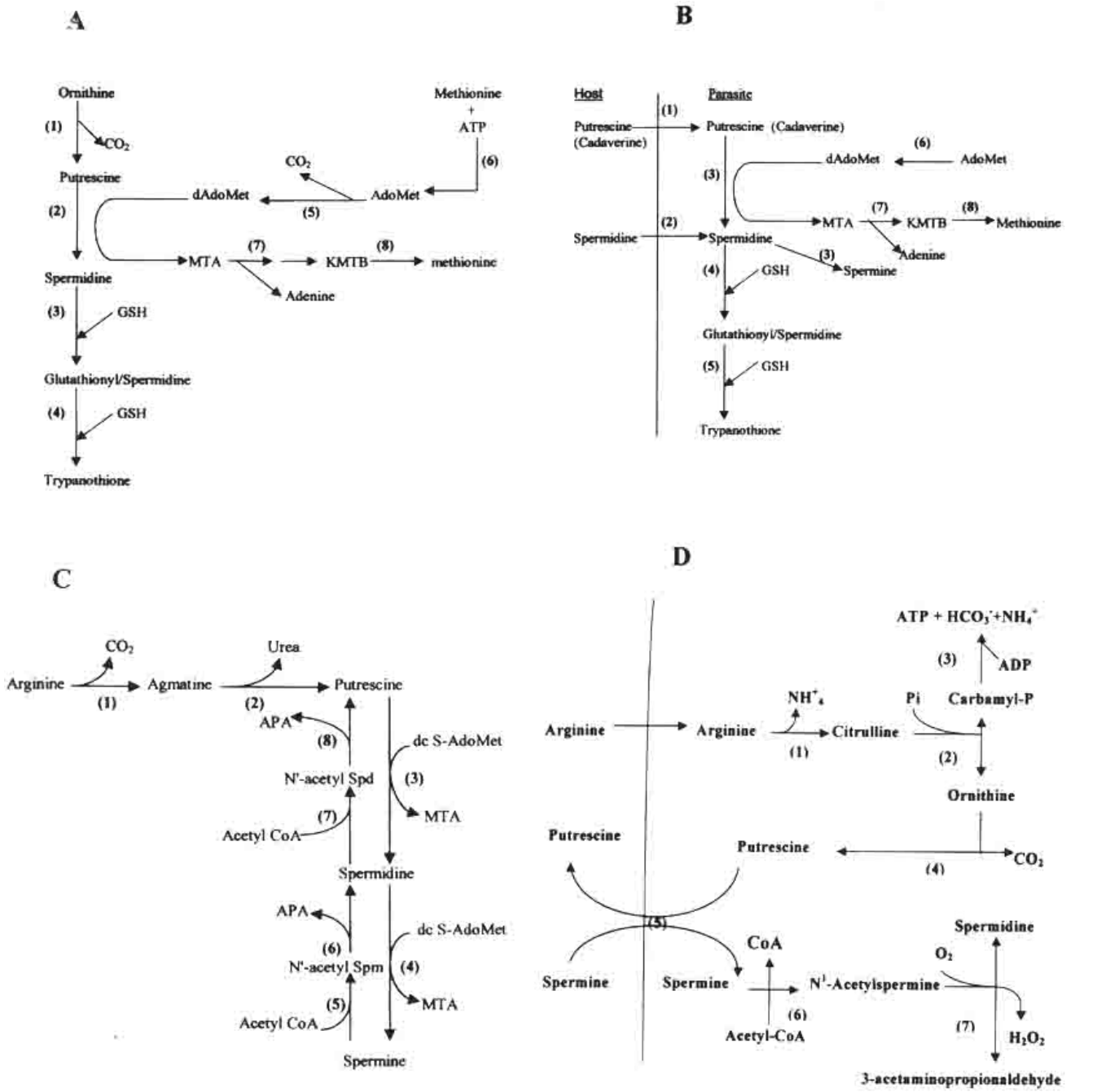

Fig. (2). Polyamine metabolic pathways of selected parasites.

A. Polyamine Metabolism in African Trypanosomes**.** Abbreviations as in Fig. 1, plus GSH, glutathione; KMTB, ketomethylthiobutyrate. Enzymes: (**1**)**,** ornithine decarboxylase; (**2**)**,** spermidine synthase; (**3**)**,** glutathionyl-spermidine synthetase; (**4**)**,** trypanothione synthase; (**5**)**,** AdoMet decarboxylase; (**6**)**,** AdoMet synthase; (**7**)**,** MTA phosphorylase; (**8**)**,** KMTB transaminase.

B. Polyamine Metabolism in *Trypanosoma cruzi***.** (**1**)**,** (**2**)**,** transport of diamines (putrescine, cadaverine) and spermidine; (**3**)**,** spermidine synthase; (**4**)**,** glutathionyl/spermidine synthase; (**5**)**,** trypanothione synthase; (**6**)**,** AdoMet decarboxylase; (**7**)**,** MTA phosphorylase; (**8**)**,** KMTB transaminase.

C. Polyamine metabolism in *Cryptosporidium parvum*. (**1**) arginine decarboxylase; (**2**) agmatine ureahydrolase; (**3**) spermidine synthase; (**4**) spermine synthase; (**5**) spermine/spermidine N'-acetyltransferase; (**6**) polyamine oxidase; (**7**) spermidine N'-acetyltransferase; (**8**) polyamine oxidase. APA, acetamido propanol; Spm, spermine; Spd, spermidine; MTA, methylthioadenosine.

D. Polyamine metabolism in *Trichomonas vaginalis*. (**1**) Arginine deiminase; (**2**) catabolic ornithine carbamyltransferase; (**3**) carbamate kinase; (**4**) ornithine decarboxylase; (**5**) putrescine/spermine antiporter; (**6**) SSAT; (**7**) PAO. Two putrescines are required to antiport with one spermine to balance the charge transfer.

i. DL-α-Difluoromethylornithine (DFMO; Ornidyl®)

This agent is an enzyme-activated irreversible inhibitor of ODC. Originally developed as an anti-tumor agent by Merrell-Dow Laboratories in the 1970's, it was minimally active in this context. However, it proved to be active in curing both early and late-stage *T. b. brucei* model infections in mice [44, 45]. It was approved by the USFDA in 1990 for treatment of human African trypanosomiasis.

There are several drawbacks to DFMO treatment: its cost (~ $500/patient), availability, and the need to administer it as an i.v. solution over 7 or 14 days [40, 46]. In a recent development, the manufacturer (Aventis) has offered to make 60,000 doses/year available free of charge to the World Health Organization for five years [40]. WHO is also conducting studies regarding oral dose eflornithine which would allow wide scale use (F. Kuzoe, pers. commun.).

Much work has been done regarding the mechanism of DFMO action, and it is clear that DFMO treatment has several effects on the parasite: reduction of polyamine and trypanothione levels, immediate blockage of ODC activity, elevation of intracellular AdoMet and dAdoMet concentrations to > 50-fold above normal, blockade of antigenic variation, and cessation of division [6, 47−49].

A major reason for the activity of DFMO lies in the long half-life of ODC (> 10 h) in trypanosomes (and Leishmania) due to the absence of an C-terminal PEST sequence which in mammalian ODC is characterized by an abundance of the amino acids Pro, Ser, Glu and Thr and which signals for rapid degradation of the enzyme by the 26S proteasome [7, 8, 50]. Thus in trypanosomes, the effect of DFMO is to shut down polyamine synthesis without concurrent increase in polyamine uptake and interconversion, a major compensatory response in mammalian cells [4]. African trypanosomes take up exogenous polyamines poorly, thus the parasite is also unable to overcome a metabolic block at ODC by importing serum polyamines which are present only in small amounts [28]. Studies with DFMO-treated trypanosomes also demonstrated that AdoMet synthetase, carefully regulated in mammalian cells, continued to function after putrescine production (and the need for AdoMet) had ceased [9]. This resulted in significant accumulation of AdoMet and dAdoMet, and was accompanied by increases in protein methylation [51].

ii) Combination Chemotherapy Using DFMO

A number of compounds have been identified in the past 7−8 years which are excellent trypanocides both *in vitro* and *in vivo*, with the exception that they do not penetrate the blood-brain barrier (BBB) and hence do not cure model CNS infections. In light of the lack of lead compounds, and the fact that even currently promising compounds have not undergone extensive pre-clinical toxicology studies which may very well eliminate them from consideration, it would be prudent to examine drug combinations for CNS activity. Which compounds however, should be the starting point? Many studies have shown that DFMO at low dose levels is active in curing model CNS infections in combination with: suramin; melarsoprol; O,O'-*bis*(1,2,dihydro-2,2-tetramethylene-4-6-diamino-S-triazin-1-yl)-16-hexanediol (SIPI 1029); 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811); 5'-hydroxyethylthioadenosine, HETA; a bicyclic analog of methylglyoxal-*bis*(guanyl)hydrazone (CGP 40215) [52−57]. Clearly, the ability of DFMO to act synergistically with so many chemically distinct agents does not imply a common biochemical basis of action. Rather, the literature on artificially induced BBB injury indicates that polyamine metabolism is significantly out of balance after injury due in part to elevated ornithine decarboxylase and that the ODC inhibitor DFMO has a role in correcting this. A related approach is the use of hyperosmotic solutions to open BBB tight junctions and permit delivery of drugs to the CNS (summarized in [58]). A recent review indicates that a number of different types of injuries to the brain result, after a complex cascade of events, in the enhanced synthesis, degradation and release of polyamines in brain tissue. Ultimately, damage may be attributable to formation of toxic reaction products as a result of the oxidative degradation of polyamines [59]. DFMO may ameliorate the injury due to CNS infection, and in doing so a situation may develop which allows passage of molecules normally impervious to the BBB to enter. Alternatively, the infusion of 400 mg/kg of DFMO may alter the permeability of the BBB, and enhance permeation of co-administered agents. Since in most combination studies with DFMO, only a single or several doses of the other agent need be given, usually within in 3−4 days of the onset of DFMO treatment [52, 55], there appears to be a short window during which the other agent will pass the BBB. There is a serious need to explore these combinations and the basis for their activity using subcurative doses of DFMO in combination with known agents, e.g. suramin and melarsoprol. It may be that other polyamine antagonists, e.g. MDL 73811, which blocks AdoMet decarboxylase and hence spermidine production, may have similar synergistic activity.

iii. Inhibition of AdoMet Decarboxylase

African trypanosomes have an AdoMet decarboxylase with an extended half-life. Diamidines, such as pentamidine and berenil were found to inhibit AdoMet decarboxylase [60]. However, the high IC_{50} values found (> 250 μM), make it unlikely that this is the sole mechanism of action. More interesting is MDL 73811, an enzyme activated inhibitor of AdoMet decarboxylase, developed by Marion-Merrell-Dow in the late 1980's. This agent cures laboratory infections of *T. b. brucei* and *T. b. rhodesiense* clinical isolates [61]. It is curative against late-stage CNS model infections when used in combination with DFMO [55], and is not toxic to mice at > 10 times the curative dosages used. It is rapidly transported by bloodforms through the P_2 adenosine site [16, 17], quickly inactivates the enzyme, and elevates AdoMet concentrations in the cytosol within an hour [61]. The resulting blockade of AdoMet decarboxylation and buildup of AdoMet lead to an increase in protein methylation, S-adenosylhomocysteine formation and excretion of intermediates of homocysteine recycling [49].

Supplies of MDL 73811 are now limited, however, the low toxicity (therapeutic index of 16−40) [62], oral dosing

and strong activity vs. *T. b. rhodesiense* isolates warrant further consideration of this agent.

CGP 40215 is a bicyclic analog of methylglyoxal *bis*(guanylhydrazone), and inhibitor of AdoMet decarboxylase. Developed by Ciba-Geigy in the 1990's as part of a series of derivatives aimed at the opportunistic pathogen *Pneumocystis carinii*, CGP resembles the trypanocidal diamidines berenil and pentamidine. It inhibited *T. b. brucei* AdoMet decarboxylase with an apparent IC_{50} of 20 μM and reduced spermidine levels by nearly 70% in intact cells incubated with 10 μM [56]. This agent was highly effective, curing acute laboratory infections of *T. b. brucei*, *T. b. rhodesiense, T. b. gambiense,* and *T. congolense* − a total of 19 isolates [56, 63]. It was not effective in CNS model infections, however, unless used in combination with DFMO.

iv. Methionine Recycling

Methionine plays an essential role in polyamine metabolism since AdoMet furnishes aminopropyl groups for spermidine formation, and the byproduct of polyamine synthesis from AdoMet is MTA. As noted earlier, African trypanosomes, like mammalian cells, cleave MTA to adenosine and methylthioribose-1-PO_4 through an MTA phosphorylase. The ribose-1-PO_4 is then recycled to methionine [3].

African trypanosomes have an MTA phosphorylase with a broad substrate specificity, cleaving 5'-substituted analogs more efficiently than the mammalian enzyme [11, 64]. *In vitro*, 5' substituted MTA analogs were 500-fold more active against *T. b. brucei* than mammalian cells [65, 66], while *in vivo*, HETA, the most active of the MTA analogs, cured laboratory model infections of *T. b. brucei* and *T. b. rhodesiense* isolates [66]. HETA is most likely metabolized through the pathway to yield an α-ketoacid derivative of ketomethylthiobutyrate, the penultimate intermediate in the pathway [12]. In mammals, the latter is then transaminated to methionine, using glutamine or asparagine as the amino donor [67]. In trypanosomes, aromatic amino acids (tyrosine, tryptophan, phenylalanine) are the preferred amino donors [68]; the deaminated byproducts (phenylpyruvate, indole-3-pyruvate, etc.) are released into the bloodstream and eventually excreted [69−71].

The activity of methionine recycling through the MTA pathway in mammalian-infective trypanosomes is significant as indicated by the finding that concentrations of aromatic amino acids are reduced in trypanosome infected livestock, while urinary concentrations of deaminated byproducts are highly elevated during infection [70, 72, 73]. HETA is taken up by both the P_2 adenosine transporter and an independent AdoMet transporter [17]. The potential for selectivity in this pathway is seen in the broad substrate specificity of MTA-Pase from trypanosomes and in the differences in transamination of the ketomethylthiobutyrate (KMTB) product.

v. Trypanothione Reductase

Much of the spermidine present in trypanosomes is in the form of bis(glutathionyl) spermidine, which is maintained in the reduced state by trypanothione reductase. Both trypanothione and trypanothione reductase function to maintain intracellular reducing environment in trypanosomes. Since trypanothione reductase is specific for trypanothione and will not reduce oxidized glutathione, as found in mammalian cells, it can be considered a logical drug target in parasitic kinetoplastids [5].

Since African trypanosomes lack catalase, inhibition of trypanothione reductase would defeat the parasites' ability to scavenge free radicals produced from peroxide, and superoxide anion. The crystaline structure and active site configuration of the *T. b. brucei* enzyme has been detailed [5]. Many inhibitors of trypanothione reductase have been developed, with some proving highly effective *in vitro* vs. blood form trypanosomes, yet thus far, more have been found to have significant activity in model infections. The classes of compounds synthesized include phenothiazines, tricyclic compounds, diphenylsulfides, phenylpropyl and naphthylmethyl-β-substituted polyamines. Problems with bioavailability, pharmacokinetics and host metabolism of these compounds need to be examined [42, 74].

2. *Trypanosoma Cruzi*

Lack of putrescine synthesis combined with the ability to transport cadaverine and spermidine and to interconvert spermine to spermidine make this organism unique among kinetoplastids [28]. Since all three forms of the parasite (epimastigotes, trypomastigotes, and amastigotes) share these qualities [25], an enticing possibility for chemotherapy is the transport and interconversion of polyamines.

The earliest attempts to probe this target in protozoan parasites utilized MDL 27695, a bis-benzyl polyamine analog (N, N'-*bis*{3-[(phenylmethyl)amino]propyl}1,7 diamino heptane). This compound was not a substrate for polyamine transport in CHO cells but was a substrate for a separate transport system [75]. MDL 27695 was a substrate for mammalian polyamine oxidase, which de-benzylated the compound to N^1,N^7-*bis*(aminopropyl)diaminoheptane. This in turn significantly down-regulated mammalian ODC and AdoMetdc activity, without directly inhibiting enzyme activity [76]. The overall effect was to block division of HTC cells. Thus MDL 27695 acted as a pro-drug which, at a low (1 μM) concentration blocked cell division. MDL 27695 blocked growth of *Plasmodium falciparum* in culture, and cured a *Plasmodium berghei* infection in mice [77]. MDL 27695 was also highly effective (> 99%) in reducing amastigote burdens of mice infected with *Leishmania donovani* after oral or parenteral treatment [78, 79]. The activity did not appear to be dependent on conversion to the de-benzylated form, since co-administration of MDL 27695 with a polyamine oxidase inhibitor did not decrease activity [78].

Tumor cells treated with DFMO become nearly depleted of polyamines, while transport and interconversion of polyamine increase to a point at which the cell eventually overcomes the block in synthesis. Thus the up-regulation of transport limits the blockade of polyamine synthesis [4]. Recently, as an alternative to the use of inhibitors of

anabolic polyamine enzymes, modified polyamine analogs have been developed which enter through polyamine uptake sites, repress ODC and AdoMetdc activity, while inducing SSAT [4, 80, 81]. The net effect is a down-regulation of synthesis combined with depletion of polyamine content caused by conversion to acetylated derivatives and enhanced release from the cell [2]. These inhibitors include N^1, N^{12}-*bis*(ethyl)spermine (BE 3-4-3), BENSpm, and N^1,N^{14}-*bis*(ethyl)homospermine (BE 4-4-4). These agents are rapidly accumulated by tumor cells and exhibit multiple effects on polyamine synthesis and interconversion [2, 4, 80].

There are several types of polyamine analogs in use, including terminally (*bis*-ethyl) modified pentamine or oligoamine analogs based on repeating aminobutyl group residues [80]. Related approaches include development of asymetric polyamine analogs [4] or addition of double bonds resulting in sterically altered molecules which have restricted rotation around the single bonds as found in natural occurring polyamines [80].

Thus there exists a rich inventory of amine analogs which may be suitable for selectively interfering with *T. cruzi* polyamine metabolism. In particular, analogs interfering with cadaverine uptake may be of particular interest because of the high capacity of uptake found in all forms of the parasite [25, 26].

A more conventional approach to antagonism of polyamine synthesis was the use of the AdoMetdc inhibitor MDL 73811 to block invasion of rat heart myoblasts by *T. cruzi* trypomastigotes [82]. This compound also prevented multiplication of amastigotes in infected host myoblasts, and inhibited AdoMetdc activity in the parasite. The effects on parasite multiplication and infectivity were not reversible by exogenous spermidine or spermine [82].

T. cruzi also recycles MTA through an MTA phosphorylase which has been shown like the enzyme from *T. b. brucei* to have a broad substrate specificity [83]. The class of 5'-substituted MTA analogs including HETA has not been examined for activity against *T. cruzi.*

3. *Cryptosporidium Parvum*

C. parvum is an opportunistic parasite potentially lethal in AIDS patients for which no treatment is currently known. The organism is one of several protozoan genera in the phylum Apicomplexa. The parasite develops in the gastrointestinal tract of vertebrates through its entire life cycle, the only exogenous stage being the sporulated oocyst. The oocyst consists of four sporozoites within a tough two-layered wall. Several outbreaks traced to infected water supplies have occurred, the largest being the 1993 outbreak in Milwaukee, WI, where an estimated 403,000 people were infected [84]. As previously mentioned *C. parvum* has a plant-like polyamine biosynthetic pathway mediated by ADC. The parasite ADC is irreversibly inhibited by DL-α-difluoromethylarginine (DFMA) with a K_i of 30 μM [29]. This agent was effective at blocking *in vitro* growth of the parasite when grown in an HCT-8 feeder cell line. In these studies 4 mM DFMA caused a 60% inhibition of growth with no observable toxicity to the feeder cells [29]. Parasite growth was also blocked by other arginine analogues effective at inhibiting plant and bacterial ADC's. The most effective of these was canavanine which caused 86% growth inhibition at 3.6 mM with no observable toxicity to the feeder cells [29]. It was found that arginine analogues when used in combination with inhibitors of the retro-conversion pathway were synergistic [29]. Hence combinations of DFMA and BENSpm at 2 and 4 mM , respectively caused 60% growth inhibition [29]. One of the most effective groups of compounds tested have been those targeting polyamine transport by the parasite. The tricyclic antidepressants, desipramine and imipramine block polyamine transport by cells due to their anti-calmodulin action [85] effectively inhibited growth of *C. parvum* at 33 μM (94%) and 310 μM (100%), respectively [29]. These results confirm the importance of uptake and retro-conversion of host derived polyamines to parasite growth and survival.

Agmatine (32 mg/kg for 7 days) alone has been demonstrated to be effective at preventing infection of *C. parvum* and at reducing the parasitemia of a mouse model infection [86]. Exogenous agmatine may disrupt polyamine metabolism of *C. parvum* in several ways. Excess agmatine may cause feedback inhibition of ADC, resulting in a buildup of arginine within the parasite. Arginine has been shown to inhibit *C. parvum* infection of adult immunocompromised mice [87]. Alternatively, excess agmatine may cause up-regulation of AUH thus causing the buildup of putrescine and possibly other polyamines. Putrescine alone has previously been shown to be effective at preventing infection in pretreated mice and also at reducing parasitemia of *C. parvum* in a neonatal mouse model [88]. Excess agmatine may also be directly toxic to the parasite. Inhibitors of the retro-conversion pathway appear to be effective growth inhibitors of *C. parvum*. One such compound [^{1}N,^{12}N]*bis*(ethyl)-cis-6,7-dehydrospermine (SL-11047), a conformationally restricted analogue of spermine, was found to cure a T-cell receptor alpha deficient mouse model infection at 134 mg/kg per day for 7 days [89].

4. *Trichomonas Vaginalis*

The trichomonads have an active ODC which is susceptible to several fluorinated analogues of ornithine, with monofluoromethylornithine and DFMO being the most effective (3.4 and 27 μM) [39]. These analogues also reduced intracellular polyamines and in a semi-defined growth medium were growth inhibitory [90]. The trichomonad ODC was fully induced after 15 hr (late log) and in common with the trypanosome enzyme [7] was found to have an extended half-life of greater than 8 hr [39]. This is in contrast to the mammalian enzyme which posses a PEST sequence and is turned over rapidly [7]. The analogue 1,4 diamino-2-butanone (DAB) was also effective at reducing intracellular polyamine levels in the cattle trichomonad *Tritrichomonas vaginalis*. Coincident with polyamine depletion were several ultrastructural alterations particularly in the redox-organelles termed hydrogenosomes [91]. These organelles were progressively degraded, resulting in the presence of large vesicles that displayed material immunoreactive to a β-succinyl-coenzyme A synthetase antibody [91].

Hydrogenosomes are the only redox organelle found in trichomonads and under aerobic conditions these structures generate free radicals such as hydroxyl, peroxide and superoxide [92]. The antioxidant properties of polyamines such as putrescine and spermine [93] may have a significant protective role. Indeed the structural damage observed in polyamine depleted trichomonads is most likely due to lipid peroxidation and protein cross-linking of components of the hydrogenosomal membrane. The hydroxyalkenal molecules that form during polyunsaturated fatty acid peroxidation could react with free sulfhydryl groups, resulting in the formation of thioether linkage-presenting adducts. This would account for the observed hydrogenosomal membrane retraction and electron dense precipitates observed in DAB-treated parasites [91]. In addition the inactivation of free sulfhydryl groups under conditions of lipid peroxidation can alter the intracellular Ca2+ distribution [94], and Ca2+ fluxes may be blocked by ODC inhibition [95]. Hydrogenosomes are considered to be archaic mitochondria and have common functions that are retained, one of this is the ability to accumulate Ca2+, particularly within the peripheral vesicles [96, 97]. It is reasonable to attribute at least part of the structural damage observed upon DAB-treatment therefore to disruption of Ca2+ homeostasis. These results suggest that polyamines in trichomonads may, as has been shown in several other cells, play an important role in the stabilization of membrane integrity. Trichomonads also have an active retro-conversion pathway which can be targeted by alkylated polyamine analogues such as BENSpm. This analogue has been shown to be readily transported by the parasite and once internalized is an effective inhibitor of SSAT resulting in a significant reduction in the intracellular concentration of spermine (84%) and spermidine (68%) [35]. Parasites treated with BENSpm also had reduced activities of ODC and SSAT and at 1mM growth was reduced by 66% [35]. Although ODC levels decrease in both *T. vaginalis* and tumor cells treated with BENSpm, SSAT levels increase 15-fold in tumor cells treated with this analogue [37], while the activity of this enzyme decreases significantly in trichomonads treated with BENSpm [35], indicating that significant differences occur in this pathway between parasite and host cells. Therefore this group of compounds has enormous clinical potential as antitrichomonad agents by blocking back conversion to the functionally active polyamine spermidine, and by decreasing synthesis of putrescine which is needed for cotransport of spermine. The *bis*-aryl analogue, MDL 27695 is also an effective trichomonacide (MIC of 0.28 to 0.56 microM: N. Yarlett unpublished), towards metronidazole-sensitive and -tolerant strains and this compound is of particular interest because it is less effective in anaerobic incubations MIC: 1.12 μM suggesting a role for oxygen in its mode of action. Two mechanisms by which MDL 27695 acts in other cells have been proposed: in *P. falciparum*, it is a substrate for polyamine oxidase forming a cytotoxic benzaldehyde derivative [77], whereas in *Leishmania* spp the intact molecule can function as a polyamine analogue [78].

PROSPECTS

Although this review focused on only four protozoan parasites, it is evident that the differences between polyamine metabolism of host and parasite extend to other parasitic protozoans. The assortment of inhibitors of polyamine synthetic reactions have largely been ineffective against mammalian cancers, the diseases for which they were designed. Thus, these agents offer potential for use against protozoan parasites with exploitable biochemical differences (Table 2). The advent of polyamine analogs which enter through amine transporters, and replace natural polyamines in cells without retaining their normal functions should allow the emergence of additional effective agents.

Table 2. Parasite Polyamine Metabolism: Likely or proven agents for chemoterapeutic targets in parasitic protozoa.

African Trypanosomes:

ODC - DFMO, 3,4dehydromonofluoromethylornithine

Trypanothione Reductase- Phenothiazones, dephenylsulfides, phenylpropyl/naphthylmethyl β-substituted polyamines.

AdoMet decarboxylase - MDL 73811, CGP 40215

MTA phosphorylase/methionine recycling - HETA and related 5'substituted-thioadenosines.

Trypanosoma cruzi

Cadaverine, Putrescine, Uptake - bisethylpolyamine analogs

AdoMet Decarboxylase - MDL 73811, CGP 40215

Trypanothione Reductase - Phenothiazenes, diphenylsulfides, phenylpropyl/napthylmethyl β-substituted polyamines.

PAO/SSAT - MDL 27695, bisethyl substituted polyamine analogs; conformationally hindered oligoamines.

MTA Phosphorylase - HETA and related 5'substituted-thioadenosines.

Cryptosporidium parvum

ADC - DFMA, canavanine

SSAT - conformationally trestricted oligoamines, e.g., SL 11047 unrestricted oligoamines, e.g, BENSpm.

PAO - MDL 72527

S-AdoMet decarboxylase - MDL 73811

Polyamine transport - imipramine, desipramine, oligoamines.

Trichomonas vaginalis

ODC - DFMO, MFMO

SSAT - BENSpm

Polyamine Transport - Imipramine, oligoamines

Polyamine oxidase - MDL 27695

ACKNOWLEDGEMENTS

We acknowledge the following grant support, which fund studies on the metabolism of polyamines in protozoan parasites, and the effects of antipolyamine agents on these parasites: AI 49785 (N.Y.); AI43094 (C.J.B.); and AI41398 (C.J.B.).

ABBREVIATIONS

ODC	=	Ornithine decarboxylase
dAdoMet	=	decarboxylated S-adenosylmethionine
AdoMet	=	S-adenosylmethionine
MTA	=	methylthioadenosine
SSAT	=	spermidine/spermine N^1-acetyltransferase
PAO	=	polyamine oxidase
APA	=	acetaminoproprionaldehyde
MTA-Pase	=	methylthioadenosine phosphorylase
ADC	=	arginine decarboxylase
AUH	=	agmatine ureohydrolase
KMTB	=	α-ketomethylthiobutyrate
MDL 27695	=	(N,N'-*bis*{3-[(phenyl-methyl) amino]propyl}1,7 diamino heptane)
BE 3-4-3	=	N^1,N^{12}-*bis*(ethyl)spermine
BENSpm	=	N^1,N^{11}-*bis*(ethyl)norspermine
BE 4-4-4	=	N^1,N^{14}-*bis*(ethyl)homospermine
DFMA	=	DL-α-difluoromethylarginine
SL 11047	=	N^1,N^{12}-*bis*(ethyl)bis-6,7,-dehydrospermine
DAB	=	1,4-diaminobutane
DFMO	=	DL-α-difluoromethylornithine
BBB	=	Blood brain barrier
SIPI 1029	=	O,O'-*bis*(1,2,dihydro-2,2-tetramethylene-4-6-diamino-S-triazin-1-yl)-16-hexanediol
MDL 5'-73811	=	5'-{[(Z)-4-amino-2-butenyl]methylamino}-deoxyadenosine
CGP 40215	=	bicyclic analog of methylglyoxal-*bis*(guanylhydrazone)

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