

Amino Acid Metabolic Routes in *Trypanosoma cruzi*: Possible Therapeutic Targets Against Chagas' Disease

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Abstract: Chagas' disease is a zoonosis caused by the parasite *Trypanosoma cruzi*, a haematic protozoan, transmitted by insects from the *Reduviidae* family. This constitutes a relevant health and socio-economic problem in the Americas, with 11 – 18 million people infected, and approximately 100 million people at risk. The therapeutic possibilities rely into two drugs, nifurtimox[®] and benznidazole[®], that were discovered more than thirty years ago, and are mainly successful during the acute phase of the disease. In the majority of the cases the disease is diagnosed in the chronic phase, when the therapy is inefficient and the probability of cure is low. In addition, these drugs are highly toxic, with systemic side effects on patients.

Trypanosoma cruzi has a metabolism largely based on the consumption of amino acids, mainly proline, aspartate and glutamate, which constitute the main carbon and energy sources in the insect stage of the parasite life cycle. These amino acids also participate in the differentiation process of the replicative non-infective form (epimastigote) to the non-replicative infective form (trypomastigote). In particular, the participation of proline in the intracellular differentiation cycle, which occurs in the mammalian host, was recently demonstrated. In addition, an arginine kinase has been described in *T. cruzi* and *T. brucei*, which converts free arginine to phosphoarginine, a phosphagen with a role as an energy reservoir. Arginine kinase seems to be an essential component of energy management during stress conditions. Taken together, these data indicate that amino acid metabolism may provide multiple as yet unexplored targets for therapeutic drugs.

Key Words: *Trypanosoma cruzi*, Chagas' disease, chemotherapy, arginine metabolism, proline metabolism, amino acid transporters, aptamers.

INTRODUCTION

Protozoans infective to humans and animals constitute an important group of parasites with medical and veterinary importance. Among them, different species of *Leishmania*, *Trypanosoma cruzi* and African trypanosomes, such as *Trypanosoma brucei brucei* (not infectious to humans), *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense* are examples of parasites of mammals. About half a million people are infected by parasites of the *T. brucei* group in Africa, 11-18 million with *T. cruzi* in the Americas and 12 million with *Leishmania* in Africa, Asia, Europe and Americas [1, 2]. The life cycles of *Leishmania* and *T. cruzi* have an obligatory intracellular stage in mammals, in contrast to the exclusively extracellular parasites of the *T. brucei* group.

Both *Leishmania* and *T. cruzi* invade host cells, but *Leishmania* lives inside parasitophorous vacuoles while *T. cruzi* escapes from the vacuole and lives in the cytoplasm of the host cell. In both cases, the parasite has to adhere to the

host cell surface in order to invade the cell and survive under harsh conditions of the host cytoplasm to establish an effective infection. The host cell is subsequently the source of nutrients for parasite metabolism. Nutrient transport from the host cell cytoplasm to the parasite (*T. cruzi*) or from the host cytoplasm and through the membrane of the parasitophorous vacuole before reaching the membrane of the parasite (*Leishmania*) are essential steps that can be targets for drug design, in addition to metabolic routes that are specific for the parasites and absent in the host.

1. THE TRYPANOSOMA CRUZI LIFE CYCLE

The flagellate protozoan *Trypanosoma cruzi* is the cause of Chagas' disease, discovered by the Brazilian scientist Carlos Chagas in 1909. The disease is primarily transmitted by triatominae insects to different mammalian hosts.

T. cruzi has a complex life cycle characterized by several developmental forms present in vertebrate and invertebrate hosts [3, 4]. Based mainly on morphological criteria, such as the spindle shape of the parasite, as well as the position of the kinetoplast (DNA of the single mitochondria of the parasite) relative to the nucleus and the flagellar emergency region, three stages are classically described: (i) amastigotes (greek, a = without; mastis = whip for flagellum) which are

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dividing round cells of 2.4-6.5 μm in diameter found in the cytoplasm of the vertebrate host cell; alternatively, some authors named these forms as spheromastigotes due to the presence of an incipient flagellum that does not emerge from the cell; (ii) trypomastigotes (trypo = to drill, referring to a property of this cell to attach to glass by one point while making rotatory movements), an infective, flagellated and non-dividing form present in vertebrate and invertebrate hosts; trypomastigotes are spindle – shaped, approximately 18 μm in length (including a 6 μm free flagellum) and 2-3 μm in breadth; (iii) epimastigotes (epi = anterior, from above), an extracellular flagellated, 20-40 μm long and 2-5 μm large, non-infective and dividing stage present typically in the invertebrate host intestine. Although the three forms are easily identified, intermediate forms can be found in the vertebrate and invertebrate hosts. A pleomorphic trypomastigote population present in the blood of an infected vertebrate is taken by the insect in a blood meal and in the insect gut, transforms to epimastigotes through intermediate forms such as the spheromastigotes [4, 5]. Trypomastigotes appear in the rectum of the insect as a differentiation product from epimastigotes. When the insect bites the vertebrate host to initiate blood sucking it concomitantly eliminates the trypomastigotes (called metacyclic trypomastigotes) in feces and urine, which are mechanically carried into the wound by the host. Trypomastigotes ultimately invade cells, residing for approximately 1 h inside a lysophagosome from which they escape into the cytoplasm where differentiation to the amastigote form occurs. After approximately 30 h all invading trypomastigotes have been transformed into amastigotes that replicate by binary fission to hundreds per invaded cell. Amastigotes start to differentiate into trypomastigotes after approximately 96 h. This step is mediated by an epimastigote-like form (intracellular epimastigote) sharing some properties with the epimastigote form, but being considerably shorter (4 μm in length). The existence of this form in the vertebrate part of the cycle has been a matter of debate since 1914 [cf. 3] but presently it was characterized using morphometric, biochemical and immunological criteria as being similar to the epimastigote forms [6]. Interestingly, proline is essential for the differentiation of intracellular epimastigotes to trypomastigotes [7], as described for the differentiation *in vitro* of epimastigotes to metacyclic trypomastigotes [8]. It seems likely that the parasite has a continuous differentiation cycle following the pattern trypomastigote – amastigote – epimastigote – trypomastigote with one or more intermediate forms in the vertebrate and invertebrate hosts [5, 6]. The predominance of a particular form would be dependent mainly on the environmental conditions surrounding the parasite.

2. TRANSMISSION

The disease is a zoonosis widespread from the southern USA to southern Argentina, affecting 11 – 18 million people with some 100 million being at risk of acquiring the disease [1, 2]. Also, more domestic animals, such as dogs, cats and pigs are reported to be infected with *T. cruzi*, increasing the possibility of parasite dissemination to humans, similar to the infection of dogs with *Leishmania* spp, presently a serious problem for the control of leishmaniasis in urban areas.

In addition to human beings and domestic animals, opossums and rats, as well as monkeys and other carnivorous wild animals, are important reservoirs of *T. cruzi*. Although all *Triatominae* species potentially can be vectors of *T. cruzi*, only *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongylus megistus*, *Triatoma brasiliensis*, *Triatoma rubrofasciata* and *Triatoma sordida* are epidemiologically important. *T. infestans* is the main vector of Chagas' disease, present in Argentina, Brazil, Chile, Uruguay, Paraguay, Peru, Bolivia and Ecuador whereas the distribution of *P. megistus* is more restricted to the Brazilian territory. In Mexico, Guatemala, Honduras, El Salvador and Nicaragua, *Triatoma dimidiata* and *Rhodnius prolixus* are, epidemiologically, the most important species. *Triatominae* also exist in the USA, mainly in California, Arizona and New Mexico. Insects contaminated with *T. cruzi* have been found in human dwellings, but some species are not good vectors since they do not readily defecate at the biting site after a blood meal. Notwithstanding, there are recent reports of domestic transmission in dogs [9, 10]. In South America, joint efforts since 1991 of the Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay), following guidance of the World Health Organization, have been successful to diminish transmission of the *Trypanosoma cruzi*. By 2001, disease transmission had been halted in Uruguay, Chile and Brazil. Canisters, which release pyrethroid insecticidal fumes when lit by householders, and insecticidal paints for use by spray teams were developed [1].

The natural portal of entry for the parasite is any region of the skin leading to an inflammation called chagoma. However, since the insect usually bites during sleeping, the face is preferred. In that case, the insect, during the blood meal deposits feces and urine and the victim transports this material to the eye provoking a unilateral swelling of both eyelids, known as Romaña's sign. Another important portal of entry is blood transfusion.

Migratory currents have always been important in transmission of Chagas' disease. In Brazil, for instance, the great number of migrants from rural and usually poorer endemic zones to developed regions of the country brought Chagas' disease to the more sophisticated urban centers. At the beginning, the lack of attention from blood bank professionals to preventing the spread of disease, associated with the inexperience in diagnosis by most physicians, made blood transfusion a major vector of disease transmission. Some concern exists in USA regarding the appearance of significant numbers of *T. cruzi* – seropositive donors contributing to the blood supply pool due to increasing immigration of citizens from South and Central American countries [11, 12]. Immigrants in the USA are estimated to be 11.4 million legal permanent residents and 5 million of illegal residents giving a total of about 16 million. Approximately 25% originated from endemic regions, mostly from Mexico [13]. In 1997, 300,000 immigrants were estimated to be infected [14].

3. PATHOLOGY

Trypanosoma cruzi induces an acute phase with patent parasitemia followed by a life-long chronic phase characterized by subpatent parasitemia and scarce tissue parasitism.

The acute phase is less studied nowadays due to the small number of detected cases and its relatively short duration (1-2 months).

In the acute phase, in addition to high parasitemia, parasites may be found in practically all tissues and organs as intracellular amastigotes, accompanied by infiltration of lymphocytes, plasma cells, and monocytes. The heart is nearly always involved and may exhibit dilatation and pericardial effusion. Lymphnode enlargement, and hepato and esplenomegaly are often found. At this phase, which in some cases can be so mild as to pass unnoticed, mortality represents less than 10%.

In the chronic phase, the heart is often involved with hypertrophy and dilatation. Chronic chagasic cardiopathy affects about a third of the infected persons [15]. Frequently the apical region looks thin with a fibrotic degenerative lesion leading to the formation of an apical aneurysm, a condition found in approximately 50% of the necropsies of chagasic patients. Also a diffuse inflammation with focal fibrosis and myocardial and neuronal destruction may be found. Some patients have macro and microscopic alterations in the digestive tract, predominantly in the esophagus and large intestine with appearance of megaviscera accompanied by a degeneration of the neurons of the mienteric plexuses and infiltration of lymphocytes. In this phase parasites are rarely found.

Clinically, there is a third form called latent or sub-clinical or indeterminate form. It will start immediately after the acute episode and may last lifelong. Patients do not have symptoms and may not have heart or digestive involvement when checked by conventional methods of exam. However, if more accurate techniques are performed, such as echocardiography or exercise testing some alterations may be found. Since 30% of these patients present cardiac and digestive complications in a late phase of the disease, the indeterminate form is, in fact, an asymptomatic chronic phase.

4. CHEMOTHERAPY

The few drugs available are limited in relation to efficacy and tolerance. The most important are Nifurtimox, a nitrofur, and Benznidazole, a nitroimidazole derivative. Production of the former [3-methyl-4-(5'-nitrofururyledene-amino tetra hydro 4H-1, 4-thiazine-1, 1 dioxide], synthesized by Bayer AG and known as Bayer 2502, has been discontinued. Adverse effects of this drug were anorexia, nausea, vomiting, polyneuropathy and, rarely, seizures. Benznidazole [N-benzyl-2-nitro-1-imidazol acetamide], from Roche is sold in the market as Rochagan (Brazil) and Radanil (Argentina) also with some adverse effects including allergic dermatopathy that usually starts around the ninth day of treatment. Prolonged use may cause peripheral sensitive neuropathy and leucopenia. Nifurtimox acts via reduction of the nitro group to unstable nitroanion radicals, which react to produce highly toxic superoxide anions and hydrogen peroxide. Benznidazole appears to modify macromolecules covalently by nitroreduction intermediates [16].

Benznidazole administration is indicated in the following situations: (1) acute phase of the infection; (2) reactivation of

the infection by immunosuppressive drugs; (3) recently acquired infection; and (4) organ transplantation procedures in chagasic patients. In the other conditions related to the chronic phase, efficacy of treatment is subject to doubt due to the persistence of positive serological reactions [17] and presence of the parasite as judged by PCR-based methods [16]. Recently, Roche gave the rights and technology to the Government of the State of Acre in Brazil which, in turn will license at least one Brazilian public laboratory to produce Benznidazole. It is hoped, in the transition period, that no discontinuity will occur in the production and distribution of Benznidazole since the drug is very effective in the treatment of children [18, 19] and remains as the only drug available to treat scientists immediately after contamination in laboratory accidents.

New approaches to specific chemotherapy are being pursued and pre-clinical trials have been performed with some new compounds. In short, attempts are being made at selectively inhibiting the pathways of *de novo* sterol biosynthesis, cruzipain-mediated proteolysis, pyrophosphate metabolism, synthesis of trypanothione, purine salvage, as well as dihydrofolate reductase, phospholipid biosynthesis and protein prenylation and acylation [16].

5. SEARCH FOR THERAPEUTIC TARGETS IN THE METABOLISM OF AMINO ACIDS

The relevance of amino acids during the *T. cruzi* life cycle is well established. The early works of Zeledón [20] and Sylvester and Krassner [21] have shown the ability of epimastigotes to oxidize certain amino acids. Addition of three amino acids to defined media promotes differentiation from epimastigote to metacyclic trypomastigote (metacyclogenesis). In addition to glucose, aspartate, glutamate and proline [22] strongly promote metacyclogenesis in a variety of *T. cruzi* strains. During the chronic infection, *T. cruzi* lives mostly restricted to specific tissues such as cardiac or striated muscle and several regions of the digestive track and kidney epithelium, maintaining its infection cycle in a restricted region of the tissue (called amastigote nests by most of pathologists) without causing major symptoms. During infection, *T. cruzi* spends a considerable time replicating as the intracellular forms (mostly amastigote, but also the transient intracellular epimastigote) and it appears that these stages have low, if any, glucose transport activity (unpublished). This fact supports the supposition that the amastigote form would have a low sensitivity to therapeutic drugs targeted to glycolytic enzymes. We have recently proposed a proline-based metabolism for the replicative amastigote forms and intracellular epimastigotes [7, 23].

Since amino acids participate in a variety of metabolic routes leading to many crucial compounds for survival of *T. cruzi*, transporters and enzymes related to their metabolism become interesting targets for drug design.

6. A GENERAL VIEW OF THE *T. CRUZI* INTERMEDIATE METABOLISM

T. cruzi has no intracellular glucose reserve such as glycogen or starch. Thus, glucose has to be imported from the environment, such as the mammalian blood, through a

glucose transport system. The amino acids can be obtained from three main sources: biosynthesis from metabolic precursors, active transport from the medium and protein degradation. Proteins could be incorporated from the surrounding medium by pynocytosis [24] and kept in reservosomes. A general schematic view of *T. cruzi* intermediate metabolism is presented in Fig. 1.

A unique characteristic of kinetoplastids is the fact that glycolysis is compartmentalized between glycosomes and cytoplasm [25]. Glucose is taken up by hexose transporters, which are present in a number varying from one to three depending on the species: for example, only one transporter was described for *T. cruzi*, while two were described for *T. brucei* and three for *Leishmania spp.* [26]. It is assumed that once in the cytoplasm, glucose is transported into the glycosomes, to be oxidized. A peculiar characteristic of glycolysis in kinetoplastids is the fact that the hexokinase and phosphofructokinase, as opposed to mammals, are not allosterically regulated [27 – 30] and the glycolytic flux it is

not influenced negatively by the presence of oxygen (Pasteur effect) [31]. It was thus proposed that compartmentalization of glycolysis may constitute an evolutionary alternative to regulate the glycolytic flux in trypanosomes at the level of glucose transport from the cytoplasm to the glycosome [32]. However, the mechanisms and kinetics of the latter are unknown and no proteins were identified that qualify for this function.

Once inside the glycosome, each molecule of glucose is rapidly converted into two molecules of glyceraldehyde - 1, 3 - bisphosphate by the glycolytic enzymes present in the organelle. The glyceraldehyde 1, 3 - bisphosphate produced in the glycosomes is transported back into the cytoplasm by another uncharacterized system and converted in phosphoenol pyruvate (PEP), which can follow two main paths: (a) conversion to pyruvate, part of which can be translocated into the mitochondria to enter the Krebs cycle and part that may be transformed to alanine by transamination in the cytoplasm [33, 34]; and (b) be transported back to the

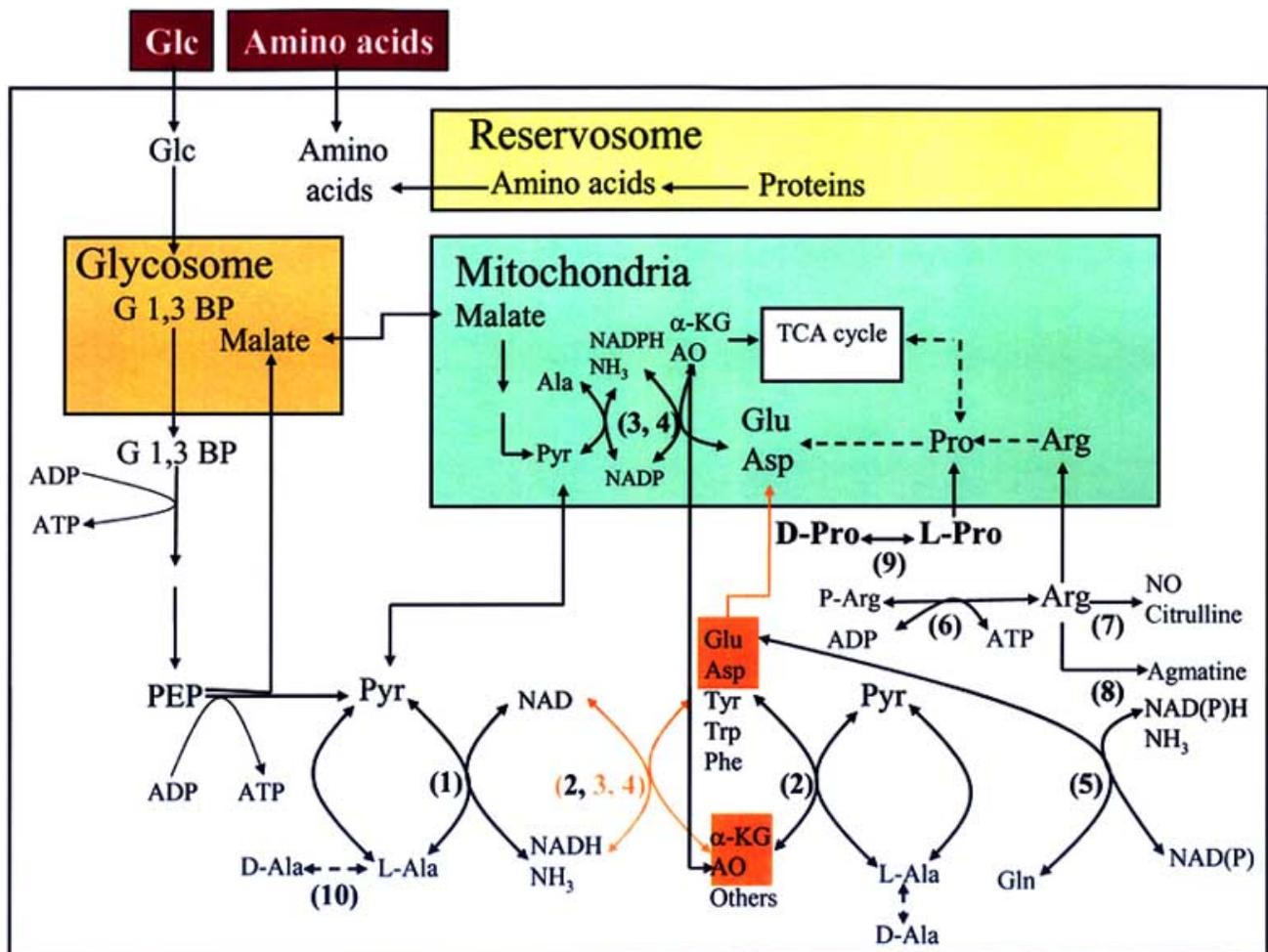


Fig. (1). Schematic representation of the amino acid metabolic pathways in *Trypanosoma cruzi*. Solid lines represent pathways or reactions that were biochemically demonstrated. Dotted lines represent putative pathways or reactions inferred from the *T. cruzi* genome project. Abbreviations and references: G 1, 3 BP: Glyceraldehyde 1, 3 biphosphate, PEP: Phosphoenol pyruvate, -KG: -ketoglutarate, OA: Oxaloacetic acid, TCA cycle: tricarboxylic acid cycle (citric acid cycle), 1: alanine aminotransferase (ALAT), 2: tyrosine aminotransferase (TAT), 3: glutamate dehydrogenase, 4: aspartate amino transferase, 5: glutamine synthetase, 6: arginine kinase, 7: nitric oxide synthase, 8: arginine decarboxylase, 9: proline racemase, 10: alanine racemase.

glycosome followed by carboxylation of PEP to oxaloacetate by phosphoenolpyruvate carboxykinase (PEPCK). The resulting oxaloacetate is finally converted to malate by malate dehydrogenase. Malate leaves the glycosome and is converted again to pyruvate by a cytoplasmic malic enzyme or is transported into the mitochondrion, where it can be converted to pyruvate by the mitochondrial malic enzyme and oxidized by the Krebs cycle. Both, cytoplasmic and mitochondrial pools of pyruvate can be aminated by amino acid transaminases yielding alanine [32-35], probably responsible for the existence of two independent pools of alanine, as observed by ^{13}C NMR [36].

7. MAJOR L-AMINO ACID ANABOLIC AND CATABOLIC PATHWAYS IN *T. CRUZI*

Early work showed that asparagine, glutamine, aspartate, glutamate, leucine, isoleucine, and proline are metabolized by *T. cruzi* [20, 21, 37]. It is believed that all these amino acids are oxidized through their conversion to glutamate or aspartate, which can be transported from the cytoplasm into the mitochondria and be processed via the Krebs cycle. As mentioned above, glutamate and alanine are directly involved in feeding the Krebs cycle with intermediates. The $-\text{NH}_2$ group of glutamate may be transferred to pyruvate by alanine aminotransferase (ALAT) and, to some extent, by tyrosine aminotransferase (TAT), yielding α -ketoglutarate and alanine. Consequently, when glycolysis is active, alanine is actively produced [38]. Another pathway by which glutamate is converted into α -ketoglutarate is by the glutamate dehydrogenase, a well-studied enzyme presenting two isoforms: one mitochondrial and the other cytoplasmic [39 – 46]. The glutamate dehydrogenase converts glutamate into α -ketoglutarate, transferring the $-\text{NH}_2$ group to H_2O . This would explain the high concentrations of NH_3 present in conditioned culture medium [47]. Aspartate is also involved in the supply of Krebs cycle intermediates through an aspartate aminotransferase. This enzyme, with biochemical characteristics similar to that from mammals, transfers the $-\text{NH}_2$ group from aspartate to α -ketoglutarate, yielding glutamate and oxaloacetate [39]. Several copies of a gene coding for a putative mitochondrial aspartate aminotransferase were recently identified in the *T. cruzi* genome (*T. cruzi* genome initiative at the Sanger Center [www.genedb.org] systematic names Tc00.1047053510945.70, Tc00.1047053503679.10 and Tc00.1047053503841.70). The glutamate obtained as a by-product can then proceed to the Krebs cycle as stated above. An aspartate aminotransferase activity was also demonstrated for the TAT. This enzyme possesses a broad specific aminotransferase activity, and can also transaminate the three aromatic amino acids and alanine. Among the acceptor groups are pyruvate (converted to alanine), α -ketoglutarate (converted to glutamate) and oxaloacetate (converted to aspartate) [48, 49] although its major role seems to be the conversion of pyruvate to alanine [36]. As TAT catalyses a reversible transamination among aspartate or alanine and the aromatic amino acids, it seems to be also a key enzyme in the biosynthesis of these amino acids. Both isoforms of glutamate dehydrogenase also participate in the biosynthesis of amino acids by their ability to incorporate NH_3 into α -ketoglutarate yielding glutamate [50]. It was also shown that

glutamine can be synthesized from glutamate and NH_3 in the presence of glutamine synthetase [50]. As this reaction is reversible, glutamine might be oxidized through the glutamate pathway. There are no biochemical data on the presence of an asparagine synthetase. However, two ORFs were found in the *T. cruzi* genome coding for a putative asparagine synthetase, which catalyzes the reversible conversion of aspartate to asparagine (*T. cruzi* genome initiative at the Sanger Center systematic names Tc00.1047053503625.10 and Tc00.1047053503899.90). In addition, an ORF corresponding to a putative asparaginase-like protein was identified, which would catalyze the conversion of asparagine to aspartate in an irreversible way (GB AAV31748). The presence of any of these two enzymes suggests that asparagine might be oxidized through the aspartate pathway.

8. ARGININE METABOLISM

Arginine is a dibasic amino acid and a key substrate for several metabolic pathways. In the urea cycle, its amidino group can be transferred to water to form urea thus allowing the elimination of excess nitrogen. The amidino group can also be transferred to amino acceptors to form substituted guanidino derivatives. These derivatives and arginine itself can be phosphorylated by specific kinases to form a variety of high-energy phosphoryl-derivatives designated as phosphagens [cf. 51-53]. L-arginine is a substrate for an alternative pathway for proline biosynthesis via ornithine and L-glutamate 5-semialdehyde [54], and can also be metabolized to polyamines via ornithine and putrescine [55]. Moreover, in tissues where nitric oxide is produced, the guanidino group in arginine is oxidized in the presence of NADPH by the nitric oxide synthase [56].

T. cruzi is unable to synthesize arginine; therefore the amino acid is obtained from the host through different transport systems [57 - 59]. In trypanosomatids, most of the well-studied enzymatic reactions involving L-arginine have been related to the ornithine-arginine pathway. Different genera of trypanosomatids have different enzymes involved in the catabolism of arginine: it was postulated that some *Trypanosoma* species, including *T. cruzi*, lack ornithine decarboxylase, arginine decarboxylase, and arginase [47, 60 - 66]. This indicates that *T. cruzi* is unable to synthesize diamines from either L-arginine or L-ornithine. Consistently, the biosynthesis of trypanothione, a glutathione-spermidine conjugate, which is involved mainly in defense of trypanosomatids against damage by oxidants, requires exogenous poly and diamines [67, 68]. However, controversial evidence was also published on the existence of these biochemical pathways. A low arginine decarboxylase (ADC) activity was measured in epimastigotes of the RA strain of *T. cruzi* in the early logarithmic phase of growth [69, 70]. On the other hand, in contrast with the biochemical data, the recently finished *Trypanosoma cruzi* genome project (<http://www.genedb.org>) identified at least four open reading frames coding for putative arginases (*T. cruzi* genome initiative at the Sanger Center systematic names Tc00.1047053507031.90, Tc00.1047053509497.30, Tc00.1047053510947.40 and Tc00.1047053507963.20). Biochemical data will have to be extended to other parasite stages since most

of the arginine metabolism was studied in epimastigotes. Other enzymes involved in arginine metabolism predicted by the *T. cruzi* genome project are: arginyl-tRNA synthetases and a previously described arginine kinase (see below). Enzymes that use polypeptide-associated arginine were also identified: arginine N-methyltransferases (putative histone protein methylases) and arginine aminopeptidases.

Arginine is the substrate for nitric oxide synthases (NOS) that have been biochemically characterized in some kinetoplastid parasites such as *Leishmania* spp. and *T. cruzi* or apicomplexa parasites i.e. *Plasmodium* spp. [71, 72]. A nitric oxide synthase (tcNOS) was partially purified and characterized from soluble extracts of *T. cruzi* epimastigote forms [73]. Two biological functions of the nitric oxide pathway in *T. cruzi* were postulated. The first one correlates the nitric oxide production with the modulation of flagellar motility in epimastigote forms [74]. The second one indicates that *T. cruzi* epimastigote apoptosis can be inhibited by L-arginine through a NO synthase-dependent NO production, in addition to an ADC-dependent production of polyamines that support parasite proliferation [75], (for a review, see ref. [76]). Interestingly, no evidence for NOS-like sequences has been found yet by any of the apicomplexa or kinetoplastid parasite genome projects, probably because of the differences on the primary structure between mammalian and unicellular eukaryotic NOS.

Phosphoarginine and phosphocreatine, generally called phosphagens, play a critical role as energy reserve because the high-energy phosphate can be transferred to adenosine diphosphate (ADP) when the renewal of adenosine triphosphate (ATP) is needed. It has been proposed that phosphoarginine supports bursts of cellular activity until metabolic events such as glycogenolysis, glycolysis and oxidative phosphorylation are switched on (for a review, see ref. [77]). Phosphoarginine synthesis also allows the cells to operate with low ATP levels since it may constitute a usable pool of the high-energy phosphate. Phosphagens act as a reservoir, not only of ATP, but also of inorganic phosphate that is mostly returned to the medium by the metabolic consumption of ATP [51]. Arginine kinase (ATP:arginine phosphotransferase; EC 2.7.3.3) catalyzes the reversible transphosphorylation between N-phospho-L-arginine and ADP [77].



Phosphagen kinases belong to the enzymatic “phosphotransfer network” that communicates the spatially separated intracellular ATP consumption and production processes [78]. From an evolutionary viewpoint, arginine kinase was included in a family of conserved proteins with phosphotransferase activity, with creatine kinase as the best known member. Arginine kinase is the most widely distributed phosphagen kinase, which is found in Annelida, Coelenterata, Platyhelminthes, Nemertea, Mollusca, Phoronida, Arthropoda, Echinodermata, Hemichordata, and Chordata [53, 79]. In addition, arginine kinase is considered the most closely related member to the ancestral guanidino kinases [80].

Recently, the molecular and biochemical characterizations of arginine kinases in *Trypanosoma cruzi* and *Trypanosoma brucei* have been reported [57, 81, 82]. Since arginine kinase, an important enzyme involved in the energy supply for the parasite, is absent from mammalian tissues, it becomes a possible target for the future development of chemotherapeutic agents against Chagas' disease and other parasitic diseases caused by related organisms. For this purpose, a rational approach would involve the validation of the enzyme as a therapeutic target and the search for specific enzyme inhibitors.

Multiple evidence indicates that *T. cruzi* arginine kinase is strongly regulated by intra and extracellular conditions: (1) the arginine kinase protein and the associated specific activity increase continuously along the epimastigote growth curve, suggesting a correlation between the enzyme activity and the nutrient availability or parasite density [83]; (2) the existence of a relationship between the arginine transport rate, arginine kinase activity and the parasite stage and replication capability was recently described, indicating a critical role of arginine kinase as a regulator of energetic reserves and cell growth [58]; and (3) the homologous overexpression of *T. cruzi* arginine kinase improves the ability of the transfectant cells to grow and resist to nutritional and pH stress conditions [84]. Arginine kinase would play a role as a stress resistance factor when expressed in organisms that lack this enzyme, such as yeast and bacteria. Recombinant yeast, expressing crab muscle arginine kinase, showed improved resistance under stress challenges that drain cellular energy, which were transient pH reduction and starvation [85, 86].

It is important to remark that the insect stage of the *T. cruzi* life cycle is frequently exposed to nutritional and pH stress conditions, depending on the feeding status of the vector. For example, the pH of excreted material of the *T. cruzi* vector *T. infestans* varies between 5.7 and 8.9, accordingly with the time after feeding [87]. All these data suggest that arginine kinase is involved in the adaptation of the parasite to environmental changes and stress conditions.

A well characterized specific inhibitor of arginine kinase has not been found although some compounds were reported in the literature. The trypanocidal action of green tea (*Camellia sinensis*) catechins against two different developmental stages of *T. cruzi* was demonstrated by Paveto *et al.* [88]. Interestingly, recombinant *T. cruzi* arginine kinase was 50% inhibited by nanomolar concentrations of these polyphenols (catechin gallate or gallicocatechin gallate). Arginine kinase was also inhibited by the arginine analogs, agmatine, canavanine, nitroarginine, and homoarginine [89]. In addition, canavanine and homoarginine also produce a significant inhibition of the epimastigote growth in culture.

9. PROLINE METABOLISM

As already mentioned, proline is a major carbon source in most trypanosomatids. However, little is known about proline degradation pathways in *T. cruzi*. Metabolic studies showed that in epimastigotes of *T. cruzi*, proline is converted

to five intermediates of the Krebs cycle (citrate, isocitrate, malate, succinate and oxaloacetate), pyruvate and the amino acids glutamate and aspartate that are rapidly metabolized [21]. A metabolic link between proline and the biosynthetic pathways leading to cysteine and lysine was also suggested. There is, as yet, no direct biochemical evidence of a proline oxidase activity in *T. cruzi*, since pyrroline-5-carboxylic acid (P5C), the product of proline oxidation by this enzyme, was not detected. However, the fact that proline is processed through the Krebs cycle *via* glutamate, and the existence of two genes coding for a putative proline oxidase recently found (*T. cruzi* genome initiative at the Sanger Center systematic names Tc00.1047053506411.30 and Tc00.1047053511237.30) support the existence of this metabolic step. The main ways by which P5C is catabolized in most eukaryotic organisms is through its conversion to glutamate by P5C dehydrogenase (two genes possibly coding for this enzyme were found, systematic names Tc00.1047053510943.50 and Tc00.1047053510943.50) or through an ornithine-oxo-acid transaminase to yield ornithine. In the case of *T. cruzi*, there is no biochemical evidence for the existence of the latter enzyme and no putative genes coding for it have been detected. These facts are consistent with early observations on the absence of an urea cycle in *T. cruzi* [47, 61]. Thus, the failure to detect P5C in parasite extracts could be explained exclusively by a rapid conversion of proline into glutamate, which would be rapidly processed in the Krebs cycle, since the proline-glutamate pathway in *T. cruzi* does not seem to be branched.

10. D-AMINO ACID METABOLISM

The description of a mitogen with a proline racemase activity raised the possibility for the existence of a D-amino acid metabolism in *T. cruzi* [90]. This protein is present in all life cycle stages, presenting many isoforms in several subcellular locations. Two major isoforms were further characterized showing, respectively, K_m values of 29 and 75 mM [91]. The role of these enzymes in proline metabolism is not clear yet, particularly taking into account that their activity might be extremely low at the intracellular proline concentration (between 0.75 and 9 mM, depending on the stage [7, 23]). It was proposed that this enzyme might take part in a chemical compartmentalization of the intracellular pool of proline [91], which is a major energy reservoir for the intracellular stages differentiation [7]. Reinforcing the idea for the existence a D-amino acids metabolism, two ORFs coding for a putative alanine racemase have been recently identified (*T. cruzi* genome initiative at the Sanger Center systematic names Tc00.1047053511179.150 and Tc00.1047053508303.10).

11. SERINE AND METHIONINE METABOLISM

In contrast to proline, aspartate and glutamate, serine and methionine did not stimulate the oxygen consumption by *T. cruzi* epimastigotes [21]. Indeed, there is no evidence supporting a role for these amino acids in the energy metabolism. However, methionine might also be involved in polyamine metabolism. The synthesis of the intermediate S-adenosyl-L-methionine might be catalyzed by a putative

methionine adenosyltransferase (TIGR *T. cruzi* gene index TC10544). The product of the previous reaction is a substrate of the S-adenosyl-L-methionine (AdoMet) decarboxylase, which yields S-adenosyl-methioninamine, a reaction that was biochemically demonstrated [92]. Finally, S-adenosyl-methioninamine might be converted in spermidine by a spermidine synthase (there are, at least, four putative genes, *T. cruzi* genome initiative at the Sanger Center systematic names Tc00.1047053510339.50, Tc00.1047053503855.20, Tc00.1047053510337.40 and Tc00.1047053504033.130). Further research is required to demonstrate biochemically the existence of that metabolic pathway.

Serine metabolism was biochemically studied to some extent. A single cytosolic serine hydroxymethyltransferase, which converts serine and tetrahydrofolate into glycine and 5, 10-methylenetetrahydrofolate was well characterized [93, 94]. Serine can also be converted to cysteine by a cystathionine -synthase, which can catalyze trans-sulfuration reactions. This enzyme is present in *T. cruzi* in at least six isoforms, all with a serine acetyltransferase activity [95]. At least two ORFs coding for the enoyl-CoA hydratase, which catalyzes the conversion of serine in pyruvate, have been found (*T. cruzi* genome initiative at the Sanger Center, systematic names Tc00.1047053508153.130 and Tc00.1047053508185.10). If this reaction really exists, labeled intermediates of the Krebs cycle and alanine should be expected when parasites are incubated with ^{14}C -U-serine. Although such intermediates have not been detected, radiolabeled CO_2 was recovered when *T. cruzi* epimastigotes were incubated with ^{14}C -serine, in addition to an extensive labeling of free serine, glycine and alanine. These results are compatible with, at least, a partial oxidation of serine through the Krebs cycle [96, 97].

12. OTHER AMINO ACIDS

Little is known about the metabolism of other amino acids. However, it is worth mentioning that phosphoenolpyruvate mutase, which catalyses the first step of the aminoethylphosphonate biosynthetic pathway, was recently characterized in *T. cruzi* [98]. The identification of three ORFs coding for putative enzymes participating of the interconversion of selenomethionine and selenocysteine (cystathionine -synthase, TIGR *T. cruzi* gene index TC9733; adenosylhomocysteinase, TIGR *T. cruzi* gene index TC8619; and methionine adenosyltransferase TIGR *T. cruzi* gene index TC10544) allows us to infer the possible existence of metabolic pathways for the synthesis of selenoamino acids in *T. cruzi*.

13. THE AMINO ACID TRANSPORTERS

The structure and molecular mechanisms of metabolite transporters are largely unknown in trypanosomatids. The only *T. cruzi* gene that was cloned and functionally expressed in a heterologous system is the glucose transporter [26]. The amino acid transporters were first biochemically described by Hampton [99, 100], and confirmed by others [101] who reported the probable presence of several transporters for amino acids, with different specificity and kinetics. Recently, the uptake of arginine, proline and

glutamate was studied in more detail. Arginine is actively transported by at least two systems, a high affinity and a low affinity system. The high affinity system seems to be highly specific and ATP dependent, while the low affinity system was partially competed by methionine, lysine and tyrosine and has characteristics of an H⁺/amino acid symporter [57, 59]. Low and high affinity systems for the transport of proline were also characterized in *T. cruzi*. Both transporters are active and powered, respectively, by the plasma membrane H⁺ gradient and ATP [23]. The glutamate transporter seems to be also powered by the cytoplasmic membrane H⁺ gradient (unpublished). Recently, a post-genomic analysis was performed to search for putative genes coding for the Amino Acid/Auxin permeases (AAP) gene family in *T. cruzi*. This family comprises permeases with characteristics of H⁺/amino acid symporters and was chosen because the use of the H⁺ gradient to energize amino acid transport in *T. cruzi* may constitute a pattern. It should be taken into account that H⁺ gradients are major components of the cytoplasmic membrane potential in these cells [102]. Accordingly, at least 60 genes coding for putative amino acid transporters belonging to the AAP family were found in *T. cruzi* [103]. The presence of such a large quantity of genes coding for a single family of amino acid transporters corroborates the notion of the relevance of these proteins in the biology of *T. cruzi*.

14. PUTATIVE THERAPY TARGETS

As mentioned, it was demonstrated that *T. cruzi* is highly dependent on the amino acid metabolism in several situations, such as the intracellular differentiation or stress resistance. The considerations above allow us to propose that targeting drugs against enzymes of the amino acid metabolism would be promising as a novel approach to define trypanocidal strategies. A summary of the main targets is presented below.

(A). Differential Metabolic Pathways

(1) Some biochemical reactions occur in *T. cruzi* but not in the mammalian host. The enzymes involved in such reactions are obvious drug targets. One of these biochemical steps is the reversible phosphorylation of arginine by the arginine kinase. As discussed, this enzyme was cloned, functionally expressed and studied in some detail [81, 82, 84].

(2) The biological meaning of the conversion of L-proline into a racemic mixture by the proline racemase is largely unknown. However, recent studies on the specific inhibition by several proline analogs and by directed mutagenesis of amino acids that are components of the active site demonstrated that the proline racemase is a valid therapeutic target [91]. In addition, the expression of a putative alanine racemase must be further investigated since this activity has not yet been described in mammals.

(3) Recently, phosphoenolpyruvate mutase, an enzyme that is absent in the mammalian host, was proposed as a potential drug target [98]. However, further validation of this enzyme as a drug target requires more studies on the biological role of the enzyme.

(B). Homologous Metabolic Pathways Catalyzed by Different Enzymes

Some enzymatic activities catalyzing conserved metabolic pathways that are central to life are present in almost all organisms. However, the enzymes and the mechanisms by which they catalyze the corresponding biochemical reactions might present differences that can be capitalized on for drug discovery. Accordingly, the differences in sequence structure may determine minor differences that can be exploited as a first criterion to elect the protein as a therapeutic target. For example, ALAT is an enzyme also present in mammals, but the identity between *T. cruzi* and the human liver enzyme is only 55% on the basis of the first 18 N-terminal amino acids [38]. *T. cruzi* TAT, which is 70% identical to the human enzyme differs from the human enzyme in substrate specificities, catalytic properties and proposed functional roles; such difference indicate that this enzyme could be a valid target [49].

The NADP⁺ dependent and NAD⁺ dependent glutamate dehydrogenases (NADP⁺-GDH and NAD⁺-GDH) of *T. cruzi* are also crucial enzymes in amino acid metabolism and show relevant differences from the corresponding mammal enzyme. While mammals and other higher eukaryotes have a single enzyme with both activities, in *T. cruzi* these activities are in separate enzymes. Only the NADP⁺-GDH was cloned and characterized at the molecular level. Biochemical characteristics and sequencing indicate that the enzyme may be more closely related to bacteria and distant from eukaryotes [104]. Thus, this enzyme is a drug target candidate for development of specific inhibitors, but further research is needed to validate it as a truly valid target. The NAD⁺-GDH activity should also be further evaluated, since its biochemical properties suggest it could be a new target [105]. An aspartate aminotransferase activity was also identified that seemed to be similar to that of mammals [39], but it is premature to assign this enzyme as a candidate for therapeutic targeting since it has not been cloned, sequence and evaluated enzymologically.

Other enzymes, such as the cystathionine γ -synthetase, involved in the cysteine metabolism and recently identified and cloned, is another candidate for therapeutic targeting, since in spite of being 50% identical to the mammalian homologous enzyme, it shows physicochemical and biochemical differences such as the absence of a heme group, and it is not activated by the presence of 1 mM S-adenosylmethionine [95].

(C). Amino Acid Transporters

Metabolite transporters are not only relevant in therapy for the transport of drugs [106] but also as drug targets. Inhibition of the arginine transport by dibromopropamide and pentamidine in *Leishmania donovani* promastigotes has been shown to correlate with growth inhibition [107]. In the case of *T. cruzi*, crystal violet has been used to treat blood in blood banks due to its efficacy in killing *T. cruzi* [108]. More than forty years later, it was demonstrated that this dye inhibits methionine and proline uptake, as well as protein synthesis [109], being the first trypanocidal drug targeted against *T. cruzi* amino acid transporters.

As mentioned, amino acid transporters of *T. cruzi* are largely unknown. In fact, only a single family of genes coding for putative amino acid transporters, has been uncovered. These genes code for putative members of the AAAP family [103]. The family is formed by, at least, 60 members having identities varying from 21 to 25% at the amino acid sequence level with the mammalian homologous enzymes. It is relevant to stress that biochemical data suggest the presence of other types of permeases [23, 57]. The amino acid transporters appear to represent valid drug targets, but further research on their characterization is needed.

T. cruzi proteases are also promising targets for drug design due to their specificities, and have been recently reviewed by Cazzulo [110].

15. COMBINATORIAL SYNTHESIS, A PROMISING METHOD FOR THERAPEUTIC INTERVENTION

Advances in molecular biology in conjunction with sophisticated screening techniques have turned combinatorial library approaches into powerful tools to develop ligands and inhibitors to parasite targets that have been shown to be important for the parasite life cycle. Target molecules of therapeutic importance include parasite enzymes necessary for host cell infection such as malarial proteases, cysteine proteases of *Leishmania mexicana* and *T. cruzi*, trypanothione reductase and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in *T. brucei*, as well as phosphoribosyltransferases in *Giardia lamblia* [111 – 116]. Polyamines, cholesterol-derivatives, aryl ureas and peptides are among the polymers that have been employed in combinatorial libraries in order to identify *T. cruzi* enzyme inhibitors [115, 117, 118].

In contrast to synthetic combinatorial libraries where all processes for the selection of target binders require chemical synthesis, random oligonucleotide and peptide libraries, used in *in vitro* selection processes (SELEX and phage-display libraries) to identify target binders, can be copied and modified *in vitro* by enzymatic reactions [119].

A). The SELEX Method

The SELEX method (systematic evolution of ligands by exponential enrichment) [120, 121] is a powerful tool for the *in vitro* selection of nucleic acids capable of a desired function from combinatorial oligonucleotide libraries (DNA or RNA) with diversities up to 10^{15} different molecules, and possible secondary and tertiary structures. For this purpose a partial random synthetic DNA template is constructed containing an inner region of 15 to 75 random positions in which all four bases (A, C, G, and T) are incorporated with equal probability. The inner random sequence is flanked on both sides by constant sequences to which complementary primers are designed for PCR amplification of the DNA pool. This pool can be either directly used for selection or is first transcribed to RNA using T7 RNA polymerase. In this case, a T7 RNA promoter site is placed on the 5' site of the DNA template. The DNA or RNA pool is exposed to its target molecule and target-bound DNA/RNA molecules are displaced from the target using a specific antagonist. These selected ligands within the SELEX pool are culled and amplified using PCR (polymerase-chain reaction) and

RT (reverse-transcription) PCR procedures. The SELEX technique involves reiterative selections with increasing stringency until the enormous diversity of the previous random pool is narrowed down to a few molecules with desired binding properties. The final DNA pool is cloned into a bacterial vector and individual aptamers are identified by DNA sequencing. It is worth mentioning that aptamers identified in a selection process often bind tighter to their targets than the antagonists that were used for the displacement process.

The SELEX technology has been used extensively to isolate high-affinity ligands for a wide variety of proteins and small molecules of therapeutic importance, including growth factors [122, 123], antibodies [124], cell surface antigens and receptors [125, 126], whole cell membranes [127], anthrax spores [128] and live trypanosomes [129, 130]. These ligands have dissociation constants for their protein targets in the picomolar to micromolar range and recognize their targets with specificity comparable to monoclonal antibodies. However, unlike antibodies, aptamers are developed by an *in vitro* selection process. Therefore, aptamers can also be evolved against toxins and antigens that present low immunogenicity, or parasites which escape the host immune response. Aptamers are capable of differentiating between isoforms of proteins [131] and different conformations of the same protein [132] and can be used for specific recognition and inhibition of parasite target proteins.

B). Targeting Parasites by Aptamers

The potential utility of aptamers for target validation *in vivo* and as therapeutic agents blocking receptor-ligand interactions on cell surfaces as well as enzymatic reactions is considerably enhanced by chemical modifications that lend resistance to nuclease attack [133]. Parasite proteins that have been targeted by RNA or DNA aptamers include aptamers binding to the *T. brucei* cell surface. Homann and Göringer [134] selected fluorescence-tagged RNA aptamers against the VSG and identified a 42-kDa flagellar protein which is constitutively expressed on the parasite cell surface. As these aptamers are internalized by the parasite, it has been assumed that aptamers can be used as a “piggy-back” molecule for the selective uptake of anti-*T. brucei* drugs [134].

The experimental outline for the identification of nuclease-resistant RNA aptamers binding to cell adhesion receptors of *T. cruzi* has been reviewed [135]. Aptamers were developed that bind to the receptors of cell matrix molecules on the *T. cruzi* cell surface and recognize specifically the infectious trypomastigote form of the parasite [130]. Following eight rounds of *in vitro* selection, four classes of RNA aptamers based on structural similarities were identified which reduced invasion of LLC-MK₂ cells by *T. cruzi* trypomastigotes *in vitro*, indicating the participation of *T. cruzi* cell surface receptors for host cell matrix molecules in cell infection [130]. Although this work did not yet result in a possible therapy for *T. cruzi* infection, it is the first example of the development of aptamers counteracting cell infection by *T. cruzi*. Promising steps towards improvement of effectiveness and *in vivo* use of

aptamers lie in the improvement of aptamer kinetics that improvement of the half time of aptamers in the blood by conjugation to high molecular weight carriers such as polyethyleneglycol or liposomes.

Taking into account the high binding affinities involved, the use of the SELEX technique is promising approach to targeting key points in the life cycle of *T. cruzi*, especially targets expressed on the cell surface. The *T. cruzi* amino acid transporters specific for proline and arginine [23, 57], previously discussed, could be targeted by a pool of aptamers followed by selection of the specific aptamer through displacement by their substrates. This approach is promising in view of the fact that RNA aptamers have been successfully developed against various cell surface proteins, including parasite proteins. Thus, it would not be surprising that these aptamers would specifically block the uptake of amino acids in *T. cruzi* without having any effect on the amino acid uptake of the host. In view of their high specificity and affinity towards their targets, aptamers could be also used instead of antibodies [133] for detection of *T. cruzi* infection.

ABBREVIATIONS

-KG	=	-ketoglutarate
AAAP	=	Amino Acid/Auxin permeases
ADC	=	Arginine decarboxylase
AdoMet	=	S-adenosyl-L-methionine
Ala	=	Alanine
ALAT	=	Alanine aminotransferase
Arg	=	Arginine
Asp	=	Aspartate
EC	=	Enzyme commission
G 1,3 BP	=	Glyceraldehydes 1, 3 bisphosphate
GAPDH	=	Glyceraldehyde-3-phosphate-dehydrogenase
GDH	=	Glutamate dehydrogenase
GB	=	GenBank accession Number
Glc	=	Glucose
Gln	=	Glutamine
Glu	=	Glutamate
NMR	=	Nuclear magnetic resonance
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
OA	=	Oxaloacetate
ORF	=	Open reading frame
P5C	=	Pyrroline-5-carboxylic acid
PCR	=	Polymerase-chain reaction
PEP	=	Phosphoenol pyruvate

PEPCK	=	Phosphoenolpyruvate carboxykinase
Phe	=	Phenylalanine
Pro	=	Proline
Pyr	=	Pyruvate
RT	=	Reverse-transcription
SELEX	=	Systematic svolution of ligands by exponential enrichment
TAT	=	Tyrosine aminotransferase
TCA cycle	=	Tricarboxylic acids cycle
tcNOS	=	<i>Trypanosoma cruzi</i> nitric oxide synthase
Trp	=	Triptophan
Tyr	=	Tyrosine
VSG	=	Variant surface glycoprotein

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