RICHARD MARCEL BRUNO MOREIRA GIRARD

Role of Alanine and Alanine Racemase in the metabolism of the *Trypanosoma cruzi*

Ph.D. Thesis presented to the Post-graduation program Biology of Hos-Pathogen Interaction at the Institute of Biomedical Sciences of the University of São Paulo, in order to obtain the degree of Doctor in Sciences

Area: Biology of Host-Pathogen Interactions

Supervior: Prof. Dr. Ariel M. Silber

Original version

São Paulo
2018

RESUMO

GIRARD, RMBM. **Papel da Alanine e da Alanine Racemase no metabolismo do *Trypanosoma cruzi*.** 2017. 167 f. Tese (Doutorado em Parasitologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

Os aminoácidos participam de vários processos importantes na biologia dos tripanossomatídeos, como osmorregulação, diferenciação celular e invasão de células hospedeiras. Alguns deles fornecem poder redutor para a síntese de ATP mitocondrial. Foi anteriormente demonstrado que a alanina, que é formada principalmente pela aminação do piruvato, é um produto final metabólico quando os parasitas estão proliferando em um meio rico em glicose e aminoácidos. Mostrou-se também que este aminoácido também pode ser usado para a regulação do volume celular e resistência ao estresse osmótico. Curiosamente, o *Trypanosoma cruzi*, agente etiológico da doença de Chagas, possui genes putativos para uma Alanina racemase (AR) que catalisam a interconversão entre L e D-alanina. Aqui mostramos, pela primeira vez, que os genes putativos TcAR_A em *T. cruzi* codificam a enzima recombinante funcional (rTcAR_A) bioquímicamente caracterizada e localizada no citoplasma. A atividade da AR foi detectada através do ciclo de vida dos parasitas. Demonstramos também que a TcAR_A racemiza a serina com parâmetros cinéticos semelhantes aos da Ala. Também demonstramos que ambos isómeros da Ala podem ser transportadas e através de um sistema ativo não estereosseletivo de baixa especificidade. Ambas, L-Ala, e D-Ala, podem ser completamente oxidadas em CO_2, fornecendo elétrons para a cadeia de transporte de elétrons, sustentando OXPHOS em epimastigotas de *T. cruzi*. Como não tem sido descrita em hospedeiros mamíferos uma AR, investigamos a atividade antiparasitária das tiadiazolidinonas, uma nova classe de inibidores de AR. Todos os inibidores testados exibiram uma inibição da replicação dos epimastigotas dependente da dose. Esses compostos também inibiram a atividade da rTcAR_A. Como o inibidor C3 mostrou uma menor concentração inibitória de 50% (IC_{50}) contra o crescimento de epimastigotas, o mecanismo de ação deste fármaco foi estudado em maior detalhe. C3 induz marcas típicas de morte celular programada em *T. cruzi*. De facto, os parasitas tratados exibiram uma exposição de fosfatidilserina na fase externa da membrana celular, diminuição do potencial de membrana mitocondrial, , um aumento da concentração intracelular de ROS, e fragmentação de DNA. C3 apresentou um efeito inibitorio sobre o ciclo de infecção celular a concentrações na faixa submicromolar , com um alto índice de seletividade. Estes compostos também mostraram um interessante efeito anti-*T. brucei*, com similares características de ação. Em conjunto, nossos dados demonstram o papel versátil de Ala na bioenergética do parasita podendo ser secretado, ou transportado para o méio intracelular para ser oxidado pelos parasitas. Além disso, o catabolismo da D-Ala, através da AR, chama a atenção sobre a flexibilidade metabólica de *T. cruzi*, bem como sobre a relevância do metabolismo dos D-aminoácidos nesses organismos. Nossos resultados também mostraram que o AR pode ser um alvo promissor para a quimioterapia.

Palavaras-chave: Transporte. L-Alanina. Metabolismo. Bioenergética. *Trypanosoma cruzi*. Alanina racemase. D-Alanina.

ABSTRACT

GIRARD, RMBM. **Role of Alanine and Alanine Racemase in the metabolism of the *Trypanosoma cruzi***. 2018. 167 p. Ph.D. Thesis (Parasitology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo 2018.

Amino acids participate in several critical processes in the biology of trypanosomatids, such as osmoregulation, cell differentiation and host cell invasion. Some of them provide reducing power for mitochondrial ATP synthesis. It was previously shown that alanine, which is formed mainly by the amination of pyruvate, is a metabolic end-product when parasites are replicating in a medium rich in glucose and amino acids. It was shown as well that this amino acid can also be used for the regulation of the cell volume and the resistance to osmotic stress. Interestingly, *Trypanosoma cruzi*, the etiological agent of Chagas's disease, possess putative Alanine racemase (AR) genes that catalyze the interconversion between L- and D-alanine. Here we describe, for the first time, that the putative TcAR_A genes in *T. cruzi* encode a functional recombinant enzyme (rTcAR_A) that was biochemically characterized and localized in the cytoplasm. AR activity was detected through parasites life cycle. We demonstrated that TcAR_A, also racemize serine with similar kinetic parameters. In this work, we also show that both isomers can be taken up and through a low specificity non-stereoselective active transport system. We show that L-Ala and as well D-Ala, through it conversion to the L-isomer by TcAR_A, can be completely oxidized to CO_2, supplying electrons for OXPHOS in *T. cruzi* epimastigotes. As AR had not been described in mammalian host, we investigated the anti-*T. cruzi* activity of the thiadiazolidinones, new class of potential AR inhibitors. All, the compounds exhibited dose-dependent inhibition of epimastigote replication and also inhibited the rTcAR_A activity. As C3 had shown a lower 50% inhibitory concentration (IC_{50}) against epimastigote growth, the mechanism of action of this drug was studied in more detail. C3 induces typical programed cell death (PCD) hallmarks in *T. cruzi*. Indeed, treated parasites exhibited a phosphatidylserine exposure in the external leaflet of the plasma membrane, a decrease of the mitochondrial membrane potential, an intracellular ROS concentration deregulation, and nuclear DNA fragmentation. C3 exhibits a submicromolar range in the intracellular cell cycle infection with a high selectivity index. These compounds also induced an interesting anti-*T. brucei* with a similar mechanism of action. Taken together, our data demonstrate the versatile role for Ala in the parasite's bioenergetics being secreted as well taken up and oxidized by the parasites. Moreover, the catabolism of D-Ala, through AR, underlines the parasites outstanding parasites metabolic flexibility as well the relevance of the D-amino acids metabolism in these organisms. Our results also showed that AR could be a promising target for chemotherapy.

Keywords: Transport. L-Alanine. Metabolism. Bioenergetics. *Trypanosoma cruzi.* Alanine racemase. D-Alanine.

1. Introduction

1.1 Chagas´ disease

The American Trypanosomiasis was also named Chagas Disease in honor of its discoverer Carlos Ribeiro Justiniano Chagas. Remarkably, in the article published in a medical journal in 1909 (2), Carlos Chagas described the etiological agent as well the vector and wild reservoirs of this disease. This parasite was named *Trypanosoma cruzi* in honor of Oswaldo Cruz. Over 100 years after its discovery, the disease is estimated to affect approximately 6 million to 7 million in all american countries and has been recognized by the World Health Organization as one of the world's neglected tropical diseases (3). Chagas disease is endemic of the Americas, from the South regions of USA to the south of Argentina and Chile. Its transmission parallel largely that of the vector insect geographical distribution (Figure 1) (3). Human population movements also spread the disease in non-endemic regions such as Europa, Asia or Oceania turning Chagas disease into a global health problem (4).

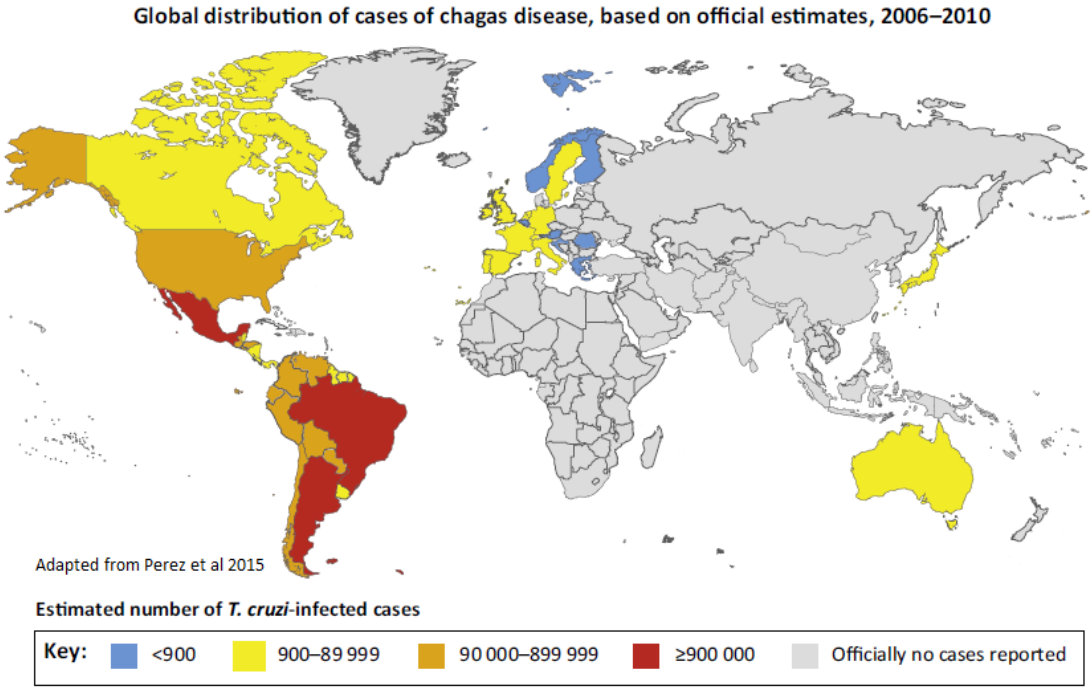

Figure 1 Global distribution of Chagas Disease. Data from World Health Organization (3).

Classically, Chagas disease is transmitted to humans through the contact of contaminated faeces from the vector insect (bugs from the *Reduviidae family*) with the mucosa or injuried skin, (5). In addition to insect transmission, Chagas disease can be transmited by ingestion of food or drinks contaminated with the vector feces containing *T. cruzi* oral or by the ingestion of raw meat from infected sylvatic reservoir (5). Alternatively, the transmission can occur by transfusions, transplants, laboratory accident and congenital mechanisms (5).

Chagas disease presents an acute and a chronic phase. In the acute phase most patients are asymptomatic. However, when symptoms are present, usually they are unspecific such as fever, headache, muscle or abdominal pain, difficulty in breathing, inflammation at the inoculation site (inoculation chancre) or unilateral palpebral oedema (Romaña sign). The acute phase lasts about lasts about 2 months after infection. In the chronic phase, two main forms of

the disease are distinguished: indeterminate (about 60–70% of the patient) and determinate or symptomatic (about 30–40% of the patient). The symptomatic form of the disease is in turn subdivided into three main forms: cardiac, digestive or cardiodigestive forms. During the indeterminate form, the patients are positive to serological tests with undetectable parasitemia; No signs and symptoms of the disease can be detected. The determinate phase is generally suspected from clinical findings, mainly in the cardiac and digestive systems, and diagnosis should be confirmed by the results of laboratory tests (3-7). The chronic phase persists for the entire host's lifes and the determinate form of chronic disease usually appears 10–30 years after the initial infection (6).

Benznidazole and nifurtimox are the only drugs with proven efficacy against Chagas disease (5, 7). Both drugs, despite various side effects, are effective during the acute phase, however their efficiencies during the chronic phase, when most cases are diagnosed, remain controversial (6, 7). These facts underline the urgent need to intensify the search of therapeutics targets against *T. cruzi*. In this sense, it is relevant to study in detail the unique features of the *T. cruzi* biology. Studies on metabolism, drugs resistance, action of new class of compounds and other general biological functions of *T. cruzi* helped to understand and to identify potential compounds against *T. cruzi*-specific targets (8-14). These finding allowed proposing new therapeutic strategies such as drug repositioning or new compounds which could result in an adequate treatment for the acute and chronic forms of Chagas disease.

1.2 Biology of *T. cruzi:* a brief revision

1.2.1 *T. cruzi* organelles linked to the energy metabolism

T. cruzi possess unique structures/organelles, characteristic of the Trypanosomatidae family (Figure 2). Herein, we briefly described some of them that are relevant to understand its metabolism.

T. cruzi possess a unique and branched **mitochondrion** which has the classical compartments: the outer and inner membrane and between them the inter membrane space and the mitochondrial matrix. The mitochondrial inner membrane forms the typical invaginations known as mitochondrial cristae (Figure 2). Studies on mitochondrial functions in *T. cruzi*, indicate that this organelle shares similar bioenergetics properties with its mammalian counterpart. Indeed, several biochemical parameters were analyzed such as respiration rates at different energy states oxidizing different metabolites, mitochondrial membrane potential, as well ions fluxes and the redox state (12, 15)(12, 15)(12, 15)(12, 15)(12, 15)(12, 15). Regarding the electron transfer system (ETS), the presence of complex II to V, in the inner membrane of the mitochondrion, has been demonstrated (Figure 3). (15, 16). In spite of the presence of a variable quantity of subunits of complex I in the ETS, there is a controversy on the contribution of complex I to energy metabolism in *T. cruzi* (Figure 2). Indeed, *T. cruzi* complex I appears to be insensitive to rotenone and *T. cruzi* naturally mutant strains for some complex I subunits did not present OxPhos alterations (12, 16-20). This facts suggest that NAD^+ regeneration occurs through a fumarate reductase (21).

Inside the mitochondrial matrix, **the kinetoplast** is **a structure containing the mitochondrial genome, and** situated in the region proximal to the nucleus. The kinetoplast is

made of kinetoplastid DNA (kDNA) composed by two type of interlocked and concatenated circular DNA: the minicircles and the maxicircles. There are several thousand minicircles (0.5 to 2.5 kb), and a few dozen maxicircles (20 to 40 kb) (22). The kDNA is a remarkable complex feature of the Trypanosomatidae family, which needs to be replicated and then equally segregated between daughter cells along the parasites life cycle. The kDNA replication is independent from the nucleus and possess a complex machinery to perform this process (22). Another special characteristic is that it presents approximately 60 % of AT-rich regions (23). These unique characteristics of kDNA has been a widely studied for potential chemotherapeutic target.

Glycosomes are an unusual type of peroxisomes which contain most of the glycolytic enzymes. This compartmentalization is a hallmark of the kinetoplastids since in most organisms this metabolic pathway is essentially cytosolic. The first six enzymes involved in the glycolytic pathway are compartmentalized within the glycosome (24). Another peculiarity is the lack the typical allosteric regulation presents in hexokinase (HK) and phosphofructokinase (PFK), which, classically tightly regulate the glycolytic pathway in many organisms (25-28). Furthermore, glycolysis only function properly with an individual pool of cofactors (NAD(H), ADP and ATP) separated from the cytosolic pool. Interestingly, the number and/or the compositions of glycosomes vary from species to species and even among developmental stages of the same species. In addition, other metabolic pathways occur within this organelle such as: purine salvage and *de novo* pyrimidine biosynthesis, fatty acid elongation, isoprenoid biosynthesis, and sterol biosynthesis (29, 30).

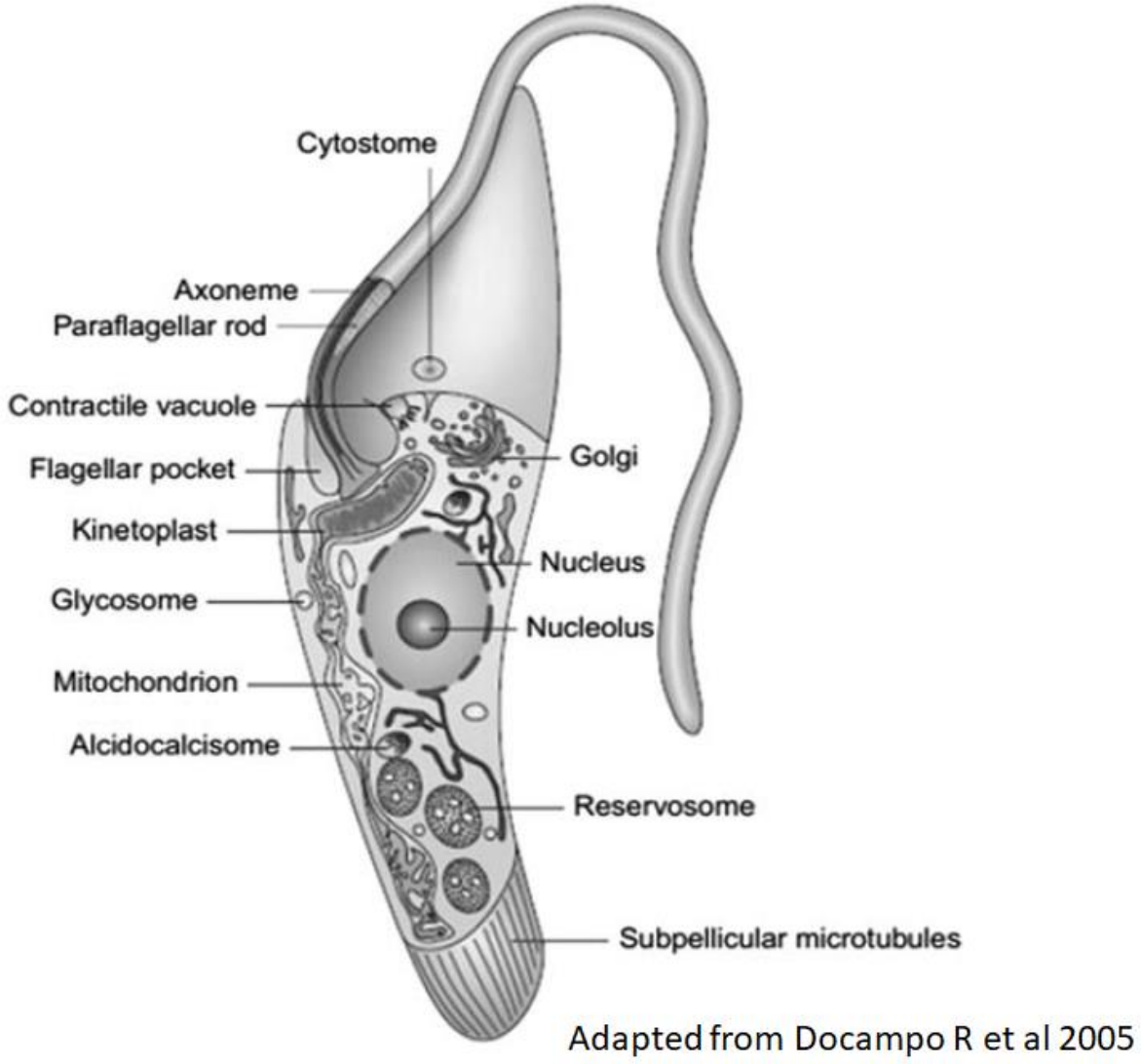

Figure 2 Schematic representation of longitudinal section of an epimastigote showing the main structures and organelles found in *T. cruzi.*

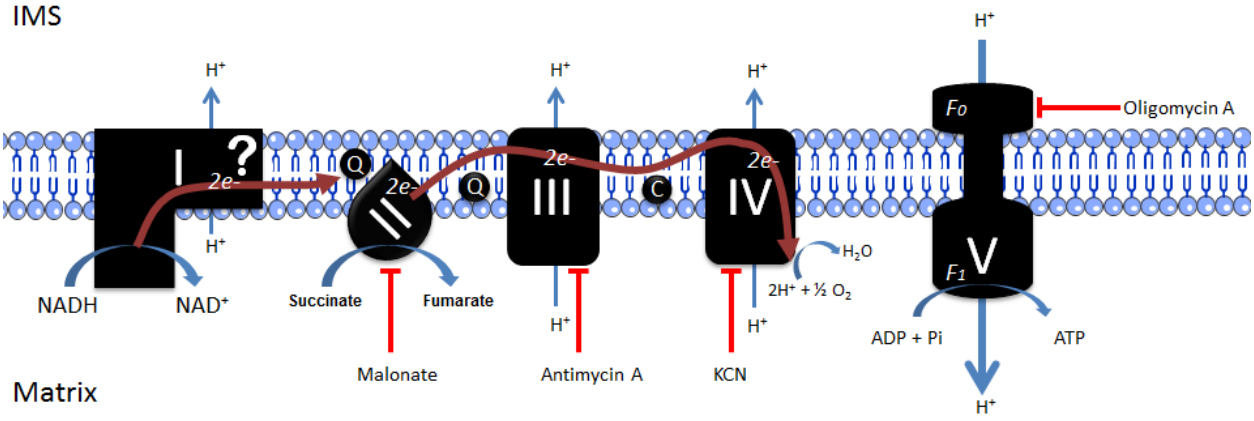

Figure 3 Schematic representation of the electron transport chain in *T. cruzi*. The major complexes of the electron transport system are present in the inner mitochondrial membrane, and some enzymes/intermediates are generated in the inter membrane space (IMS) or mitochondrial matrix. Q: ubiquinone; C: cytochrome c. The F_0-F_1 ATP synthase is responsible for ADP phosphorylation resulting from the proton-motive force generated through an electrochemical gradient.

1.2.2 *T. cruzi* life cycle

This protist affront a myriad of environmental conditions during its complex life cycle, which occurs inside the entire digestive tube of triatomine insect vectors, the blood of more than 100 species of mammals, and the cytoplasm of (potentially) every mammalian nucleated cell in every tissue and organ (31). Inside the insects, parasites proliferate in the gut as non-infective forms, named epimastigotes, and later develop into infective non-replicating metacyclic trypomastigotes (32). During the insect bloodmeal, the metacyclic forms are expelled together with the insect feces and potentially being in contact with the skin of the mammalian host. The metacyclic forms eventually enter into the host through mucous or the abrasion caused by the host when scratching after being bitted (33). Once inside the host, the metacyclic trypomastigotes must invade the host-cells, differentiate into intracellular amastigotes and reach the cytoplasm to initiate the cell proliferation (34). After several rounds of binary divisions, amastigotes, differentiate to trypomastigotes forms passing through a transient replicative form

called intracellular epimastigotes (35, 36). Trypomastigotes lyse the infected cells and burst into the extracellular environment. Trypomastigote forms can infect neighboring cells or eventually reach the bloodstream, from where they can infect cells in remote tissues or infect an uninfected Triatomine bug during its bloodmeal (Figure 4) (37-39). As a consequence of its complex life cycle, *T. cruzi* faces different environments with markedly different biochemical characteristics and availability of nutrients.

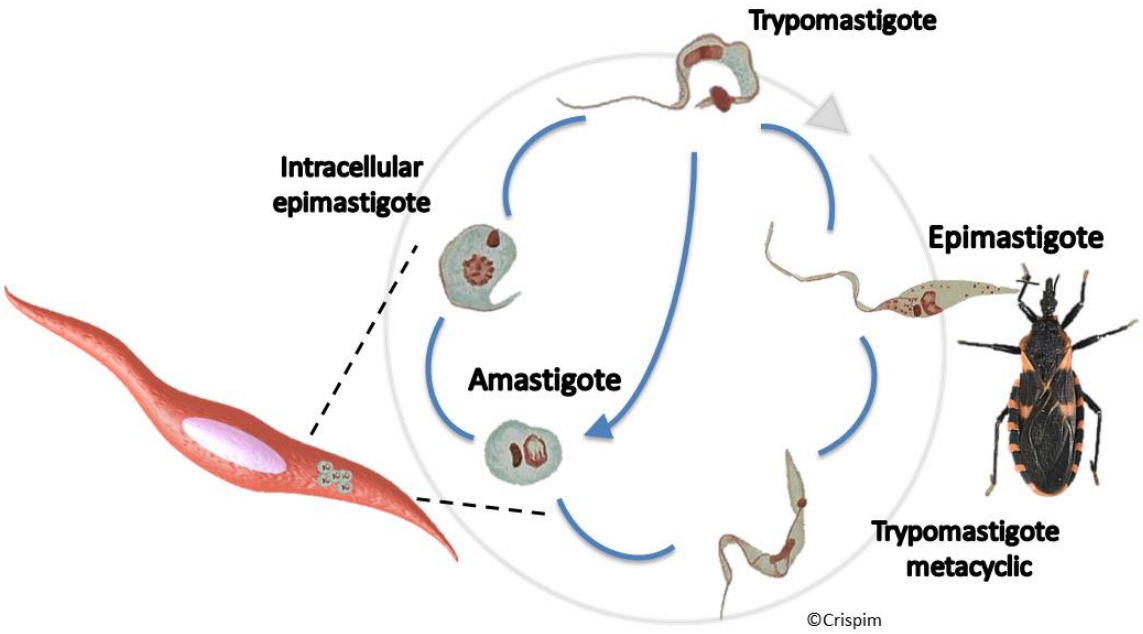

Figure 4 Schematic representation of the *Trypanosoma cruzi* life cycle. Replicative, non-infective epimastigotes forms, differentiate to the non-replicative, infective metacyclic trypomastigotes inside the insect gut. Metacyclic forms invade the mammalian host cells and differentiate into replicative amastigote forms to establish the infection. These forms give rise to a transient stage called intracellular epimastigotes, which differentiate into trypomastigotes. After cells lysis, trypomastigotes can disseminate in the mammalian host through the bloodstream. The ingested trypomastigotes, by uninfected insect vector during its bloodmeal, differentiate again into epimastigotes, which can colonize the insect digestive tract.

1.2.3 The journey of *T. cruzi* inside the insect vector

T. cruzi infection occurs essentially in the intestinal tract of the vector insect. The insect digestive system is composed by the anterior midgut (stomach, AM), the posterior midgut (PM) and the rectum. Classically, the blood is hemolysed in the AM, then the nutrients are digested in the PM, finally the rectum stores the remains of the digestion process. Bloodstream trypomastigotes enter first in the AM where they face to inhospitable environment where parasite population decreases dramatically (40). The remaining trypomastigotes quickly reach the PM to differentiate in epimastigote and sustain the infection. *T. cruzi* preferentially growth in the insect rectum (41, 42), however it also can be found in the other intestinal region depending of the infection process. Nevertheless, parasites, through the digestive system, have to rapidly adapt to tremendous stress in theses environments such as temperature and pH variation, osmotic pressure, nutritional deprivation, and sudden interaction with insect immune defence and microbiota system. Temperature variation has been shown to affect the parasites infection *in vitro* as well *in vivo* (43, 44) because the temperature modifies several biochemical processes such as molecular transport, enzyme activity but also the vector feeding pattern (33). During the triatominae feeding process the excreta pH exhibits change of almost 3 fold from an acidic to an alkaline pH, associated with an increase in osmolality in the same proportion (45). *T. cruzi* has to endure striking period of starvation as its vector, the triatominae, can affront fasting periods up to one years (45). Throughout the insect vector starvation process, the parasites show an astonishing resilience to the insect nutritional deprivation condition (42, 45, 46). Indeed, the *T. cruzi* colonization pattern remains unaltered up to four month and living parasites can be find 200days after the last blood meal in triatonimae rectum (41). Furthermore, *T. cruzi* must confront to the insect immune system which produces several molecules such as lysozymes (47),

defensins or nitric oxide (32). Additionally, an increase number of studies indicate an intimate interplay between *T. cruzi* and the triatomine microbiota (48). The gut microbiota can have an impact in the parasite development and growth (32, 40, 44). For example, *Serratia marcescens* can impair the *T. cruzi* infection (49). On the other hand, *T. cruzi* successful infection also affects microbiota composition, with an increase of it diversity (48) while other failed to show a clear correlation between triatomine, *T. cruzi* and microbiota (44, 50). The complex interactions and fitness mechanisms among the parasites, the insect and it microbiota remains largely unsolved as this interplay appears to be specific to parasites strains and invertebrates species and/or population (50, 51). Taken together, *T. cruzi* have to exhibit outstanding adaptation mechanisms to establish a successful infection along it journey inside the insect.

1.3 *T. cruzi* metabolism

1.3.1 Carbohydrate metabolism in *T. cruzi*

The ATP synthesis in *T. cruzi* can occur at substrate level phosphorylation through the glycolytic pathway and through oxidative phosphorylation. Trypanomosatids have a high rate of glucose incorporation and consumption which is associated with the production and excretion of reduced compounds from glucose catabolism instead of its complete oxidation. This metabolic particularity occurs even under aerobic conditions and is called “aerobic fermentation of glucose” (52). In the case of *T. cruzi*, the major products of aerobic glucose catabolism by epimastigotes are the alanine (Ala) and to a lesser extent succinate (53-55). Ala production might be linked to reoxidation of glycolytically produced NADH, even under aerobic conditions (55-57). Furthermore, trypanosomatids did not present Pasteur effect (a considerable decrease in

glucose consumption in aerobiosis) which is related to the lack of major controls on the glycolysis as described above.

1.3.2 Amino acids (AA) metabolism

Beyond the carbohydrates consumption, early works showed that several AA are metabolized by *T. cruzi* (58). Classically AA should be oxidized through their conversion to glutamate or aspartate, and then be processed via the Krebs cycle into the mitochondrion. Since, various studies appoint that AA transport and metabolism are relevant processes to supply the derived metabolites to many biological processes in *T. cruzi* (11, 12, 14, 59). These AA could be produced by proteolysis of proteins, biosynthesized from metabolic precursors or to be taken up from the extracellular medium (14).

1.3.2.1 AA Uptake

The metabolite incorporation is the first essential step for various metabolic routes, as it allows the entry of essential nutrients into the cell and regulate it intracellular concentrations. In the case of the AA, their availability and metabolism results from an equilibrium among protein degradation, their uptake and their biosynthesis. Since AA participate in broad variety of metabolic pathways, they are important for *T. cruzi* survival (14). As AA are available in most of environments that *T. cruzi* encounters throughout its life cycle, the uptake of various AA has been biochemically characterized. Briefly, two systems for Arg and for Pro (59-62), one for Glu (63), one for Lys (64, 65), one for Cys (66), one for Asp (67), one for the branched-chain AAs (BCAA) Val Ile and Leu (68), one for His (69), one for Gln (70) and one for GABA (71) has been already biochemically characterized (Table 6). Most of these processes has been associated to members of the AAAP (AA/Auxin Permease), a family of transporters grouping H^+/AAs and

auxin permeases (72-75). Some transport system has been associated to specific transport functions in *T. cruzi* (76-79). The gene TcAAP7 was identified, through heterologous expression and overexpression in epimastigotes, to encode for the Lys transport in *T. cruzi* (64, 65). Furthermore, the Arg high-affinity as well as the non stereopecific Pro transport system has also been associated to the genes encoding for TcAAAP411/TcAAP3 and TcAAP24 respectively (76-79).

1.3.2.2 AA as energy source

First evidence of AA consumption was reported by Chang et al who observed an increase of pH at the end of epimastigotes exponential growth. The alkalization of the culture medium was due to the excretion of significant amounts of NH_4^+, a hallmark of AA degradation. This fact was confirmed by other authors, whose studies showed that asparagine, glutamine, aspartate, glutamate, leucine, isoleucine, and proline can be metabolized by *T. cruzi* (11, 12, 25, 58, 69, 80-84) . More recently, Pro degradative pathway has been characterized in detail and it was shown that it is able to sustain energetically the *T. cruzi* growth and host cells infection process (82, 83). His can be completely oxidized by *T. cruzi* powering ATP production through the oxidative phosphorylation (69). It has been shown that His is relevant in the epimastigote bioenergetics and persistence within the invertebrate host (69). Arg has been proposed to be involved in the management of the energetic metabolism through an arginine kinase (ArgK) (EC 2.7.3.3). The phosphorylation of Arg to phospho-Arg (P-Arg) using ATP would act as an "energetic reservoir", similar to phosphocreatine in mammalian, restoring ATP levels when it is needed (61).

1.3.2.3 Alanine metabolism in *T. cruzi*

As described above, Ala (together with succinate), is one of the main end-products from glucose degradation by epimastigote (55). It has been proposed that Ala production could be necessary to reoxidate the NADH glycolytically produced, even under aerobic conditions (55-57). Ala is at the same time the main intracellular and secreted AA which appears be produced separately and kept compartmentalized (54). Ala possess a central role in the AA catabolism, through its participation in the Glu conversion into α-KG. This reaction can be considered a the main gates to entry the TCA cycle for several AA that are converted into Glu to be fully oxidized, such as His, Gln, Pro, degradation via the Krebs cycle (Figure5). Indeed, the Glu dehydrogenase can convert Glu into α-KG producing NH_4^+ or alternatively, the -NH_2 group from Glu can be transferred to pyruvate by aminotransferase, such as Ala (ALAT) or a Tyr aminotransferase (TAT), producing Ala (Figure 5). The significant amounts of NH_4^+ produced (25, 53, 80), from the AA catabolism (11, 12, 25, 58, 69, 80-84) needs to be detoxified. A main detoxifying pathway fr this excretion product is the urea cycle. However, *T. cruzi* lacks it (85, 86). In thise sense, the parasite requires a specific metabolic network to address NH_4^+ accumulation. Recent works showed that NH_4^+ storage in acidic compartments (reservosome and lysosomes) by intracellular NH_4^+ transporter (87) as well as its condensation with Glu by the glutamine synthetase (88) are essential for NH_4^+ detoxification and participate of various *T. cruzi* biological functions. The deficiency of the intracellular NH_4^+ transporter leads to alterations in the replication, differentiation and respond to starvation and osmotic stress (87). The glutamine synthetase inhibition impairs the epimastigote replication under excess of ammonium and the establishment of the host-cell infection (88). In this context, it is important to underline that as α-KG-Glu interconversion is reversible, the concerted action of a NAD-linked Glu dehydrogenase, and aminotransferases should contribute as well with the detoxification of the excess of NH_4^+

produced by the AA consumption. Taken together, the robust transamination system, yielding to accumulation of Ala instead of NH_4^+ (55), indicates the central role of Ala in the nitrogen detoxification. Furthermore, early studies suggested that, despite of being an end-product of the metabolism, Ala could be metabolized by *T. cruzi* since it was able to trigger O_2 consumption (58). Indeed, depending on the relative quantity of substrates and products, Ala could be re-converted into pyruvate by the same aminotransferases or Ala dehydrogenases that produce it (57, 89). In addition, the influx and efflux of Ala has been shown to participate in the response to osmotic stress (90-92). The osmoregulation process is present in all *T. cruzi* stages and is complete about 5 min (92). The AA efflux, where Ala is the principal AA, responsible for approximately 50% of the regulatory volume response.

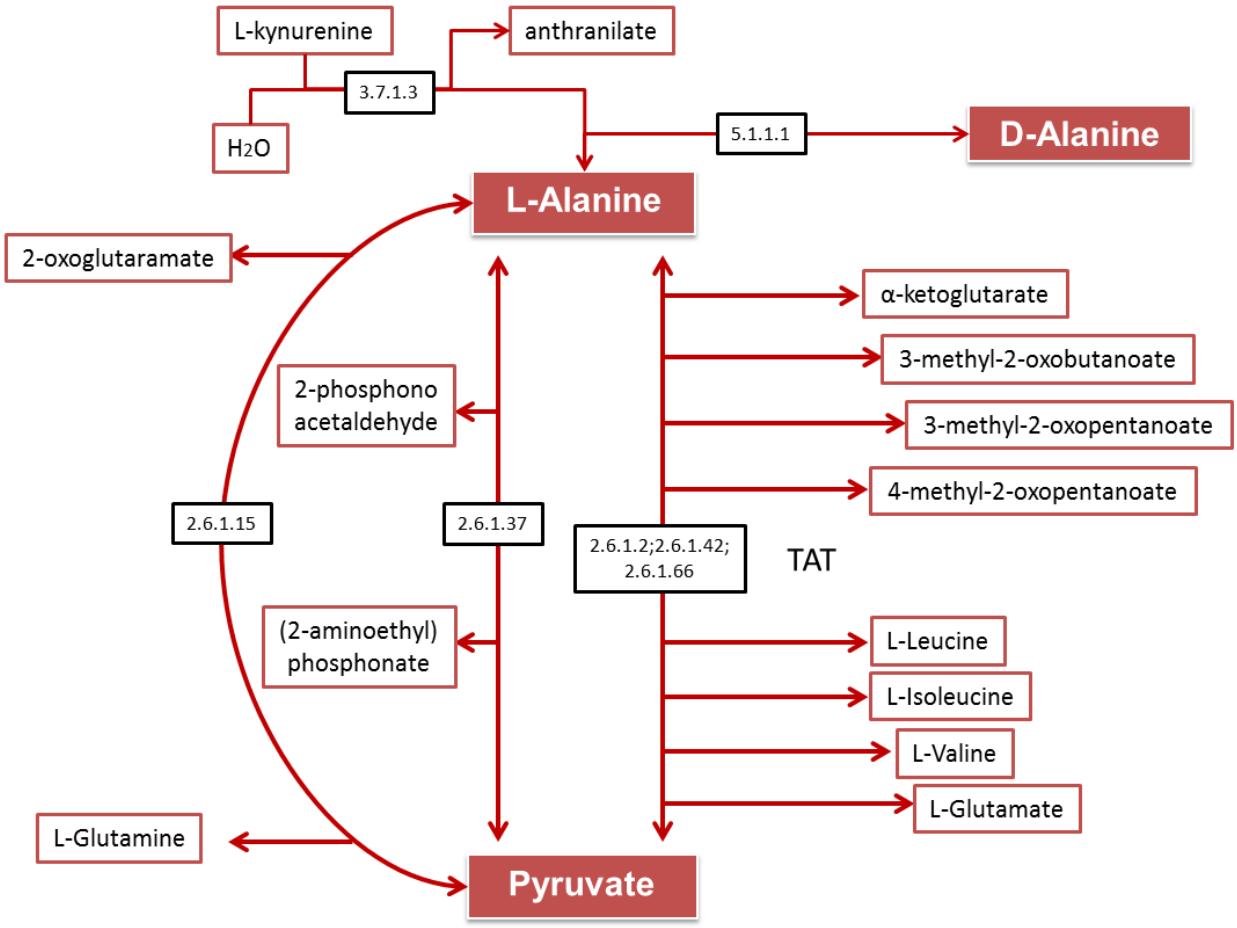

Figure 5 Schematic representation of the Alanine metabolism.. ECs numbers correspond to: 3.7.1.3: kynureninase, 5.1.1.1: alanine racemase, 2.6.1.15: glutamine-pyruvate transaminase, 2.6.1.37: 2-aminoethylphosphonate-pyruvate transaminase, 2.6.1.2: alanine transaminase and tyrosine transaminase (TAT), 2.6.1.42/2.6.1.66: TAT.

1.3.2.4 D-AA metabolism

D-AAs (D-AA) functions remains little studied. The AA α-carbon which is connected to an amine group (-NH2), a carboxyl group (-COOH), a hydrogen (75) and a side chain (-R) is a stereocenter. Effectively, depending on the spatial arrangement of these four different groups, it exist two stereoisomers: the levorotatory (L) and the dextrorotatory (D) which are not superimposable mirror images. Living organisms principally employ L-AA as proteins building block. Several hypotheses have addressed the asymmetry origin of the biomolecules. The abiotic hypothesis suggests that the molecular symmetry may have been broken, through physical induction, before the appearance of life. On the other hand, biotic origin hypothesis suggest biomolecules symmetric selection based on some biological advantage (93). Ultimately, the asymmetric origin of life remains open. Nevertheless, D-AAs are key constituents of the bacterial peptidoglycan (PG) (94). Indeed, D-AAs presence within PG confer resistance to proteolysis, additionally it presence has been associated to resistance to antimicrobial agents (95, 96). D-AA also possess critical roles in various other bacterial physiological processes, such as biofilm formation, peptidoglycan remodeling, energy catabolism, small peptides production and sporulation (97-101). D-AA has been considered biologically irrelevant in higher eukaryote for a long time. However, recent studies elucidated the presence of various D-AAs, such as D-Ser, D-Asp or D-Ala and appoint a tightly regulated D-AA metabolism in the nervous system but also in the mammalian endocrine system (102, 103). For example, D-Ser and D-Asp and it respective

racemases were shown to have a central role in mammalian brain neurotransmitters system (104, 105).

It is believed that D-AAs are principally originated in nature from L-AA racemization by AA racemases (106). A large broad of racemases and epimerases has been cataloged and characterized in the bacteria and fungi kingdom. Briefly, some racemases classically use, with some exceptions, the pyridoxal phosphate (PLP) as cofactor such as the serine racemase (107), alanine racemase (AR), and aspartate racemase (AsR), while glutamate racemase (GR) and proline racemase (PR) are generally PLP-independent. Regardless of the type of racemase, the AA α-proton is removed then reprotonated on the same or the opposite side of the Cα (108). This reaction is reversible and works in both directions: L to D and D to L. PR is, until now, the first and only biochemically characterized racemase in *T. cruzi* (109, 110). Two PR isoforms are present in all life cycle stages, and are involved in parasites differentiation and infections as well as acting as a B-cell mitogen (110, 111). The Ala racemase (AR) (EC 5.1.1.1) catalyzes the interconversion of L-Ala and D-Ala, the bacterial AR is classically a PLP dependent enzymes (Figure 5) (108). AR is a key enzyme in bacteria to generate D-Ala for the biosynthesis of PG. Indeed, the AR absence could be lethal in absence of exogenous D-Ala in bacteria (97, 112, 113). Other studies appoint that prokaryotic ARs are also implicated in regulating spore germination regulation (101, 114) and D-Ala catabolism (115, 116). AR has been also found in some eukaryotic microorganisms associated with the formation nonribosomal peptide such as cyclosporin A (117) or HC-toxin (118) or as well D-Ala catabolism (119). Furthermore, AR was also found in aquatic animals (120-124). Various eukaryotic ARs from aquatic invertebrate were characterized (120, 124-126) and it has been hypothesized that AR is involved in the osmotic regulation (127). Regarding kinetoplastids, AR activity was only reported in *Leishmania*

amazonensis associated with D-Ala release and a possible role in response to hypotonic stress (128). Interestingly, significant amount of free D-AA and as well D-AA-bearing peptides were found in the *T. cruzi* insect forms (129). These evidences stress the existence and the relevance of D-AA metabolism in *T. cruzi*. Putative AR have been found in *T. cruzi* genome, appointing the relevance of the D-AAs metabolism (11, 14).

5. Conclusions

- Beyond being a main end-product of the metabolism of glucose and AAs, L-Ala can:

  - ✓ Be taken up by the cells through a low specificity non-stereoselective active transport system with a main driving force being a transmembrane H^+ gradient, most likely created by a plasma membrane located proton-pumping ATPase
  - ✓ Be oxidized with production of CO_2, triggering O_2 consumption, contributing to the maintenance of the inner mitochondrial membrane potential and powering ATP production through the oxidative phosphorylation.

- Here we describe, for the first time, that the putative TcAR_A genes in *T. cruzi* encode at least a functional recombinant enzyme (rTcAR_A):

  - ✓ rTcAR_A biochemical parameters were determined, being the enzyme able to perform its activity across in a large spectrum of pH and temperature. Furthermore, TcAR_A also racemize Ser with similar kinetic parameters when compared to Ala racemization.
  - ✓ AR activity was detected through all the parasites life cycle stages and TcAR_A is localized in the cytoplasm.

-D-Ala :

  - ✓ Is likely transported by the same transport system of L-Ala.
  - ✓ Can be catabolized, through its conversion to L-Ala by TcAR_A, producing CO_2 and used for ATP production by OxPhos to feed the energy metabolism

- Thiadiazolidinones compounds a new class of AR inhibitors:

  - ✓ Inhibited TcAR_A activity.

✓ Presented promising anti-parasitic activity, inducing the PCD features in *T. cruzi* and *T. brucei*.

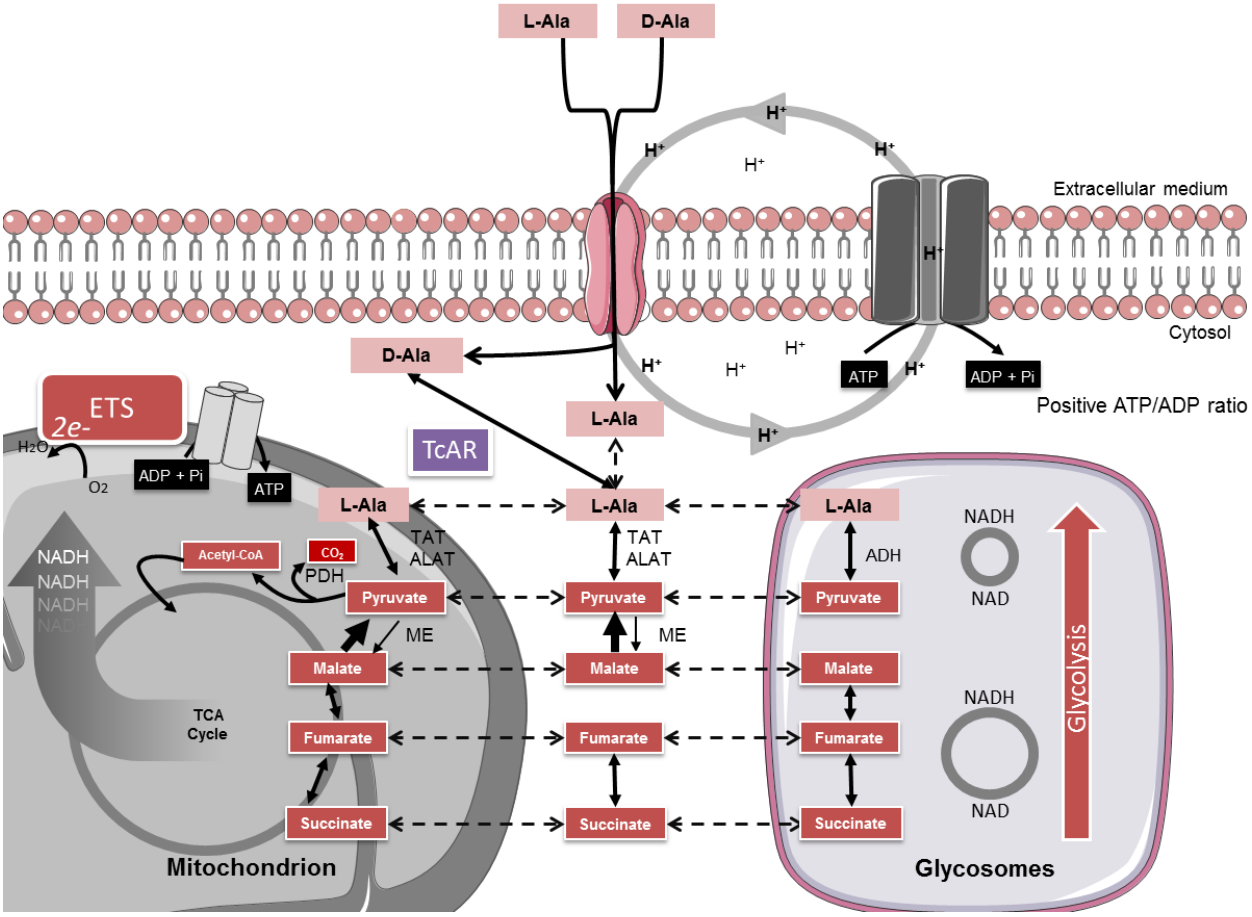

Figure 38 Schematic proposal for the uptake and catabolism of L-Ala and D-Ala in *T. cruzi*. The glycosomal and mitochondrial compartments and the TCA cycle are indicated. The metabolic flux at each enzymatic step is represented by arrows of different thicknesses. Dashed arrows indicate the intracellular shuttle of the molecules between different compartments. ETC: Electron Transport Chain; TAT: tyrosine aminotransferase; ALAT: Ala aminotransferase; ADH: Ala dehydrogenase; ME: malic enzyme; PDH: pyruvate dehydrogenase; TcAR_A Alanine racecemase.

REFERENCES

1. Arias CA, Weisner J, Blackburn JM, Reynolds PE. Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in Enterococcus gallinarum BM4174. Microbiology. 2000 Jul;146 (Pt 7):1727-34. PubMed PMID: 10878136.
2. Chagas C. Nova tripanozomiaze humana: estudos sobre a morfolojia e o ciclo evolutivo do Schizotrypanum cruzi n. gen., n. sp., ajente etiolojico de nova entidade morbida do homem. Mem Inst Oswaldo Cruz. 1909;1:159-218.
3. WHO. Chagas Disease (American trypanosomiasis) 2017. Available from: http://www.who.int/mediacentre/factsheets/fs340/en/.
4. Perez-Molina JA, Perez AM, Norman FF, Monge-Maillo B, Lopez-Velez R. Old and new challenges in Chagas disease. Lancet Infect Dis. 2015 Nov;15(11):1347-56. PubMed PMID: 26231478.
5. Perez-Molina JA, Molina I. Chagas disease. Lancet. 2018 Jan 6;391(10115):82-94. PubMed PMID: 28673423.
6. Bern C. Chagas' Disease. N Engl J Med. 2015 Nov 5;373(19):1882. PubMed PMID: 26535522.
7. Sales Junior PA, Molina I, Fonseca Murta SM, Sanchez-Montalva A, Salvador F, Correa-Oliveira R, et al. Experimental and Clinical Treatment of Chagas Disease: A Review. Am J Trop Med Hyg. 2017 Nov;97(5):1289-303. PubMed PMID: 29016289. Pubmed Central PMCID: 5817734.
8. Moyersoen J, Choe J, Fan E, Hol WG, Michels PA. Biogenesis of peroxisomes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. FEMS Microbiol Rev. 2004 Nov;28(5):603-43. PubMed PMID: 15539076.
9. Barros-Alvarez X, Gualdron-Lopez M, Acosta H, Caceres AJ, Graminha MA, Michels PA, et al. Glycosomal targets for anti-trypanosomatid drug discovery. Curr Med Chem. 2014;21(15):1679-706. PubMed PMID: 23834165.
10. Girard RM, Crispim M, Stolic I, Damasceno FS, Santos da Silva M, Pral EM, et al. An Aromatic Diamidine That Targets Kinetoplast DNA, Impairs the Cell Cycle in Trypanosoma cruzi, and Diminishes Trypomastigote Release from Infected Mammalian Host Cells.

Antimicrob Agents Chemother. 2016 Oct;60(10):5867-77. PubMed PMID: 27431229. Pubmed Central PMCID: 5038236.

11. Silber AM, Colli W, Ulrich H, Alves MJ, Pereira CA. Amino acid metabolic routes in *Trypanosoma cruzi*: possible therapeutic targets against Chagas' disease. Curr Drug Targets Infect Disord. 2005 Mar;5(1):53-64. PubMed PMID: 15777198.

12. Paes LS, Mantilla BS, Barison MJ, Wrenger C, Silber AM. The uniqueness of the *Trypanosoma cruzi* mitochondrion: opportunities to identify new drug target for the treatment of Chagas disease. Curr Pharm Des. 2011;17(20):2074-99. PubMed PMID: 21718252.

13. Damasceno FS, Barison MJ, Pral EM, Paes LS, Silber AM. Memantine, an antagonist of the NMDA glutamate receptor, affects cell proliferation, differentiation and the intracellular cycle and induces apoptosis in Trypanosoma cruzi. PLoS Negl Trop Dis. 2014 Feb;8(2):e2717. PubMed PMID: 24587468. Pubmed Central PMCID: 3937314.

14. Marchese L, Nascimento J, Damasceno F, Bringaud F, Michels P, Silber A. The uptake and metabolism of amino acids, and their unique role in the biology of pathogenic trypanosomatids. Pathogens. 2018;7(2):36. PubMed PMID: doi:10.3390/pathogens7020036.

15. Vercesi AE, Bernardes CF, Hoffmann ME, Gadelha FR, Docampo R. Digitonin permeabilization does not affect mitochondrial function and allows the determination of the mitochondrial membrane potential of *Trypanosoma cruzi* in situ. J Biol Chem. 1991 Aug 5;266(22):14431-4. PubMed PMID: 1860850.

16. Opperdoes FR, Michels PA. Complex I of Trypanosomatidae: does it exist? Trends Parasitol. 2008 Jul;24(7):310-7. PubMed PMID: 18534909.

17. Carranza JC, Kowaltowski AJ, Mendonca MA, de Oliveira TC, Gadelha FR, Zingales B. Mitochondrial bioenergetics and redox state are unaltered in *Trypanosoma cruzi* isolates with compromised mitochondrial complex I subunit genes. J Bioenerg Biomembr. 2009 Jun;41(3):299-308. PubMed PMID: 19618257.

18. Westenberger SJ, Cerqueira GC, El-Sayed NM, Zingales B, Campbell DA, Sturm NR. *Trypanosoma cruzi* mitochondrial maxicircles display species- and strain-specific variation and a conserved element in the non-coding region. BMC Genomics. 2006 Mar 22;7:60. PubMed PMID: 16553959. Pubmed Central PMCID: 1559615.

19. Denicola-Seoane A, Rubbo H, Prodanov E, Turrens JF. Succinate-dependent metabolism in *Trypanosoma cruzi* epimastigotes. Mol Biochem Parasitol. 1992 Aug;54(1):43-50. PubMed PMID: 1518531.

20. Hernandez FR, Turrens JF. Rotenone at high concentrations inhibits NADH-fumarate reductase and the mitochondrial respiratory chain of *Trypanosoma brucei* and *T. cruzi*. Mol Biochem Parasitol. 1998 May 15;93(1):135-7. PubMed PMID: 9662035.

21. Boveris A, Hertig CM, Turrens JF. Fumarate reductase and other mitochondrial activities in *Trypanosoma cruzi*. Mol Biochem Parasitol. 1986 May;19(2):163-9. PubMed PMID: 3523239.

22. Jensen RE, Englund PT. Network news: the replication of kinetoplast DNA. Annu Rev Microbiol. 2012;66:473-91. PubMed PMID: 22994497.

23. Brack C, Delain E, Riou G. Replicating, convalently closed, circular DNA from kinetoplasts of Trypanosoma cruzi. Proceedings of the National Academy of Sciences of the United States of America. 1972 Jun;69(6):1642-6. PubMed PMID: 4556467. Pubmed Central PMCID: 426766.

24. Opperdoes FR, Borst P. Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome. FEBS Lett. 1977 Aug 15;80(2):360-4. PubMed PMID: 142663.

25. Urbina JA, Azavache V. Regulation of energy metabolism in *Trypanosoma (Schizotrypanum) cruzi* epimastigotes. II. NAD^+-dependent glutamate dehydrogenase. Mol Biochem Parasitol. 1984 Apr;11:241-55. PubMed PMID: 6379448.

26. Urbina JA, Crespo A. Regulation of energy metabolism in *Trypanosoma (Schizotrypanum) cruzi epimastigotes*. I. Hexokinase and phosphofructokinase. Mol Biochem Parasitol. 1984 Apr;11:225-39. PubMed PMID: 6235452.

27. Aguilar Z, Urbina JA. The phosphofructokinase of *Trypanosoma (Schizotrypanum) cruzi:* purification and kinetic mechanism. Mol Biochem Parasitol. 1986 Nov;21(2):103-11. PubMed PMID: 2946951.

28. Urbina JA, Ysern X, Mildvan AS. Involvement of a divalent cation in the binding of fructose 6-phosphate to *Trypanosoma cruzi* phosphofructokinase: kinetic and magnetic resonance studies. Arch Biochem Biophys. 1990 Apr;278(1):187-94. PubMed PMID: 2138869.

29. Michels PA, Bringaud F, Herman M, Hannaert V. Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta. 2006 Dec;1763(12):1463-77. PubMed PMID: 17023066.

30. Haanstra JR, Gonzalez-Marcano EB, Gualdron-Lopez M, Michels PA. Biogenesis, maintenance and dynamics of glycosomes in trypanosomatid parasites. Biochim Biophys Acta. 2016 May;1863(5):1038-48. PubMed PMID: 26384872.

31. Fernandes MC, Andrews NW. Host cell invasion by *Trypanosoma cruzi*: a unique strategy that promotes persistence. FEMS Microbiol Rev. 2012 May;36(3):734-47. PubMed PMID: 22339763. Pubmed Central PMCID: 3319478.

32. Guarneri AA, Lorenzo MG. Triatomine physiology in the context of trypanosome infection. J Insect Physiol. 2017 Feb - Mar;97:66-76. PubMed PMID: 27401496.

33. Catala S, Crocco LB, Morales GF. Trypanosoma cruzi transmission risk index (TcTRI): an entomological indicator of Chagas disease vectorial transmission to humans. Acta Trop. 1997 Dec;68(3):285-95. PubMed PMID: 9492913.

34. de Souza W, de Carvalho TM, Barrias ES. Review on Trypanosoma cruzi: Host Cell Interaction. International journal of cell biology. 2010;2010. PubMed PMID: 20811486. Pubmed Central PMCID: 2926652.

35. Almeida-de-Faria M, Freymuller E, Colli W, Alves MJ. Trypanosoma cruzi: characterization of an intracellular epimastigote-like form. Exp Parasitol. 1999 Aug;92(4):263-74. PubMed PMID: 10425154. Epub 1999/07/30. eng.

36. Alves MJ, Colli W. Trypanosoma cruzi: adhesion to the host cell and intracellular survival. IUBMB life. 2007 Apr-May;59(4-5):274-9. PubMed PMID: 17505965.

37. Tyler KM, Engman DM. The life cycle of *Trypanosoma cruzi* revisited. Int J Parasitol. 2001 May 1;31(5-6):472-81. PubMed PMID: 11334932.

38. Bern C. Chagas' Disease. The New England journal of medicine. 2015 Jul 30;373(5):456-66. PubMed PMID: 26222561.

39. Brener Z. Biology of Trypanosoma cruzi. Annu Rev Microbiol. 1973;27:347-82. PubMed PMID: 4201691. Epub 1973/01/01. eng.

40. Ferreira RC, Kessler RL, Lorenzo MG, Paim RM, Ferreira Lde L, Probst CM, et al. Colonization of *Rhodnius prolixus* gut by *Trypanosoma cruzi* involves an extensive parasite killing. Parasitology. 2016 Apr;143(4):434-43. PubMed PMID: 26818093.

41. Schaub GA, Boker CA. Colonization of the rectum of *Triatoma infestans* by *Trypanosoma cruzi:* influence of starvation studied by scanning electron microscopy. Acta Trop. 1986 Dec;43(4):349-54. PubMed PMID: 2882662.

42. Kollien AH, Schaub GA. Development of *Trypanosoma cruzi* after starvation and feeding of the vector - a review. The Tokai journal of experimental and clinical medicine. 1998 Dec;23(6):335-40. PubMed PMID: 10622631.

43. Elliot SL, Rodrigues Jde O, Lorenzo MG, Martins-Filho OA, Guarneri AA. *Trypanosoma cruzi*, etiological agent of Chagas disease, is virulent to its triatomine vector *Rhodnius prolixus* in a temperature-dependent manner. PLoS neglected tropical diseases. 2015 Mar;9(3):e0003646. PubMed PMID: 25793495. Pubmed Central PMCID: 4368190.

44. de Fuentes-Vicente JA, Gutierrez-Cabrera AE, Flores-Villegas AL, Lowenberger C, Benelli G, Salazar-Schettino PM, et al. What makes an effective Chagas disease vector? Factors underlying Trypanosoma cruzi-triatomine interactions. Acta Trop. 2018 Jul;183:23-31. PubMed PMID: 29625091.

45. Kollien AH, Schaub GA. The development of *Trypanosoma cruzi* in triatominae. Parasitol Today. 2000 Sep;16(9):381-7. PubMed PMID: 10951597.

46. Kollien AH, Schaub GA. *Trypanosoma cruzi* in the rectum of the bug *Triatoma infestans*: effects of blood ingestion by the starved vector. Am J Trop Med Hyg. 1998 Jul;59(1):166-70. PubMed PMID: 9684647.

47. Buarque DS, Gomes CM, Araujo RN, Pereira MH, Ferreira RC, Guarneri AA, et al. A new antimicrobial protein from the anterior midgut of *Triatoma infestans* mediates *Trypanosoma cruzi* establishment by controlling the microbiota. Biochimie. 2016 Apr;123:138-43. PubMed PMID: 26905205.

48. Diaz S, Villavicencio B, Correia N, Costa J, Haag KL. Triatomine bugs, their microbiota and *Trypanosoma cruzi*: asymmetric responses of bacteria to an infected blood meal. Parasites & vectors. 2016 Dec 9;9(1):636. PubMed PMID: 27938415. Pubmed Central PMCID: 5148865.

49. Azambuja P, Feder D, Garcia ES. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. Exp Parasitol. 2004 May-Jun;107(1-2):89-96. PubMed PMID: 15208042.

50. Gumiel M, da Mota FF, Rizzo Vde S, Sarquis O, de Castro DP, Lima MM, et al. Characterization of the microbiota in the guts of *Triatoma brasiliensis* and *Triatoma*

pseudomaculata infected by *Trypanosoma cruzi* in natural conditions using culture independent methods. Parasites & vectors. 2015 Apr 24;8:245. PubMed PMID: 25903360. Pubmed Central PMCID: 4429471.

51. Castro DP, Moraes CS, Gonzalez MS, Ratcliffe NA, Azambuja P, Garcia ES. Trypanosoma cruzi immune response modulation decreases microbiota in Rhodnius prolixus gut and is crucial for parasite survival and development. PloS one. 2012;7(5):e36591. PubMed PMID: 22574189. Pubmed Central PMCID: 3344921.

52. von Brand T, Johnson EM, Rees CW. Observations on the Respiration of *Trypanosoma cruzi* in Culture. J Gen Physiol. 1946 Nov 20;30(2):163-75. PubMed PMID: 19873484. Pubmed Central PMCID: 2142820.

53. Cazzulo JJ, Franke de Cazzulo BM, Engel JC, Cannata JJ. End products and enzyme levels of aerobic glucose fermentation in trypanosomatids. Mol Biochem Parasitol. 1985 Sep;16(3):329-43. PubMed PMID: 3903497.

54. Frydman B, de los Santos C, Cannata JJ, Cazzulo JJ. Carbon-13 nuclear magnetic resonance analysis of [1-^{13}C]glucose metabolism in *Trypanosoma cruzi*. Evidence of the presence of two alanine pools and of two CO_2 fixation reactions. Eur J Biochem. 1990 Sep 11;192(2):363-8. PubMed PMID: 2120054.

55. Cazzulo JJ. Intermediate metabolism in *Trypanosoma cruzi*. J Bioenerg Biomembr. 1994 Apr;26(2):157-65. PubMed PMID: 8056782.

56. Duschak VG, Cazzulo JJ. Subcellular localization of glutamate dehydrogenases and alanine aminotransferase in epimastigotes of *Trypanosoma cruzi*. FEMS Microbiol Lett. 1991 Oct 1;67(2):131-5. PubMed PMID: 1778428.

57. Acosta H, Dubourdieu M, Quinones W, Caceres A, Bringaud F, Concepcion JL. Pyruvate phosphate dikinase and pyrophosphate metabolism in the glycosome of *Trypanosoma cruzi* epimastigotes. Comp Biochem Physiol B Biochem Mol Biol. 2004 Aug;138(4):347-56. PubMed PMID: 15325334.

58. Zeledon R. Comparative physiological studies on four species of hemoflagellates in culture. II. Effect of carbohydrates and related substances and some amino compounds on the respiration. J Parasitol. 1960 Oct;46:541-51. PubMed PMID: 13788134.

59. Silber AM, Tonelli RR, Martinelli M, Colli W, Alves MJ. Active transport of L-proline in *Trypanosoma cruzi*. J Eukaryot Microbiol. 2002 Nov-Dec;49(6):441-6. PubMed PMID: 12503677.

60. Hampton JR. Arginine transport in the culture form of *Trypanosoma cruzi*. The Journal of protozoology. 1971 Nov;18(4):701-3. PubMed PMID: 4943764.

61. Pereira CA, Alonso GD, Paveto MC, Flawia MM, Torres HN. L-arginine uptake and L-phosphoarginine synthesis in *Trypanosoma cruzi*. J Eukaryot Microbiol. 1999 Nov-Dec;46(6):566-70. PubMed PMID: 10568030.

62. Canepa GE, Silber AM, Bouvier LA, Pereira CA. Biochemical characterization of a low-affinity arginine permease from the parasite Trypanosoma cruzi. FEMS Microbiol Lett. 2004 Jul 1;236(1):79-84. PubMed PMID: 15212794.

63. Silber AM, Rojas RL, Urias U, Colli W, Alves MJ. Biochemical characterization of the glutamate transport in *Trypanosoma cruzi*. Int J Parasitol. 2006 Feb;36(2):157-63. PubMed PMID: 16373069.

64. Hampton JR. Lysine uptake in cultured *Trypanosoma cruzi*: interactions of competitive inhibitors. The Journal of protozoology. 1970 Nov;17(4):597-600. PubMed PMID: 5505361.

65. Inbar E, Canepa GE, Carrillo C, Glaser F, Suter Grotemeyer M, Rentsch D, et al. Lysine transporters in human trypanosomatid pathogens. Amino Acids. 2012 Jan;42(1):347-60. PubMed PMID: 21170560.

66. Canepa GE, Bouvier LA, Miranda MR, Uttaro AD, Pereira CA. Characterization of *Trypanosoma cruzi* L-cysteine transport mechanisms and their adaptive regulation. FEMS Microbiol Lett. 2009 Mar;292(1):27-32. PubMed PMID: 19175408. Epub 2009/01/30. eng.

67. Canepa GE, Bouvier LA, Urias U, Miranda MR, Colli W, Alves MJ, et al. Aspartate transport and metabolism in the protozoan parasite *Trypanosoma cruzi*. FEMS Microbiol Lett. 2005 Jun 1;247(1):65-71. PubMed PMID: 15927749. Epub 2005/06/02. eng.

68. Manchola NC, Rapado LN, Barison MJ, Silber AM. Biochemical characterization of branched chain amino acids uptake in *Trypanosoma cruzi*. J Eukaryot Microbiol. 2016 May;63(3):299-308. PubMed PMID: 26496801.

69. Barison MJ, Damasceno FS, Mantilla BS, Silber AM. The active transport of histidine and its role in ATP production in *Trypanosoma cruzi*. J Bioenerg Biomembr. 2016 Aug;48(4):437-49. PubMed PMID: 27222029.

70. Damasceno FS, Barison MJ, Crispim M, Souza ROO, Marchese L, Silber AM. L-Glutamine uptake is developmentally regulated and is involved in metacyclogenesis in Trypanosoma cruzi. Mol Biochem Parasitol. 2018 Jul 17;224:17-25. PubMed PMID: 30030130.

71. Galvez Rojas RL, Ahn IY, Suarez Mantilla B, Sant'Anna C, Pral EM, Silber AM. The Uptake of GABA in *Trypanosoma cruzi*. J Eukaryot Microbiol. 2015 Sep-Oct;62(5):629-36. PubMed PMID: 25851259.

72. Bouvier LA, Silber AM, Galvao Lopes C, Canepa GE, Miranda MR, Tonelli RR, et al. Post genomic analysis of permeases from the amino acid/auxin family in protozoan parasites. Biochem Biophys Res Commun. 2004 Aug 27;321(3):547-56. PubMed PMID: 15358142.

73. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, et al. The genome of the African trypanosome *Trypanosoma brucei*. Science. 2005 Jul 15;309(5733):416-22. PubMed PMID: 16020726.

74. Jackson AP. Origins of amino acid transporter loci in trypanosomatid parasites. BMC Evol Biol. 2007 Feb 23;7:26. PubMed PMID: 17319943. Pubmed Central PMCID: 1810246.

75. Saier MH, Jr., Reddy VS, Tsu BV, Ahmed MS, Li C, Moreno-Hagelsieb G. The Transporter Classification Database (TCDB): recent advances. Nucleic Acids Res. 2016 Jan 4;44(D1):D372-9. PubMed PMID: 26546518. Pubmed Central PMCID: 4702804.

76. Carrillo C, Canepa GE, Giacometti A, Bouvier LA, Miranda MR, Camara MM, et al. *Trypanosoma cruzi* amino acid transporter TcAAAP411 mediates arginine uptake in yeasts. FEMS Microbiol Lett. 2010 May;306(2):97-102. PubMed PMID: 20337715.

77. Saye M, Miranda MR, di Girolamo F, Camara MM, Pereira CA. Proline modulates the *Trypanosoma cruzi* resistance to reactive oxygen species and drugs through a novel D, L-proline transporter. PloS one. 2014;9(3):e92028. PubMed PMID: 24637744. Pubmed Central PMCID: 3956872.

78. Saye M, Miranda MR, Reigada C, Pereira CA. *Trypanosoma cruzi p*roline transport presents a cell density-dependent regulation. J Eukaryot Microbiol. 2016 Jul;63(4):516-23. PubMed PMID: 26750517.

79. Inbar E, Schlisselberg D, Suter Grotemeyer M, Rentsch D, Zilberstein D. A versatile proline/alanine transporter in the unicellular pathogen *Leishmania donovani* regulates amino acid homoeostasis and osmotic stress responses. Biochem J. 2013 Jan 15;449(2):555-66. PubMed PMID: 22994895.

80. Wynne de Martini GJ, Abramo Orrego L, de Rissio AM, Alvarez M, Mujica LP. Culture of *Trypanosoma cruzi* in a monophasic medium. Application to large-scale cultures in fermentation processes. Medicina (B Aires). 1980;40 Suppl. 1:109-14. PubMed PMID: 7005593. Cultivo de Trypanosoma cruzi en un medio monofasico. Su aplicacion en cultivos en gran escala en procesos de fermentacion.

81. Sylvester D, Krassner SM. Proline metabolism in *Trypanosoma cruzi* epimastigotes. Comparative biochemistry and physiology B, Comparative biochemistry. 1976;55(3B):443-7. PubMed PMID: 789007.

82. Paes LS, Mantilla BS, Zimbres FM, Pral EM, Diogo de Melo P, Tahara EB, et al. Proline dehydrogenase regulates redox state and respiratory metabolism in *Trypanosoma cruzi*. PloS one. 2013;8(7):e69419. PubMed PMID: 23894476. Pubmed Central PMCID: 3718742.

83. Mantilla BS, Paes LS, Pral EM, Martil DE, Thiemann OH, Fernandez-Silva P, et al. Role of Delta1-Pyrroline-5-Carboxylate-dehydrogenase supports mitochondrial metabolism and host-cell invasion of *Trypanosoma cruzi*. J Biol Chem. 2015 Jan 26;290:7767-90. PubMed PMID: 25623067.

84. Barison MJ, Rapado LN, Merino EF, Furusho Pral EM, Mantilla BS, Marchese L, et al. Metabolomic profiling reveals a finely tuned, starvation-induced metabolic switch in *Trypanosoma cruzi* epimastigotes. J Biol Chem. 2017 May 26;292(21):8964-77. PubMed PMID: 28356355. Pubmed Central PMCID: 5448128.

85. Camargo EP, Coelho JA, Moraes G, Figueiredo EN. Trypanosoma spp., Leishmania spp. and Leptomonas spp.: enzymes of ornithine-arginine metabolism. Exp Parasitol. 1978 Dec;46(2):141-4. PubMed PMID: 569593.

86. Yoshida N, Camargo EP. Ureotelism and ammonotelism in trypanosomatids. J Bacteriol. 1978 Dec;136(3):1184-6. PubMed PMID: 721777. Pubmed Central PMCID: 218555.

87. Cruz-Bustos T, Potapenko E, Storey M, Docampo R. An intracellular ammonium transporter Is necessary for replication, differentiation, and resistance to starvation and osmotic stress in *Trypanosoma cruzi*. mSphere. 2018;3(1).

88. Crispim M, Damasceno FS, Hernandez A, Barison MJ, Pretto Sauter I, Souza Pavani R, et al. The glutamine synthetase of *Trypanosoma cruzi* is required for its resistance to ammonium accumulation and evasion of the parasitophorous vacuole during host-cell infection. PLoS neglected tropical diseases. 2018 Jan;12(1):e0006170. PubMed PMID: 29320490.

89. Cazzulo JJ, Arauzo S, Franke de Cazzulo BM, Cannata JJB. On the production of glycerol and l-alanine during the aerobic fermentation of glucose by trypanosomatids. FEMS Microbiol Lett. 1988;51(2-3):187-91.

90. Rohloff P, Rodrigues CO, Docampo R. Regulatory volume decrease in *Trypanosoma cruzi* involves amino acid efflux and changes in intracellular calcium. Mol Biochem Parasitol. 2003 Feb;126(2):219-30. PubMed PMID: 12615321.

91. Rohloff P, Montalvetti A, Docampo R. Acidocalcisomes and the contractile vacuole complex are involved in osmoregulation in *Trypanosoma cruzi*. J Biol Chem. 2004 Dec 10;279(50):52270-81. PubMed PMID: 15466463.

92. Rohloff P, Docampo R. A contractile vacuole complex is involved in osmoregulation in *Trypanosoma cruzi*. Exp Parasitol. 2008 Jan;118(1):17-24. PubMed PMID: 17574552. Pubmed Central PMCID: 2243178.

93. Morozov L. Mirror symmetry breaking in biochemical evolution. Origins of life. 1979 Jul;9(3):187-217. PubMed PMID: 481872.

94. Armstrong JJ, Baddiley J, Buchanan JG. Structure of the ribitol teichoic acid from the walls of Bacillus subtilis. Biochem J. 1960 Sep;76:610-21. PubMed PMID: 13684313. Pubmed Central PMCID: 1204841.

95. Reynolds PE, Courvalin P. Vancomycin resistance in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine. Antimicrob Agents Chemother. 2005 Jan;49(1):21-5. PubMed PMID: 15616270. Pubmed Central PMCID: 538887.

96. Sieradzki K, Tomasz A. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of Staphylococcus aureus. J Bacteriol. 1997 Apr;179(8):2557-66. PubMed PMID: 9098053. Pubmed Central PMCID: 179004.

97. Aliashkevich A, Alvarez L, Cava F. New Insights Into the Mechanisms and Biological Roles of D-Amino Acids in Complex Eco-Systems. Front Microbiol. 2018;9:683. PubMed PMID: 29681896. Pubmed Central PMCID: 5898190.

98. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. Science. 2010 Apr 30;328(5978):627-9. PubMed PMID: 20431016. Pubmed Central PMCID: 2921573.

99. Radkov AD, McNeill K, Uda K, Moe LA. D-Amino Acid Catabolism Is Common Among Soil-Dwelling Bacteria. Microbes Environ. 2016 Jun 25;31(2):165-8. PubMed PMID: 27169790. Pubmed Central PMCID: 4912152.

100. Radkov AD, Moe LA. Bacterial synthesis of D-amino acids. Appl Microbiol Biotechnol. 2014 Jun;98(12):5363-74. PubMed PMID: 24752840.

101. Hills GM. Chemical factors in the germination of spore-bearing aerobes; the effects of amino acids on the germination of Bacillus anthracis, with some observations on the relation of optical form to biological activity. Biochem J. 1949;45(3):363-70. PubMed PMID: 15392864. Pubmed Central PMCID: 1275005.

102. Fuchs SA, Berger R, Klomp LW, de Koning TJ. D-amino acids in the central nervous system in health and disease. Mol Genet Metab. 2005 Jul;85(3):168-80. PubMed PMID: 15979028.

103. Kiriyama Y, Nochi H. D-Amino Acids in the Nervous and Endocrine Systems. Scientifica. 2016;2016:6494621. PubMed PMID: 28053803. Pubmed Central PMCID: 5178360.

104. Kim PM, Duan X, Huang AS, Liu CY, Ming GL, Song H, et al. Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2010 Feb 16;107(7):3175-9. PubMed PMID: 20133766. Pubmed Central PMCID: 2840285.

105. Wolosker H. Serine racemase and the serine shuttle between neurons and astrocytes. Biochim Biophys Acta. 2011 Nov;1814(11):1558-66. PubMed PMID: 21224019.

106. Bodanszky M, Perlman D. Origin of D-amino-acids in microbial peptides: rule of alpha-epimerization. Nature. 1968 Apr 20;218(5138):291-2. PubMed PMID: 5648233.

107. Corrigan JJ, Srinivasan NG. The occurrence of certain D-amino acids in insects. Biochemistry. 1966 Apr;5(4):1185-90. PubMed PMID: 5958195.

108. Conti P, Tamborini L, Pinto A, Blondel A, Minoprio P, Mozzarelli A, et al. Drug discovery targeting amino acid racemases. Chemical reviews. 2011 Nov 9;111(11):6919-46. PubMed PMID: 21913633.

109. Chamond N, Gregoire C, Coatnoan N, Rougeot C, Freitas-Junior LH, da Silveira JF, et al. Biochemical characterization of proline racemases from the human protozoan parasite *Trypanosoma cruzi* and definition of putative protein signatures. J Biol Chem. 2003 May 2;278(18):15484-94. PubMed PMID: 12735293.

110. Reina-San-Martin B, Degrave W, Rougeot C, Cosson A, Chamond N, Cordeiro-Da-Silva A, et al. A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase. Nat Med. 2000 Aug;6(8):890-7. PubMed PMID: 10932226.

111. Chamond N, Goytia M, Coatnoan N, Barale JC, Cosson A, Degrave WM, et al. *Trypanosoma cruzi* proline racemases are involved in parasite differentiation and infectivity. Mol Microbiol. 2005 Oct;58(1):46-60. PubMed PMID: 16164548.

112. Hols P, Defrenne C, Ferain T, Derzelle S, Delplace B, Delcour J. The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. J Bacteriol. 1997 Jun;179(11):3804-7. PubMed PMID: 9171436. Pubmed Central PMCID: 179184.

113. Milligan DL, Tran SL, Strych U, Cook GM, Krause KL. The alanine racemase of *Mycobacterium smegmatis* is essential for growth in the absence of D-alanine. J Bacteriol. 2007 Nov;189(22):8381-6. PubMed PMID: 17827284. Pubmed Central PMCID: 2168708.

114. Shrestha R, Lockless SW, Sorg JA. A Clostridium difficile alanine racemase affects spore germination and accommodates serine as a substrate. J Biol Chem. 2017 Jun 23;292(25):10735-42. PubMed PMID: 28487371. Pubmed Central PMCID: 5481577.

115. Franklin FC, Venables WA. Biochemical, genetic, and regulatory studies of alanine catabolism in *Escherichia coli* K12. Mol Gen Genet. 1976 Dec 8;149(2):229-37. PubMed PMID: 13292.

116. Wasserman SA, Daub E, Grisafi P, Botstein D, Walsh CT. Catabolic alanine racemase from *Salmonella typhimurium*: DNA sequence, enzyme purification, and characterization. Biochemistry. 1984 Oct 23;23(22):5182-7. PubMed PMID: 6391537.

117. Hoffmann K, Schneider-Scherzer E, Kleinkauf H, Zocher R. Purification and characterization of eucaryotic alanine racemase acting as key enzyme in cyclosporin biosynthesis. J Biol Chem. 1994 Apr 29;269(17):12710-4. PubMed PMID: 8175682.

118. Cheng YQ, Walton JD. A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. J Biol Chem. 2000 Feb 18;275(7):4906-11. PubMed PMID: 10671527.

119. Uo T, Yoshimura T, Tanaka N, Takegawa K, Esaki N. Functional characterization of alanine racemase from *Schizosaccharomyces pombe*: a eucaryotic counterpart to bacterial alanine racemase. J Bacteriol. 2001 Apr;183(7):2226-33. PubMed PMID: 11244061. Pubmed Central PMCID: 95128.

120. Nomura T, Yamamoto I, Morishita F, Furukawa Y, Matsushima O. Purification and some properties of alanine racemase from a bivalve mollusc *Corbicula japonica*. J Exp Zool. 2001 Jan 1;289(1):1-9. PubMed PMID: 11169488.

121. Shibata K, Shirasuna K, Motegi K, Kera Y, Abe H, Yamada R. Purification and properties of alanine racemase from crayfish *Procambarus clarkii*. Comp Biochem Physiol B Biochem Mol Biol. 2000 Aug;126(4):599-608. PubMed PMID: 11026672.

122. Yoshikawa N, Ashida W, Hamase K, Abe H. HPLC determination of the distribution of D-amino acids and effects of ecdysis on alanine racemase activity in kuruma prawn *Marsupenaeus japonicus*. J Chromatogr B Analyt Technol Biomed Life Sci. 2011 Nov 1;879(29):3283-8. PubMed PMID: 21592870.

123. Yoshikawa N, Dhomae N, Takio K, Abe H. Purification, properties, and partial amino acid sequences of alanine racemase from the muscle of the black tiger prawn *Penaeus monodon*. Comp Biochem Physiol B Biochem Mol Biol. 2002 Nov;133(3):445-53. PubMed PMID: 12431412.

124. Yoshikawa N, Okada S, Abe H. Molecular characterization of alanine racemase in the Kuruma prawn *Marsupenaeus japonicus*. J Biochem. 2009 Feb;145(2):249-58. PubMed PMID: 19060308.

125. Uo T, Ueda M, Nishiyama T, Yoshimura T, Esaki N. Purification and characterization of alanine racemase from hepatopancreas of black-tiger prawn, *Penaeus monodon*. Journal of Molecular Catalysis B: Enzymatic. 2001 2001/02/28/;12(1):137-44.

126. Yokoyama T, Mimura Y, Sato M, Nagahisa E. Purification and some properties of alanine racemase from the marine gastropod *Cellana grata*. Fish Sci. 2006 January 01;72(1):195-201.

127. Abe H, Yoshikawa N, Sarower MG, Okada S. Physiological function and metabolism of free D-alanine in aquatic animals. Biol Pharm Bull. 2005 Sep;28(9):1571-7. PubMed PMID: 16141518.

128. Panizzutti R, de Souza Leite M, Pinheiro CM, Meyer-Fernandes JR. The occurrence of free D-alanine and an alanine racemase activity in *Leishmania amazonensis*. FEMS Microbiol Lett. 2006 Mar;256(1):16-21. PubMed PMID: 16487314.

129. Coatnoan N, Berneman A, Chamond N, Minoprio P. Proline racemases: insights into *Trypanosoma cruzi* peptides containing D-proline. Mem Inst Oswaldo Cruz. 2009 Jul;104 Suppl 1:295-300. PubMed PMID: 19753488.

130. Brener Z, Chiari E. Aspects of early growth of different *Trypanosoma cruzi* strains in culture medium. J Parasitol. 1965 Dec;51(6):922-6. PubMed PMID: 5848818.

131. Camargo EP. Growth and Differentiation in Trypanosoma Cruzi. I. Origin of Metacyclic Trypanosomes in Liquid Media. Rev Inst Med Trop Sao Paulo. 1964 May-Jun;6:93-100. PubMed PMID: 14177814.

132. Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S. In vitro differentiation of *Trypanosoma cruzi* under chemically defined conditions. Mol Biochem Parasitol. 1985 Sep;16(3):315-27. PubMed PMID: 3903496.

133. de Sousa MA. A simple method to purify biologically and antigenically preserved bloodstream trypomastigotes of *Trypanosoma cruzi* using DEAE-cellulose columns. Mem Inst Oswaldo Cruz. 1983 Jul-Sep;78(3):317-33. PubMed PMID: 6361445.

134. Tonelli RR, Silber AM, Almeida-de-Faria M, Hirata IY, Colli W, Alves MJ. L-proline is essential for the intracellular differentiation of Trypanosoma cruzi. Cell Microbiol. 2004 Aug;6(8):733-41. PubMed PMID: 15236640.

135. Brun R, Schonenberger. Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop. 1979 Sep;36(3):289-92. PubMed PMID: 43092.

136. Hirumi H, Hirumi K. Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. J Parasitol. 1989 Dec;75(6):985-9. PubMed PMID: 2614608.

137. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America. 1977 Dec;74(12):5463-7. PubMed PMID: 271968. Pubmed Central PMCID: 431765.

138. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5. PubMed PMID: 5432063.

139. Cazzulo Franke MC, Vernal J, Cazzulo JJ, Nowicki C. The NAD-linked aromatic alpha-hydroxy acid dehydrogenase from Trypanosoma cruzi. A new member of the cytosolic malate

dehydrogenases group without malate dehydrogenase activity. Eur J Biochem. 1999 Dec;266(3):903-10. PubMed PMID: 10583384.

140. Gutheil WG, Stefanova ME, Nicholas RA. Fluorescent coupled enzyme assays for D-alanine: application to penicillin-binding protein and vancomycin activity assays. Anal Biochem. 2000 Dec 15;287(2):196-202. PubMed PMID: 11112264.

141. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983 Dec 16;65(1-2):55-63. PubMed PMID: 6606682.

142. Girard R, Crispim M, Alencar MB, Silber AM. Uptake of l-Alanine and Its Distinct Roles in the Bioenergetics of Trypanosoma cruzi. mSphere. 2018 Jul 18;3(4). PubMed PMID: 30021880. Pubmed Central PMCID: 6052336.

143. Manchola NC, Silber AM, Nowicki C. The non-canonical substrates of *Trypanosoma cruzi t*yrosine and aspartate aminotransferases: branched-chain amino acids. J Eukaryot Microbiol. 2018 Jan;65(1):70-6. PubMed PMID: 28618210.

144. Magdaleno A, Ahn IY, Paes LS, Silber AM. Actions of a proline analogue, L-thiazolidine-4-carboxylic acid (T4C), on *Trypanosoma cruzi*. PloS one. 2009;4(2):e4534. PubMed PMID: 19229347. Pubmed Central PMCID: 2645137.

145. Huang G, Vercesi AE, Docampo R. Essential regulation of cell bioenergetics in *Trypanosoma brucei* by the mitochondrial calcium uniporter. Nature communications. 2013;4:2865. PubMed PMID: 24305511. Pubmed Central PMCID: 3868461.

146. LeMagueres P, Im H, Dvorak A, Strych U, Benedik M, Krause KL. Crystal structure at 1.45 A resolution of alanine racemase from a pathogenic bacterium, *Pseudomonas aeruginosa*, contains both internal and external aldimine forms. Biochemistry. 2003 Dec 23;42(50):14752-61. PubMed PMID: 14674749.

147. LeMagueres P, Im H, Ebalunode J, Strych U, Benedik MJ, Briggs JM, et al. The 1.9 A crystal structure of alanine racemase from *Mycobacterium tuberculosis* contains a conserved entryway into the active site. Biochemistry. 2005 Feb 8;44(5):1471-81. PubMed PMID: 15683232.

148. Yoshimura T, Esak N. Amino acid racemases: functions and mechanisms. Journal of bioscience and bioengineering. 2003;96(2):103-9. PubMed PMID: 16233494.

149. Llorente P, Marco R, Sols A. Regulation of liver pyruvate kinase and the phosphoenolpyruvate crossroads. Eur J Biochem. 1970 Mar 1;13(1):45-54. PubMed PMID: 4314710.

150. Barros EGd, Caldas RdA. Partial purification and characterization of glutamic-pyruvic transaminase from *Trypanosoma cruzi*. Comparative biochemistry and physiology B, Comparative biochemistry. 1983 1983/01/01/;74(3):449-52.

151. Montemartini M, Bua J, Bontempi E, Zelada C, Ruiz AM, Santome JA, et al. A recombinant tyrosine aminotransferase from *Trypanosoma cruzi* has both tyrosine aminotransferase and alanine aminotransferase activities. FEMS Microbiol Lett. 1995 Nov 1;133(1-2):17-20. PubMed PMID: 8566704.

152. Montemartini M, Santome JA, Cazzulo JJ, Nowicki C. Purification and partial structural and kinetic characterization of tyrosine aminotransferase from epimastigotes of *Trypanosoma cruzi*. Biochem J. 1993 Jun 15;292 (Pt 3):901-6. PubMed PMID: 8100416. Pubmed Central PMCID: 1134199.

153. Nowicki C, Montemartini M, Duschak V, Santome JA, Cazzulo JJ. Presence and subcellular localization of tyrosine aminotransferase and p-hydroxyphenyllactate dehydrogenase in epimastigotes of *Trypanosoma cruzi*. FEMS Microbiol Lett. 1992 Apr 15;71(2):119-24. PubMed PMID: 1351016.

154. Zelada C, Montemartini M, Cazzulo JJ, Nowicki C. Purification and partial structural and kinetic characterization of an alanine aminotransferase from epimastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol. 1996 Aug;79(2):225-8. PubMed PMID: 8855559.

155. Maugeri DA, Cannata JJ, Cazzulo JJ. Glucose metabolism in *Trypanosoma cruzi*. Essays Biochem. 2011;51:15-30. PubMed PMID: 22023439.

156. Raw I. Some aspects of carbohydrate metabolism of cultural forms of *Trypanosoma cruzi*. Revista do Instituto de Medicina Tropical de Sao Paulo. 1959;1(3):192-4. not specified.

157. Cannata JJ, Frasch AC, Cataldi de Flombaum MA, Segura EL, Cazzulo JJ. Two forms of 'malic' enzyme with different regulatory properties in *Trypanosoma cruzi*. Biochem J. 1979 Nov 15;184(2):409-19. PubMed PMID: 393256. Pubmed Central PMCID: 1161776.

158. Cazzulo JJ, Juan SM, Segura EL. The malic enzyme from *Trypanosoma cruzi*. J Gen Microbiol. 1977 Mar;99(1):237-41. PubMed PMID: 16984.

159. Avilan L, Garcia P. Hysteresis of cytosolic NADP-malic enzyme II from *Trypanosoma cruzi*. Mol Biochem Parasitol. 1994 Jun;65(2):225-32. PubMed PMID: 7969264.

160. Duncan K, Faraci WS, Matteson DS, Walsh CT. (1-Aminoethyl)boronic acid: a novel inhibitor for Bacillus stearothermophilus alanine racemase and Salmonella typhimurium D-alanine:D-alanine ligase (ADP-forming). Biochemistry. 1989 Apr 18;28(8):3541-9. PubMed PMID: 2663072.

161. Fenn TD, Stamper GF, Morollo AA, Ringe D. A side reaction of alanine racemase: transamination of cycloserine. Biochemistry. 2003 May 20;42(19):5775-83. PubMed PMID: 12741835.

162. Panesso D, Reyes J, Rincon S, Diaz L, Galloway-Pena J, Zurita J, et al. Molecular epidemiology of vancomycin-resistant Enterococcus faecium: a prospective, multicenter study in South American hospitals. J Clin Microbiol. 2010 May;48(5):1562-9. PubMed PMID: 20220167. Pubmed Central PMCID: 2863891.

163. Au K, Ren J, Walter TS, Harlos K, Nettleship JE, Owens RJ, et al. Structures of an alanine racemase from Bacillus anthracis (BA0252) in the presence and absence of (R)-1-aminoethylphosphonic acid (L-Ala-P). Acta Crystallogr Sect F Struct Biol Cryst Commun. 2008 May 1;64(Pt 5):327-33. PubMed PMID: 18453697. Pubmed Central PMCID: 2376406.

164. Parsons WH, Patchett AA, Bull HG, Schoen WR, Taub D, Davidson J, et al. Phosphinic acid inhibitors of D-alanyl-D-alanine ligase. J Med Chem. 1988 Sep;31(9):1772-8. PubMed PMID: 3137344.

165. Ciustea M, Mootien S, Rosato AE, Perez O, Cirillo P, Yeung KR, et al. Thiadiazolidinones: a new class of alanine racemase inhibitors with antimicrobial activity against methicillin-resistant Staphylococcus aureus. Biochem Pharmacol. 2012 Feb 1;83(3):368-77. PubMed PMID: 22146584. Pubmed Central PMCID: 3478562.

166. Anthony KG, Strych U, Yeung KR, Shoen CS, Perez O, Krause KL, et al. New classes of alanine racemase inhibitors identified by high-throughput screening show antimicrobial activity against Mycobacterium tuberculosis. PLoS One. 2011;6(5):e20374. PubMed PMID: 21637807. Pubmed Central PMCID: 3102704.

167. Lee Y, Mootien S, Shoen C, Destefano M, Cirillo P, Asojo OA, et al. Inhibition of mycobacterial alanine racemase activity and growth by thiadiazolidinones. Biochem Pharmacol. 2013 Jul 15;86(2):222-30. PubMed PMID: 23680030. Pubmed Central PMCID: 3700342.

168. Smirlis D, Soteriadou K. Trypanosomatid apoptosis: 'Apoptosis' without the canonical regulators. Virulence. 2011 May-Jun;2(3):253-6. PubMed PMID: 21566464.

169. Smirlis D, Duszenko M, Ruiz AJ, Scoulica E, Bastien P, Fasel N, et al. Targeting essential pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. Parasites & vectors. 2010 Nov 17;3:107. PubMed PMID: 21083891. Pubmed Central PMCID: 3136144.

170. Irigoin F, Inada NM, Fernandes MP, Piacenza L, Gadelha FR, Vercesi AE, et al. Mitochondrial calcium overload triggers complement-dependent superoxide-mediated programmed cell death in *Trypanosoma cruzi*. Biochem J. 2009 Mar 15;418(3):595-604. PubMed PMID: 19053945.

171. Blum JJ. Energy metabolism in *Leishmania*. J Bioenerg Biomembr. 1994 Apr;26(2):147-55. PubMed PMID: 8056781.

172. Mallinson DJ, Coombs GH. Biochemical characteristics of the metacyclic forms of *Leishmania major* and *L. mexicana mexicana*. Parasitology. 1989 Feb;98 (Pt 1):7-15. PubMed PMID: 2717219.

173. Darling TN, Blum JJ. Changes in the shape of *Leishmania major* promastigotes in response to hexoses, proline, and hypo-osmotic stress. The Journal of protozoology. 1990 Jul-Aug;37(4):267-72. PubMed PMID: 2258829.

174. Burrows C, Blum JJ. Effect of hyper-osmotic stress on alanine content of *Leishmania major* promastigotes. The Journal of protozoology. 1991 Jan-Feb;38(1):47-52. PubMed PMID: 1997677.

175. Rainey PM, MacKenzie NE. A carbon-13 nuclear magnetic resonance analysis of the products of glucose metabolism in *Leishmania pifanoi* amastigotes and promastigotes. Mol Biochem Parasitol. 1991 Apr;45(2):307-15. PubMed PMID: 1903845.

176. Castilla JJ, Sanchez-Moreno M, Mesa C, Osuna A. *Leishmania donovani:* in vitro culture and [1H] NMR characterization of amastigote-like forms. Mol Cell Biochem. 1995 Jan 26;142(2):89-97. PubMed PMID: 7770070.

177. Keegan FP, Sansone L, BLUM JJ. Oxidation of glucose, ribose, alanine, and glutamate by *Leishmania braziliensis panamensis*. The Journal of protozoology. 1987;34(2):174-9.

178. Blum JJ. Oxidation of alanine, acetate, glutamate, and succinate by digitonin-permeabilized *Leishmania major* promastigotes. J Eukaryot Microbiol. 1996 Mar-Apr;43(2):144-50. PubMed PMID: 8720944.

179. Saunders EC, Ng WW, Chambers JM, Ng M, Naderer T, Kromer JO, et al. Isotopomer profiling of *Leishmania mexicana* promastigotes reveals important roles for succinate fermentation and aspartate uptake in tricarboxylic acid cycle (TCA) anaplerosis, glutamate synthesis, and growth. J Biol Chem. 2011 Aug 5;286(31):27706-17. PubMed PMID: 21636575. Pubmed Central PMCID: 3149361.

180. Saunders EC, Ng WW, Kloehn J, Chambers JM, Ng M, McConville MJ. Induction of a stringent metabolic response in intracellular stages of *Leishmania mexicana* leads to increased dependence on mitochondrial metabolism. PLoS Pathog. 2014 Jan;10(1):e1003888. PubMed PMID: 24465208. Pubmed Central PMCID: 3900632.

181. Saunders EC, Naderer T, Chambers J, Landfear SM, McConville MJ. *Leishmania mexicana* can utilize amino acids as major carbon sources in macrophages but not in animal models. Mol Microbiol. 2018 Apr;108(2):143-58. PubMed PMID: 29411460.

182. Spitznagel D, Ebikeme C, Biran M, Nic a' Bhaird N, Bringaud F, Henehan GT, et al. Alanine aminotransferase of *Trypanosoma brucei*--a key role in proline metabolism in procyclic life forms. FEBS J. 2009 Dec;276(23):7187-99. PubMed PMID: 19895576.

183. Mantilla BS, Marchese L, Casas-Sanchez A, Dyer NA, Ejeh N, Biran M, et al. Proline Metabolism is Essential for *Trypanosoma brucei brucei* Survival in the Tsetse Vector. PLoS Pathog. 2017 Jan;13(1):e1006158. PubMed PMID: 28114403. Pubmed Central PMCID: 5289646.

184. Harington JS. Studies of the amino acids of *Rhodnius prolixus* I. Analysis of the haemolymph. Parasitology. 1961 Nov;51:309-18. PubMed PMID: 13904705.

185. Harington JS. Studies of the amino acids of *Rhodnius prolixus* II. Analysis of the excretory material. Parasitology. 1961 Nov;51:319-26. PubMed PMID: 13904706.

186. Antunes LC, Han J, Pan J, Moreira CJ, Azambuja P, Borchers CH, et al. Metabolic signatures of triatomine vectors of *Trypanosoma cruzi* unveiled by metabolomics. PloS one. 2013;8(10):e77283. PubMed PMID: 24204787. Pubmed Central PMCID: 3813737.

187. Baydoun AR, Emery PW, Pearson JD, Mann GE. Substrate-dependent regulation of intracellular amino acid concentrations in cultured bovine aortic endothelial cells. Biochem Biophys Res Commun. 1990 Dec 31;173(3):940-8. PubMed PMID: 2268354.

188. El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science. 2005 Jul 15;309(5733):409-15. PubMed PMID: 16020725.

189. Schofield PJ, Edwards MR, Grossman G, Tutticci EA. Amino acid exchange activity of the alanine transporter of *Giardia intestinalis*. Exp Parasitol. 1995 Feb;80(1):124-32. PubMed PMID: 7821401.

190. Nygaard T, Bennett CC, Grossman G, Edwards MR, Schofield PJ. Efflux of alanine by *Giardia intestinalis*. Mol Biochem Parasitol. 1994 Mar;64(1):145-52. PubMed PMID: 8078517.

191. Edwards MR, Knodler LA, Wilson JR, Schofield PJ. The transport and metabolism of alanine by *Giardia intestinalis*. Mol Biochem Parasitol. 1993 Sep;61(1):49-57. PubMed PMID: 8259132.

192. Hansen BD. *Trypanosoma gambiense*: membrane transport of amino acids. Exp Parasitol. 1979 Oct;48(2):296-304. PubMed PMID: 477819.

193. Southworth GC, Read CP. Absorption of some amino acids by the haemoflagellate, *Trypanosoma gambiense*. Comp Biochem Physiol A. 1972 1972/04/01/;41(4):905-11.

194. dos Santos MG, Paes LS, Zampieri RA, da Silva MF, Silber AM, Floeter-Winter LM. Biochemical characterization of serine transport in Leishmania (Leishmania) amazonensis. Mol Biochem Parasitol. 2009 Feb;163(2):107-13. PubMed PMID: 19041672.

195. Bonay P, Cohen BE. Neutral amino acid transport in Leishmania promastigotes. Biochim Biophys Acta. 1983 Jun 10;731(2):222-8. PubMed PMID: 6849919.

196. Zilberstein D, Dwyer DM. Protonmotive force-driven active transport of D-glucose and L-proline in the protozoan parasite *Leishmania donovani*. Proceedings of the National Academy of Sciences of the United States of America. 1985 Mar;82(6):1716-20. PubMed PMID: 2984665. Pubmed Central PMCID: 397343.

197. Ryley JF. Studies on the metabolism of the Protozoa. 7. Comparative carbohydrate metabolism of eleven species of trypanosome. Biochem J. 1956 Feb;62(2):215-22. PubMed PMID: 13293175. Pubmed Central PMCID: 1215895.

198. Chang SL. Studies on hemoflagellates; observations concerning some biochemical activities in culture, and respiration of three species of leishmanias and *Trypanosoma cruzi*. J Infect Dis. 1948 Mar-Apr;82(2):109-18. PubMed PMID: 18905209.

199. Gonzalez-Marcano E, Acosta H, Mijares A, Concepcion JL. Kinetic and molecular characterization of the pyruvate phosphate dikinase from *Trypanosoma cruzi*. Exp Parasitol. 2016 Jun;165:81-7. PubMed PMID: 27003459.

200. Marciano D, Maugeri DA, Cazzulo JJ, Nowicki C. Functional characterization of stage-specific aminotransferases from trypanosomatids. Mol Biochem Parasitol. 2009 Aug;166(2):172-82. PubMed PMID: 19443056.

201. Cannata JJ, Cazzulo JJ. Glycosomal and mitochondrial malate dehydrogenases in epimastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol. 1984 Apr;11:37-49. PubMed PMID: 6379451.

202. Takashima E, Inaoka DK, Osanai A, Nara T, Odaka M, Aoki T, et al. Characterization of the dihydroorotate dehydrogenase as a soluble fumarate reductase in *Trypanosoma cruzi*. Mol Biochem Parasitol. 2002 Jul;122(2):189-200. PubMed PMID: 12106873.

203. de Boiso JF, Stoppani AO. The mechanism of acetate and pyruvate oxidation by *Trypanosoma cruzi*. The Journal of protozoology. 1973 Nov;20(5):673-8. PubMed PMID: 4587232.

204. Hampton JR. Serine metabolism in the culture form of *Trypanosoma cruzi:* synthesis of other amino acids. Comparative biochemistry and physiology B, Comparative biochemistry. 1971 Aug 15;39(4):999-1003. PubMed PMID: 4943661.

205. Hampton JR. Serine metabolism in the culture form of *Trypanosoma cruzi*: distribution of 14C from serine into metabolic fractions. Comp Biochem Physiol A Comp Physiol. 1971 Mar 1;38(3):535-40. PubMed PMID: 4396825.

206. Marciano D, Santana M, Nowicki C. Functional characterization of enzymes involved in cysteine biosynthesis and H(2)S production in Trypanosoma cruzi. Mol Biochem Parasitol. 2012 Oct;185(2):114-20. PubMed PMID: 22898136.

207. Nozaki T, Shigeta Y, Saito-Nakano Y, Imada M, Kruger WD. Characterization of transsulfuration and cysteine biosynthetic pathways in the protozoan hemoflagellate, *Trypanosoma cruzi*. Isolation and molecular characterization of cystathionine beta-synthase and

serine acetyltransferase from Trypanosoma. J Biol Chem. 2001 Mar 2;276(9):6516-23. PubMed PMID: 11106665.

208. Sasabe J, Miyoshi Y, Rakoff-Nahoum S, Zhang T, Mita M, Davis BM, et al. Interplay between microbial d-amino acids and host d-amino acid oxidase modifies murine mucosal defence and gut microbiota. Nat Microbiol. 2016 Jul 25;1(10):16125. PubMed PMID: 27670111. Pubmed Central PMCID: 5074547.

209. Miller MM, Popova LB, Meleshkevitch EA, Tran PV, Boudko DY. The invertebrate B(0) system transporter, D. melanogaster NAT1, has unique d-amino acid affinity and mediates gut and brain functions. Insect Biochem Mol Biol. 2008 Oct;38(10):923-31. PubMed PMID: 18718864. Pubmed Central PMCID: 2676678.

210. Vollero A, Imperiali FG, Cinquetti R, Margheritis E, Peres A, Bossi E. The D-amino acid transport by the invertebrate SLC6 transporters KAAT1 and CAATCH1 from *Manduca sexta*. Physiol Rep. 2016 Feb;4(4). PubMed PMID: 26884475. Pubmed Central PMCID: 4759042.

211. Geer BW. Utilization of D-amino acids for growth by *Drosophila melanogaster* larvae. J Nutr. 1966 Sep;90(1):31-9. PubMed PMID: 5918848.

212. Yurimoto H, Hasegawa T, Sakai Y, Kato N. Physiological role of the D-amino acid oxidase gene, DAO1, in carbon and nitrogen metabolism in the methylotrophic yeast *Candida boidinii*. Yeast. 2000 Sep 30;16(13):1217-27. PubMed PMID: 10992285.

213. Lobocka M, Hennig J, Wild J, Klopotowski T. Organization and expression of the *Escherichia coli* K-12 dad operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. J Bacteriol. 1994 Mar;176(5):1500-10. PubMed PMID: 7906689. Pubmed Central PMCID: 205218.

214. Pioli D, Venables WA, Franklin FC. D-Alanine dehydrogenase. Its role in the utilisation of alanine isomers as growth substrates by *Pseudomonas aeruginosa* PA01. Arch Microbiol. 1976 Nov 2;110(23):287-93. PubMed PMID: 13755.

215. Kishimoto K, Yoshimura T, Esaki N, Sugio S, Manning JM, Soda K. Role of leucine 201 of thermostable D-amino acid aminotransferase from a thermophile, Bacillus sp. YM-1. J Biochem. 1995 Apr;117(4):691-6. PubMed PMID: 7592528.

216. Jimenez V. Dealing with environmental challenges: mechanisms of adaptation in *Trypanosoma cruzi*. Res Microbiol. 2014 Apr;165(3):155-65. PubMed PMID: 24508488. Pubmed Central PMCID: 3997592.

217. Reece SE, Pollitt LC, Colegrave N, Gardner A. The meaning of death: evolution and ecology of apoptosis in protozoan parasites. PLoS Pathog. 2011 Dec;7(12):e1002320. PubMed PMID: 22174671. Pubmed Central PMCID: 3234211.

218. Kaczanowski S, Sajid M, Reece SE. Evolution of apoptosis-like programmed cell death in unicellular protozoan parasites. Parasites & vectors. 2011 Mar 25;4:44. PubMed PMID: 21439063. Pubmed Central PMCID: 3077326.

219. Proto WR, Coombs GH, Mottram JC. Cell death in parasitic protozoa: regulated or incidental? Nat Rev Microbiol. 2013 Jan;11(1):58-66. PubMed PMID: 23202528.

220. Duszenko M, Figarella K, Macleod ET, Welburn SC. Death of a trypanosome: a selfish altruism. Trends Parasitol. 2006 Nov;22(11):536-42. PubMed PMID: 16942915.

221. van Zandbergen G, Luder CG, Heussler V, Duszenko M. Programmed cell death in unicellular parasites: a prerequisite for sustained infection? Trends Parasitol. 2010 Oct;26(10):477-83. PubMed PMID: 20591738.

222. Ameisen JC, Idziorek T, Billaut-Mulot O, Loyens M, Tissier JP, Potentier A, et al. Apoptosis in a unicellular eukaryote (*Trypanosoma cruzi*): implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival. Cell Death Differ. 1995 Oct;2(4):285-300. PubMed PMID: 17180034.

223. Marcinkeviciene J, Rogers MJ, Kopcho L, Jiang W, Wang K, Murphy DJ, et al. Selective inhibition of bacterial dihydroorotate dehydrogenases by thiadiazolidinediones. Biochem Pharmacol. 2000 Aug 1;60(3):339-42. PubMed PMID: 10856428.

224. Turner EM, Blazer LL, Neubig RR, Husbands SM. Small Molecule Inhibitors of Regulator of G Protein Signalling (RGS) Proteins. ACS medicinal chemistry letters. 2012 Feb 9;3(2):146-50. PubMed PMID: 22368763. Pubmed Central PMCID: 3285107.

225. Blazer LL, Storaska AJ, Jutkiewicz EM, Turner EM, Calcagno M, Wade SM, et al. Selectivity and anti-Parkinson's potential of thiadiazolidinone RGS4 inhibitors. ACS chemical neuroscience. 2015 Jun 17;6(6):911-9. PubMed PMID: 25844489.

226. Swinney ZT, Haubrich BA, Xia S, Ramesha C, Gomez SR, Guyett P, et al. A Four-Point Screening Method for Assessing Molecular Mechanism of Action (MMOA) Identifies Tideglusib as a Time-Dependent Inhibitor of *Trypanosoma brucei* GSK3beta. PLoS neglected tropical diseases. 2016 Mar;10(3):e0004506. PubMed PMID: 26942720. Pubmed Central PMCID: 4778863.

227. Martinez A, Alonso M, Castro A, Perez C, Moreno FJ. First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. J Med Chem. 2002 Mar 14;45(6):1292-9. PubMed PMID: 11881998.