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THE MICROBICIDAL DEFENSES INVOLVED IN INTRACELLULAR *TOXOPLASMA GONDII, TRYPANOSOMA CRUZI* AND *LEISHMANIA AMAZONENSIS* ELIMINATION IN THE PRESENCE OF SEMICARBAZONES, THIOSEMICARBAZONES, AND THIAZOLIDINONES

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ABSTRACT

Some pathogens that cause diseases of medical and veterinary importance settle down and utilize the intracellular environment to replicate and disseminate, which establish an infection on several vertebrate hosts. In this context, *Toxoplasma gondii*, *Trypanosoma cruzi* and *Leishmania sp* are obligate parasites that infect host cells of vertebrates, including humans, which cause toxoplasmosis, Chagas disease, and leishmaniasis, respectively. These parasites handle many mechanisms to evade the microbicidal defenses of the host cell that includes avoiding the endocytic pathway and lysosome fusion. The success of the parasite evasion mechanisms will allow the infection development, with the parasite within a parasitophorous vacuole or free in the cytoplasm, which limits the activity of chemotherapeutic agents. Studies about the mechanisms of parasite survival are fundamental to develop new drugs to combat the intracellular pathogens. Compounds of semicarbazone, thiosemicarbazone, and thiazolidinone classes have been extensively tested against these three parasites, and some studies showed that they were able to arrest the replication cycle of *T. gondii*, *T. cruzi* and *L. amazonensis*, controlling the cell infection, which allows the recovery of microbicidal defenses of host cells. Consequently, the parasites are identified and eliminated through the parasite-containing vacuole - lysosome fusion or autophagic pathway.

KEYWORDS: Anti-proliferative drugs; intracellular parasites; *Toxoplasma gondii; Trypanosoma cruzi;* vacuole-lysosome fusion.

1. INTRODUCTION

Some pathogens, including viruses, bacteria, fungi, and protozoans that cause diseases of medical and veterinary importance in vertebrate hosts settle down and utilize the intracellular host environment as part of their life cycle. Those pathogens that can either replicate or reside inside these vacuoles throughout their entire intracellular life cycle are termed professional vacuolar pathogens, whereas those that escape and invade the cytosol are referred to as transient vacuolar pathogens [Casadeval 2008; Cemma and Brumell 2012; Kumar and Valdivia 2009]. The vacuolar lifestyle protects the pathogens against extracellular immune responses of the hosts. Also, pathogens have developed essential evasion mechanisms to overcome intracellular microbicidal reactions [Liehl 2015] and establish an infection. Nonetheless, the host cells have a wide variety of microbicide responses [Radtke and O'Riordan 2006] of which the induction of programmed cell death types I (apoptosis) and II (autophagy) are primary mechanisms, resulting in parasite-containing vacuole-lysosome fusion.

Apoptosis is a genetically programmed mechanism of cell death, enabling multicellular organisms to survive eliminating damaged or infected cells that might interfere with the homeostasis [Portt et al. 2011]. Recently, autophagy was described as an essential microbicidal defense that is triggered by intracellular receptors and plays a crucial role in both the innate and adaptive immunity [Orvedahl and Levine 2009]. However, parasites have developed processes to evade the standard routes of elimination, such as inhibiting cellular apoptosis and escaping autophagosome-lysosome fusion, structurally rearranging the host cell. The success of evasion mechanisms and exploitation of the host cell`s metabolism will allow the infection to develop, limiting the activity of chemotherapeutic agents.

1.2 Cellular microbicide responses

Endocytosis is a form of active transport that occurs in most eukaryotic cell types. During endocytosis, a cell engulfs molecules and drives them to the digestive vacuole for lysosome fusion [Bonazzi and Cossart 2006]. This process can be divided into at least five or six classes: (a) clathrin-dependent endocytosis; (b) caveolae-dependent endocytosis; (c/d) clathrin- and caveolae-independent endocytosis (which can be either lipid rafts-dependent or independent); (e) micropinocytosis; and (f) phagocytosis [Conner and Schmid 2003].

Phagocytosis, a kind of endocytosis, is defined as an orchestrated cascade of events, which involves particle recognition and uptake through signal transduction, cytoskeleton rearrangement, and membrane remodeling [Groves et al. 2008]. The steps that follow are phagosome selective fusion with primary lysosomes or the product of the endoplasmic reticulum and Golgi complex, thereby forming a secondary phagolysosome [Huynh et al. 2007]. In higher organisms, phagocytosis is mainly performed by professional phagocytes (macrophages, neutrophils, and dendritic cells) to eliminate pathogens; however, non-professional phagocytic cells can perform phagocytosis under certain conditions [Kim et al. 2010].

Autophagy is an endogenous mechanism that occurs constitutively in all cells to maintain homeostatic functions, such as protein and organelle turnover, and works to generate intracellular nutrients and energy in harsh situations [Glick, Barth and Macleod 2010]. Recent studies have shown that autophagy is a remarkable intracellular defense used to abolish intracellular pathogens (named xenophagy) [Kikegaard, Taylor and Jackson 2004], as reviewed in [Levine and Deretic 2007; Schmid and Munz 2007].

These latter three mechanisms (apoptosis, endocytosis, and autophagy) will culminate in the formation (vesicle nucleation) and expansion of an isolated membrane, named the phagophore, whose edges fuse to form the autophagosome, a double-membrane vesicle that sequesters the cytoplasmic material. Subsequently, the fusion of the autophagosome with the lysosome forms the autophagolysosome, where the sequestered material is degraded [Levine, Mizushima and Virgin 2011]. Nonetheless, pathogens have evolved strategies to avoid or subvert these mechanisms to promote their survival and replication.

1.3 Strategies of parasitism

Once the microorganisms have entered the host cell, they have developed sophisticated tools that enable them to overcome host cell defenses and replicate successfully [Galán and Cossart 2005; Phalipon and Sansonetti 2007]. Although each parasite has its features and molecular framework to establish an intracellular infection, the strategies of parasitism overlapped, as reviewed in [Hackstadt 2000]. The possible mechanisms include: (A) to reside within a parasitophorous vacuole, where parasite reorganized structures of the host and to divert the microbicide system to avoid lysosomal fusion (as *Toxoplasma gondii, Chlamydia psittaci, Legionella pneumophila, Mycobacterium tuberculosis*) [Mordue et al. 1999; Bastidas et al 2003; Xu and Luo 2013; Ernst 2012]. Other mechanisms include: (B) to promote the

parasitophorous vacuole-lysosome fusion to escape to the cytoplasm (*Trypanosoma cruzi*, *Listeria monocytogenes*) [Andrews and Webster 1991; Campoy and Colombo 2009], (C) or to maintain itself within the parasitophorous vacuole (*Leishmania sp.*, *Coxiella burnetii* and *Yersinia petis*) [Le Moal and Loiseau 2016; Baca and Paretsky 1983; Ke, Chen and Yang 2013]. The pathogens can also (D) disrupt the vacuolar membrane to quickly escape to cytoplasm before lysosome fusion (*Rickettsia sp.*, *Shigella flexneri*, *Theileria parva* and *Babesia bovis)* [Phalipon and Sansonetti 2007; Le Moal and Loiseau 2016] or can (E) invade lysosome-free cells (*Plasmodium sp*.) [Chaal et al. 2010]. In this regard, *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Leishmania amazonensis* have different mechanisms to evade the microbicide system of the host cell (A, B and C, respectively), and, therefore, are often used as parasitism models.

1.3.1. *Toxoplasma gondii* – avoiding parasitophorous vacuole-lysosome fusion (A)The causative agent of toxoplasmosis belongs to the phylum Apicomplexa and has a digenetic cycle involving felines as definitive hosts and other vertebrates as intermediate hosts. This parasite can infect all eukaryotic cells residing within a PV, where it avoids oxidative stress [Joiner et al. 1990], and vacuole-lysosome fusion [Garcia-del-Portillo and Finlay 1995] (Figure 1). The parasite secretes products that, together with the outer cell plasma membrane, form the PV membrane, thus avoiding host cell recognition. The cytoplasmic face of the PV-membrane is associated with host cell mitochondria and endoplasmic reticulum [Melo and Souza 1997]. In this regards, the host cell undergoes ultrastructural (microtubules depolymerization, endoplasmic reticulum and mitochondria redistribution) and functional (lipid traffic to PV) reorganization to guarantee parasite survival, replication, and infective success [Melo, Carvalho and Souza 1992; Melo and Souza 1996; Clough and Frickel 2017].

The non-fusogenic nature of the vacuole has benn related to the active penetration of parasites [Charron and Sibley 2004] and blockage of Fc receptors recognition, thus evading PV-membrane-endocytic pathway fusion [Hall and Joiner 1991]. Therefore, intracellular *T. gondii* survival is dependent on the mechanism of parasite entry, forming a specialized PV, which avoids non-oxidative defense mechanisms [[Hall and Joiner 1991]. Secretory organelles as dense granules play a crucial role in maintaining the PV after an invasion and during parasite development [Joiner et al. 1990; Black and Boothroyd 2000] (Fig. 1). However, Carvalho and Melo [2006] found that the features of the vacuole must be maintained during all stages of infection to preserve its non-fusogenic ability.

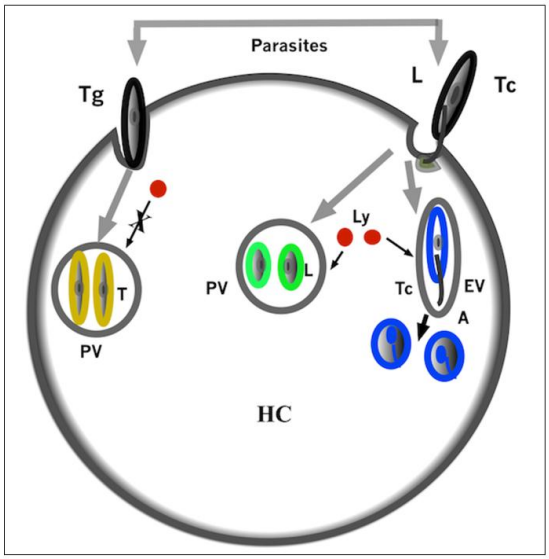

Fig. 1: Representation of entry mechanisms of *Toxoplasma gondii, Leishmania sp*. and *Trypanosoma cruzi* on the host cell. Tachyzoites (T) of *T. gondii* (Tg) actively penetrate the host cell and reside within a parasitophorous vacuole (PV) where they multiply without recognition. Promastigotes of *Leishmania* (L) are phagocytosed by macrophages and reside within a parasitophorous vacuole where utilize the lysosomal enzymes to survive. Cells endocytosis trypomastigote (Tc) of *T. cruzi*, which remains inside the endocytic vacuole (EV) until lysosomal (Ly) fusion, when the parasite escapes to the cytoplasm (HC), transforms into amastigote (A) and replicates until host cell lysis.

This mode of parasite-containing PV is of great interest to the development of chemotherapeutic drugs since other intracellular pathogens such as *Chlamydia psittaci, Legionella pneumophila,* and *Mycobacterium tuberculosis* also use these same mechanisms for evasion of the host cellular defenses.

1.3.2 *Trypanossoma cruzi* – parasitophorous vacuole-lysosome fusion to escape to cytoplasm (B)

T. cruzi is the parasite causative of Chaga's disease or American trypanosomíase. *T. cruzi* has two flagellated forms – trypomastigote and epimastigote – both present at the triatomine vector. The trypomastigotes follow sequential steps (a–f) to invade the vertebrate host cells. First, trypomastigotes interact with plasma membrane constituents of the host cells to promote recognition and adhesion (a) [Eptin, Coates and Engman 2010]; thereby triggering signaling cascades culminating in parasite entry through the endocytic pathway, and thus forming a parasite-containing vacuole (b). Next, a fusion of the vacuole-endocytic organelles occurs (c), followed by membrane lysis and parasite escape to the host cytoplasm (d). In the

host cytoplasm, the trypomastigotes transform to amastigotes (replicative form) and proceed through the replicative stage (e) [Alves and Colli 2007]. (f) After many proliferative cycles (f), the amastigotes transform to trypomastigotes, host cell lysis occurs, and the parasite reaches the bloodstream [Tyler and Engman 2001; Cueto et al. 2017] (Fig. 1).

Unlike *T. gondii*, *T. cruzi* depends on the vacuole acidification to succeed in its intracellular development [Andrade and Andrews 2004]. In this regard, trypomastigotes benefit from the vacuole-lysosome fusion to secrete the enzymes TcTOX and Perforin, both membrane pore-forming proteins that are activated at pH 5.5 [Ley et al. 1990]. Subsequently, the vacuole membrane breaks, and the parasites establish within the cytosol, thus perpetuating the infection [Ley et al. 1990; Barrias, Carvalho and Souza 2013].

1.3.3 *Leishmania sp.* – parasitophorous vacuole-lysosome fusion to survive (C)

Leishmania is a trypanosomatid protozoan that causes various manifestations of leishmaniasis (e.g., cutaneous, visceral) in mammals, including humans. *L. amazonensis* produces the cutaneous form of the disease [Ashford 2000]. The promastigote form, coming from the insect vector (a phlebotomine), infects mammalian macrophages (host cells), where they turn into the proliferative – amastigote – form inside the phagosome [Teixeira et al. 2002; McConville and Naderer 2011] (Fig. 1).

Promastigotes use complement receptors, mainly types I and II (CR1 and CR3), to be phagocytosed [Antoine et al. 1998] as well as Gp63, a zinc-containing surface metalloproteinase, which binds to C3bi, favoring the parasite opsonization and internalization [Hall and Joiner 1991; Oliver, Gregory and Forget 2005]. The surface molecules of promastigotes can block lysis by the complement system. Lipophosphoglycan, the major constituent of parasite surface membranes, prevents the binding of C5b-C9 subunits and cell lysis [Oliver, Gregory and Forget 2005] and modulates the phagosomal maturation. Amastigotes have a favorable metabolism inside the phagolysosomes because of their adaptations, which include the expression of glycoinositolphospholipids and glycosphingolipids at their plasma membrane, which seem to protect them from an adverse vacuole environment. Fusion with secondary lysosomes allows the parasite to access a high quantity of acid hydrolases, which are neutralized and digested by parasitic proteases to provide amino acid sources [Burchmore and Barret 2001; Real and Mortara 2012]. Another defense mechanism of amastigotes is to reduce the quantity of complexed antigen-MHC II molecules at the surface of macrophages [Hall and Joiner 1991] through cysteine peptidase-

dependent degradation. Macrosialin, Lamp-1, Lamp-2, Rab-7, MHCII, and H2M [Oliver, Gregory and Forget 2005] are molecules found at the vacuolar membrane. Inside of this vacuole, there are acid hydrolases, lysosome membrane glycoproteins [Antoine et al. 1990], acid phosphatase, trimetaphosphatase, arylsulphatase A and B, and cathepsin B, D, H and L [Oliver, Gregory and Forget 2005]. Therefore, the presence of Rab 7 and macrosialin suggests that the vacuole is a mixed structure, formed by late endosomes and lysosomes [Antoine et al. 1990].

As cited above, the parasites have developed a sophisticated mechanism to guarantee their intracellular infection. However, the host cell metabolism supports the establishment of the infection and parasite multiplication [Caradonna et al. 2013]. For this reason, the metabolic coupling of intracellular pathogens with host cells is tightly regulated. Nonetheless, the self-limiting damages caused by unbalanced infections in human often leads to death [Santos et al. 2012]. As the intracellular lifestyles limit the efficacy of the chemotherapy, most of these pathogens cause diseases following incomplete and harmful treatment. Because of this, many drugs have been delineated and tested to reach different targets on the parasites.

1.4 Anti-proliferative compounds on intracellular parasitism

Many parasite structures and cell phenomenon have been studied as possible drug candidates. Among them, the enzymes involved in synthesis and replication of DNA identified as promising targets for new chemotherapeutic agents [Crews and Mohan 2000]. Cell cycle control can occur through the direct targeting of the nuclei and its constituents, mainly DNA, or targeting major cytoplasmic organelles during the prior period for cell division, thus avoiding replication. Hydroxyurea (HU) is a well-known DNA-synthesis inhibitor – an anticancer drug that acts preferentially by blocking the enzyme ribonucleotide reductase (RNR), thus impairing DNA synthesis. Dressler and Stein, in Germany in 1869, first synthesized HU, which is a urea-hydroxylated analog, as reviewed in [Yarbro 1992]. Molecular and functional analysis between HU and urea suggests that the active molecular form is due to a ketone adjacent hydroxylate nitrogen atom. The adjacent ketone refunds the hydrogen atom - weak amino group hydroxyl binding (comparing it with the standard group) so that HU is susceptible to a non-enzymatic free-radical formation, which seems to be the active form of the drug [Gwilt and Tracewell 1998] (Fig. 2 a).

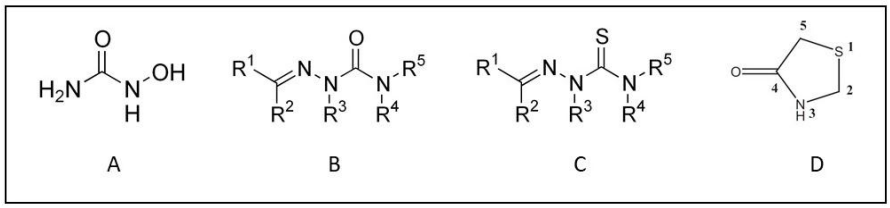

Fig. 2: (a) Representative structures of Hydroxyurea, (b) Semicarbazones, (c) Thiosemicarbazone and (d) the radical hydrazine of thiazolidinone.

Because HU is more specific to the S phase (since the RNR is activated), it enters the cell through passive diffusion and reaches the cells that are synthesizing DNA. Therefore, in the presence of HU, the proliferative cycle arrests at S phase and stays there until cell death. The cells at G1 phase do not die, and those that survive at S phase are partially synchronized [Yarbro 1992; Gwilt and Tracewell 1998]. In kinetoplastids, high doses of HU synchronize extracellular parasites without causing visible toxic effects [Galanti et al. 1994].

Those studies using HU against the intracellular parasites *T. gondii*, *L. amazonensis*, and *T. cruzi* have confirmed that drugs targeting cell cycle regulators can be essential source of new drugs [Melo, Mayerhoffer, and Souza, 2000; Kasper and Pfefferkorn 1982; Melo and Beiral 2003]. From this idea, many HU derivatives were synthesized to improve its efficacy and to avoid parasite resistance, including Semicarbazone, Thiosemicarbazone, and Thiazolidinone compounds.

1.4.1 Semicarbazones (SC)

SC derivatives are obtained by a condensation reaction between a ketone or aldehyde and hydrazine (Semicarbazide) moiety, having [R_2C=NNHC(=O)NH_2] as the structure (Fig. 2 b). The SCs and their analogs are flexible substrates, from which various heterocyclic compounds can be derived. The SC class of compounds has multiple pharmacological activities, including anticonvulsant, antitumor, and antimicrobial activity [Nain et al. 2015; Beraldo and Gambino 2004].

1.4.2 Thiosemicarbazones (TSC)

TSCs are molecules that have the basic structure C=N-NH-CS-NHR (Fig. 2 c). TSCs have a wide pharmacological profile, constituting an important class of compounds with a high property of metal chelation [Casas, García-Tasende and Sordo 2000]. These compounds act as clinical anti-tumor, antiviral, antifungal, antibacterial, and antimalarial drugs. Despite the

vast pharmacological versatility of this class of compounds, structural specificities can lead to manifestations of other biological and clinical activities [Santos et al. 2016]. French and collaborators [French and Blanz 1965], verified that the mode of action of α (N) -heterocyclic TSC was through chelating Fe ion and that anti-neoplastic activity would only be manifested in compounds in which the side chain was in position α concerning hetero-aromatic nitrogen [Santos et al. 2016]. This lateral chain position was also crucial for the antiviral effect of heterocyclic TSC, derived from pyridine, isoquinoline, purine, and isatin against herpes simplex virus (HSV), as reviewed in [Beraldo and Gambino 2004]. For example, when TSC chelates Fe, its prevents the RNR R2 subunit activity that catalyzes the conversion of ribonucleotide to deoxyribonucleotides (dNTPs). Thus, TCS inhibits the generation of cytosine, adenine, and guanine deoxyribonucleotide 5'triphosphate in the construction of the DNA, arresting the cell cycle and inducing the death of the organism. Thus, TSCs prove to be promising compounds for the treatment of many diseases [Beraldo and Gambino 2004].

1.4.3 Thiazolidinones (TZD)

The 4-TZDs are TSC secondary structures, where two hydrazine radicals are added to each side of the molecule (Fig. 2 d). The molecular composition of the TZD structures is of great interest in medicinal chemistry because their wide range of biological activities, such as anti-inflammatory, anticonvulsant, and antiproliferative (antiviral, antifungal, antibacterial, antiprotozoal, anti-tumoral) activity. Therefore, many 4-TZDs have been identified as promising drug candidates, as reviewed in [Tripathi et al. 2014].

1.5 Intracellular infections in the presence of SC, TSC, and TZD

Many HU, SC, TSC, and TZD derivatives have been synthesized from the insertion of different radicals. These news compounds were tested against various pathogens, including *T. gondii*, *T. cruzi*, and *Leishmania sp* [Tenório et al 2005; Aquino et al. 2008; Liesen et al. 2010; Carvalho et al. 2010; Aquino et al. 2011; Gomes et al. 2012; Dzitko et al. 2014; D'Ascenzio et al. 2014; Du et al. 2002; Pizzo et al. 2011; Caputto et al. 2011; Moreira et al. 2014; Greenbaum et al. 2004; Merlino et al. 2010; Soares et al. 2011; Carvalho et al. 2014; Moreira et al. 2014b; Espíndola et al. 2015; Filho et al. 2015; Britta et al. 2012; Britta et al. 2014; Melos et al. 2015; Manzano et al. 2016; Silva et al. 2017; Scariot et al. 2017; Schroder et al 2013]. The majority of these studies, however, have examined only the inhibitory or toxic effects of the compounds against *T. gondii* without describing possible mechanisms of actions and parasite elimination [Tenório et al 2005; Aquino et al. 2008; Liesen et al. 2010;

Carvalho et al. 2010; Aquino et al. 2011; Gomes et al. 2012; Dzitko et al. 2014; D'Ascenzio et al. 2014] and extra- [Pizzo et al. 2011; Caputto et al. 2011; Moreira et al. 2014; Greenbaum et al. 2004] and intracellular forms of trypanosomatids [Soares et al. 2011; Carvalho et al. 2014; Moreira et al. 2014b; Espíndola et al. 2015; Filho et al. 2015; Britta et al. 2012; Melos et al. 2015; Manzano et al. 2016; Silva et al. 2017; Scariot et al. 2017; Schroder et al 2013].

The toxic activities of HU and derivatives against *T. gondii* were described some sutidies [Tenório et al 2005; Aquino et al. 2008; Liesen et al. 2010; Carvalho et al. 2010; Aquino et al. 2011; Gomes et al. 2012; Dzitko et al. 2014; D'Ascenzio et al. 2014]. Nonetheless, the analyzes of the mechanisms involved in parasite elimination were performed in only one study [Carvalho and Melo 2010]. These analyzes show that lysosomes co-localize with *T. gondii* – containing PV (Fig. 3 a and b) and acid phosphatase within the vacuole after treatment with HU, TSC, and TZD. These data suggest that, in the presence of these compounds, the parasite cell cycle is arrested and the innate defenses of the host cells (endocytic vacuole – lysosome fusion and autophagy) are restored, resulting in parasite digestion and elimination from the intracellular environment.

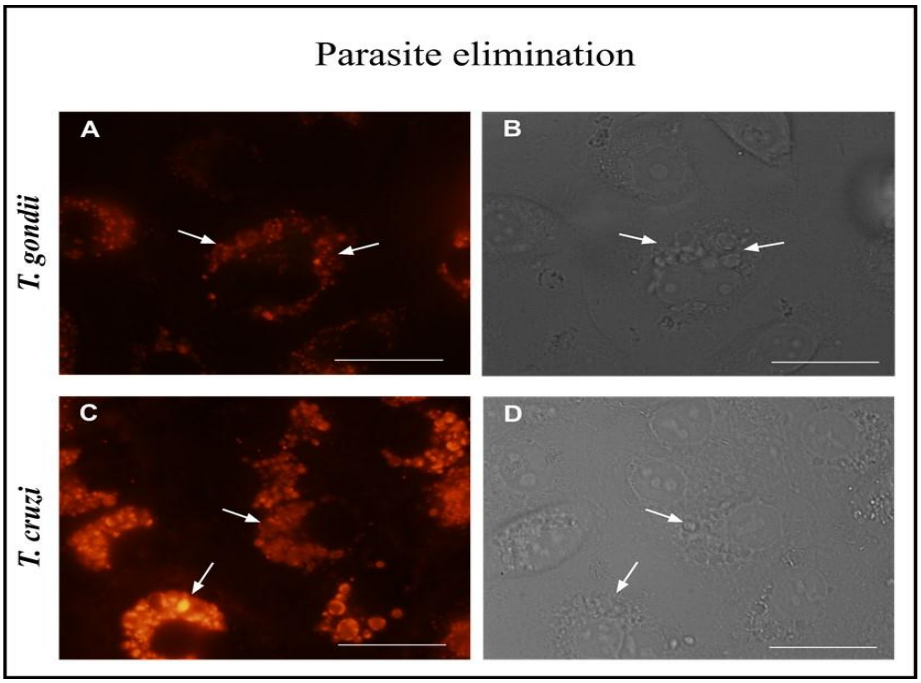

Fig. 3: Mechanisms of parasite elimination after treatment with HU and thiosemicarbazone compound. (a and b) Lysosomes concentrated at destroyed *T. gondii*-containing parasitophorous vacuole after HU treatment, or (c and d) destroyed *T. cruzi*-containing autophagic vacuole stained by Lyso Tracker Red after C_6H_5-C=N-NH-CS-NHH treatment.

In the last decade, only a few studies using SC, TSC or TZD have addressed the molecular or cellular targets of trypomastigotes *in vitro*. Some of these studies described the inhibition of *T. cruzi*-NOS [Soares et al. 2011] or cruzain activity, the major cysteine protease of trypanosomatids [Du et al. 2002; Pizzo et al. 2011; Caputto et al. 2011; Moreira et al. 2014; Greenbaum et al. 2004; Merlino et al. 2010; Espíndola et al. 2015; Filho et al. 2015; Schroder et al. 2013]. Other studies have described the toxic effect of TZD derivatives against trypomastigotes and observed atypical dilatation of the Golgi complex and endoplasmatic reticulum, enlargement of the ER perinuclear membrane, and atypical vacuoles, suggesting parasite autophagy [Moreira et al. 2014]. Regarding *L. amazonensis*, Britta et al. [Britta et al. 2012] analyzed TSC derivatives against the promastigotes. Electron microscopy showed morphological changes in cell shape and size, as well as cellular disintegration and severe organelle damage. The toxic effects were also related to the appearance of lipid bodies and alteration in $\Delta\Psi m$, but not in the integrity of the cell membrane or mitochondrial production of O^{2-} [Britta et al. 2014]. De Melos et al. [2015] have shown that TSC derivatives had antileishmanial effects against promastigotes and amastigotes, and molecular studies have suggested that the compounds bind to the NO synthase site, decreasing parasite defense [Silva et al. 2017]. Although some molecular and cellular targets described as SC, TSC, and TZD targets, the mechanisms of elimination of killed parasites remain unclear, especially for *T. cruzi*, which replicates in the host cytoplasm (Fig. 3).

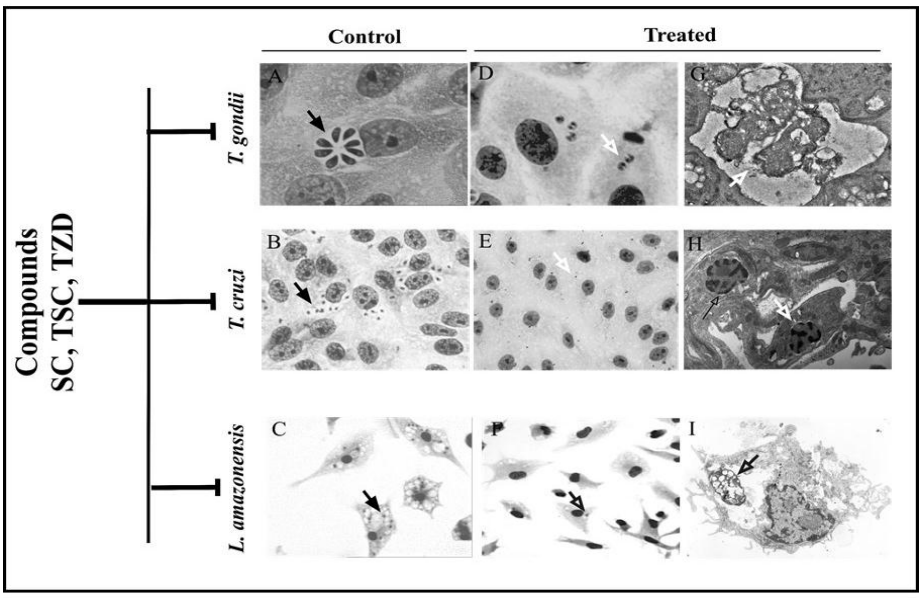

Fig. 4: A representative scheme of parasite elimination after treatments with C_6H_5-C=N-NH-CS-NHH. (a, b and c) Optical microscopy of untreated cultures infected with T. gondii, T. cruzi and L. amazonensis, respectively. (d, e and f) Optical microscopy of

treated infected host cells showing parasite destruction and elimination. (g, h and i) Transmission electron microscopy of treated infected host cells showing ultrastructural damage of the parasite before elimination. Black arrows: common parasites. White arrows: destroyed parasites.

In this context, only one study has investigated the toxic effects of TSC and TZD derivatives in intracellular *T. cruzi* and some cellular events related to death parasite elimination. Carvalho et al. found that TSC and 4-TZD derivatives reduced infection and eliminated intracellular parasites. After treatment with C6H5-C=N-NH-CS-NHH, it revealed that the increase of acidic organelles, such as lysosomes (Fig. 4 c and d) and functional autophagic vacuoles co-located with morphologically disorganized parasites, were involved in the parasite elimination. Ultrastructural studies also shown damaged intracellular parasites located within double-membrane vacuoles, suggesting autophagy. The disorganization and elimination of the parasite occurred asynchronously, indicating that the drugs reach the parasites at different stages of the intracellular life cycle. For *Leishmania sp.*, further studies should be performed to identify the mechanism that overcomes the parasite's resistance to lysosome-macrophage fusion, allowing subsequent digestion and elimination.

In summary, the drugs led to a blockage in parasite development, as well as progressive disorganization within a vacuole that fuses with lysosomes, making it possible to eliminate the parasite without toxic effects on the host cell. Dead parasites are eliminated by multiple mechanisms, involving acidification of the host cytoplasm, vacuole-lysosome fusion, and, in the case of *T. cruzi*, activation of the autophagy pathway. These results suggest that the host cells have recovered their innate microbicide mechanism (autophagolysosome formation and vacuole-lysosome fusion) to eliminate intracellular pathogens. In the case of *T. cruzi*, it was the first description of a double-membrane vacuole positive for autophagy surrounding the parasite, since its natural location is free in the host cytoplasm [Carvalho et al. 2014] (Fig. 5).

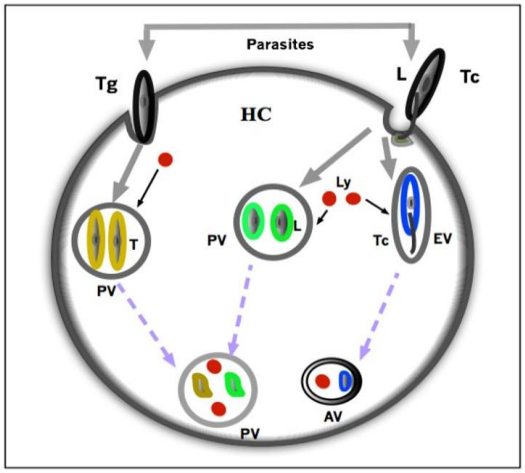

Fig. 5: Suggested mechanisms involved in parasite elimination after treatment with the drugs. After the establishment of the infections, the compounds were incubated (dotted arrows), arresting parasite multiplication, leading them to death and enabling the host cell to recover its microbicide responses. Then, the events of parasitophorous vacuole-lysosome fusion occur in case of *T. gondii* and *Leishmania* or an autophagic vacuole forms surrounding the *T. cruzi*. T: tachyzoite. Pv: parasitophorous vacuole. Ly: lysosomes. EV: endocytic vacuole. Tc: *Trypanosoma cruzi*. Tg: *Toxoplasma gondii*. L: *Leishmania.* Av: autophagic vacuole. HC: host cytoplasm.

CONCLUSION

Many studies have shown that many SC, TSC, and TZD class compounds lead to *T. gondii*, *T. cruzi*, and *L. amazonensis* death and elimination. Arresting the parasite replicative cycles has often been shown to lead to parasite death. Consequently, the host cell microbicidal responses were able to recognize and eliminate the intracellular parasites residing within a vacuole or cytoplasm. These events suggest that parasites must maintain an unrecognizable environment within the intracellular environment during the entire infection and, that the host cells eliminate these soon after the parasite lose their viability. The cellular mechanisms that lead to pathogen-containing vacuole-lysosome fusion is especially important to cellular immunity because they culminate in antigen exposure of destroyed parasites on the cell surface, increasing the host defense. Also, it has been shown that the same compounds could eliminate a variety of pathogens, and the ability of many compounds to eliminate intracellular pathogens is important to avoid resistance.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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REFERENCES

1. Casadevall A. Evolution of intracellular pathogens. Annu Rev Microbiol, 2008; 62: 19–33.
2. Cemma M, Brumell JH. Interactions of pathogenic bacteria with autophagy systems. Curr Biol, 2012; 22: R540-5.
3. Kumar Y, Valdivia RH. Leading a sheltered life. Cell Host Microbe, 2009; 5: 593–601.
4. Liehl P, Zuzarte-Luis V, Mota MM. Unveiling the pathogen behind the vacuole. Nat Rev Microbiol, 2015; 13: 1–10.
5. Radtke AL, O'Riordan MX. Intracellular innate resistance to bacterial pathogens. Cell Microbiol, 2006; 8: 1720–1729.
6. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. Biochim Biophys Acta - Mol Cell Res, 2011; 1813: 238–259.
7. Orvedahl A, Levine B. Eating the enemy within: autophagy in infectious diseases. Cell Death Differ, 2009; 16: 57–69.
8. Bonazzi M, Cossart P. Bacterial entry into cells: a role for the endocytic machinery. FEBS Lett, 2006; 580: 2962–67.
9. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature, 2003; 422: 37–44.
10. Groves E, Dart AE, Covarelli V, Caron E. Molecular mechanisms of phagocytic uptake in mammalian cells. Cell Mol Life Sci, 2008; 65: 1957–76.
11. Huynh KK, Eskelinen E-L, Scott CC, Malevanets A, Saftig P, Grinstein S. LAMP proteins are required for fusion of lysosomes with phagosomes. The EMBO J, 2007; 26: 313-24.
12. Kim M, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H,Sasakawa C. Bacterial interactions with the host epithelium. Cell Host Microbe, 2010; 8: 20–35.
13. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol, 2010; 221: 3–12.
14. Kirkegaard K, Taylor MP, Jackson WT. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat Rev Microbiol, 2004; 2: 301–14.
15. Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity.

Nat Rev Immunol, 2007; 7: 767-77.

16. Schmid D, Münz C. Innate and adaptive immunity through autophagy. Immunity, 2008; 27: 11–21.
17. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature, 2011; 469: 323–35.
18. Galán JE, Cossart P. Host-pathogen interactions: a diversity of themes, a variety of molecular machines. Curr Opin Microbiol, 2005; 8: 1–3.
19. Phalipon A, Sansonetti PJ. *Shigella*'s ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? Immunol Cell Biol, 2007; 85: 119–29.
20. Hackstadt T. Redirection of host vesicle trafficking pathways by intracellular parasites. Traffic, 2000; 1: 93–9.
21. Mordue DG, Håkansson S, Niesman I, Sibley LD. *Toxoplasma gondi*i resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. Exp Parasitol, 1999; 92: 87–99.
22. Bastidas RJ, Elwell CA, Engel NJ, Valdivia RH. Chlamydial intracellular survival strategies. Cold Spring Harb Perspect Med, 2003; 3: 1–20.
23. Xu L, Luo Z-Q. Cell biology of infection by *Legionella pneumophila.* Microbes Infect, 2013; 15: 157-67.
24. Ernst JD. The immunological cycle of tuberculosis. Nature Rev, 2012; 12: 581-91.
25. Andrews NW, Webster P. Phagolysosomal escape by intracellular pathogens. Parasitol Today, 1991; 7: 335-40.
26. Campoy E, Colombo MI. Autophagy in intracellular bacterial infection, Biochim. Biophys. Acta, 2009; 1793: 1465–77.
27. Le Moal VL, Loiseau PM. *Leishmania* hijacking of the macrophage intracellular compartments. FEBS J, 2016; 283: 598–7.
28. Baca OG, Paretsky D. Q fever and *Coxiella burnetii*: a model for host-parasite interactions. Microbiol Rev, 1983; 47: 127–49.
29. Ke Y, Chen Z, Yang R. *Yersinia pestis*: mechanisms of entry into and resistance to the host cell. Front Cell Infect Microbiol, 2013; 3: 106-18.
30. Chaal BK, Gupta AP, Wastuwidyaningtyas DB, Luah Y-H, Bozdech Z. Histone deacetylases play a major role in the transcriptional regulation of the *Plasmodium falciparum* life cycle. Plos Pathog, 2010; 6: 1000373.
31. Joiner KA, Fuhrman SA, Miettinen HM, Kasper LH, Mellman I. *Toxoplasma gondii*: fusion competence of parasitophorous vacuole. Science, 1990; 249: 641-46.

32. Garcia-del-Portillo F, Finlay BB. The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. Trends Microbiol, 1995; 3: 373–80.
33. Melo EJT, Souza W. A cytochemistry study of the inner membrane complex of the pellicle of tachyzoites of *Toxoplasma gondi*i. Parasitol Res, 1997; 83: 252–56.
34. Melo EJT, Carvalho TMU, Souza W. Penetration of T*oxoplasma gondii* into host cells induces changes in the distribution of the mitochondria and the endoplasmic reticulum. Cell Struct Funct, 1992; 17: 311–17.
35. Melo EJT, Souza W. Pathway of C6-NBD-Ceramide on the host cell infected with *Toxoplasma gondii*. Cell Struct Funct, 1996; 21: 47–52.
36. Clough B, Frickel EM. The *Toxoplasma* parasitophorous vacuole: an evolving host-parasite frontier. Trends Parasitol, 2017; 33: 473–88.
37. Charron AJ, Sibley LD. Molecular partitioning during host cell penetration by *Toxoplasma gondii*. Traffic, 2004; 5: 855-67.
38. Hall BF, Joiner KA. Strategies of obligate intracellular parasites for evading host defences. Immunol Today, 1991; 12: 22–7.
39. Black MW, Boothroyd JC. Lytic cycle of *Toxoplasma gondii*. Microbiol Mol Biol Rev, 2000; 64: 607–23.
40. Carvalho CS, Melo EJT. Acidification of the parasitophorous vacuole containing *Toxoplasma gondii* in the presence of hydroxyurea. An Acad Bras Cienc, 2006; 78: 475–84.
41. Eptin CL, Coates BM, Engman DM. Molecular mechanisms of host cell invasion by *Trypanosoma cruzi*. Exp Parasitol, 2010; 126: 283-91.
42. Alves MJ, Colli W. *Trypanosoma cruzi*: adhesion to the host cell and intracellular survival. IUBMB Life, 2007; 59: 274–79.
43. Tyler KM, Engman DM. The life cycle of *Trypanosoma cruzi* revisited. Int J Parasitol, 2001; 31: 472–81.
44. Cueto JA, Vanrell MC, Salassa BN, Nola S, Galli T, Colombo MI, Romano PS. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors required during *Trypanosoma cruzi* parasitophorous vacuole development. Cell Microbiol, 2017 19: 1–14.
45. Andrade LO, Andrews NW. Lysosomal fusion is essential for the retention of *Trypanosoma cruzi* inside host cells. J Exp Med, 2004; 200: 1135–43.
46. Ley V, Robbins ES, Nussenzweig V, Andrews NW. The exit of *Trypanosoma cruzi* from the phagosome is inhibited by raising the pH of acidic compartments. J Exp Med, 1990; 171: 401-13.

47. Barrias ES, Carvalho TMU, Souza W. *Trypanosoma cruzi*: entry into mammalian host cells and parasitophorous vacuole formation. Front Immunol, 2013; 4: 186.
48. Ashford RW. The leishmaniasis as emerging and reemerging zoonoses. Int J Parasitol, 2000; 30: 1269–81.
49. Teixeira MC, Santos RJ, Sampaio RB, Pontes-de-Carvalho L, Santos WL. A simple and reproducible method to obtain large numbers of axenic amastigotes of different *Leishmania species*. Parasitol Res, 2002; 88: 963–68.
50. McConville MJ, Naderer T. Metabolic pathways required for the intracellular survival of *Leishmania*. Annu Rev Microbiol, 2011; 65: 543–61.
51. Antoine JC, Prina E, Lang T, Courret N. The biogenesis and properties of the parasitophorous vacuoles that harbour *Leishmania* in murine macrophages. Trends Microbiol, 1998; 6: 392–1.
52. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev, 2005; 18: 293–5.
53. Burchmore RJ, Barrett MP. Life in vacuoles-nutrient acquisition by *Leishmania* amastigotes. Int J Parasitol, 2001; 31: 1311–20.
54. Real F, Mortara RA. The diverse and dynamic nature of *Leishmania* parasitophorous vacuoles studied by multidimensional imaging. PLoS Negl Trop Dis, 2012; 6: e1518.
55. Antoine JC, Prima E, Jouanne C, Bongrand P. Parasitophorous vacuoles of *Leishmania amazonensis* infected macrophages maintain an acidic pH. Infect Immun, 1990; 58: 779-87.
56. Caradonna KL, Engel JC, Jacobi D, et al. Host metabolism regulates intracellular growth of *Trypanosoma cruzi*. Host Cell Microbe, 2013; 13: 108-17.
57. Santos D, Parajón-Costa B, Rossi M, et al. Activity on *Trypanosoma cruzi*, erythrocytes lysis and biologically relevant physicochemical properties of Pd(II) and Pt(II) complexes of thiosemicarbazones derived from 1-indanones. J Inorg Biochem, 2012; 117: 270–76.
58. Crews CM, Mohan R. Small-molecule inhibitors of the cell cycle. Cur Opin Chem Biol, 2000; 4: 47-53.
59. Yarbro JW. Mechanism of action of Hydroxyurea. Semin Oncol, 1992; 19: 1-10.
60. Gwilt PR, Tracewell JG. Pharmacokinetics and pharmacodynamics of hydroxyurea. Clin Pharmacokinetics, 1998; 34: 347-58.
61. Galanti N, Dvorak JA, Grenet J, McDaniel JP. Hydroxyurea-induced synchrony of DNA replication in the Kinetoplastida. Exp Cell Res, 1994; 214: 225–30.

62. Melo EJT, Mayerhoffer RO, Souza W. Hydroxyurea inhibits intracellular *Toxoplasma gondii* multiplication. FEMS Microbiol Lett, 2000; 185: 79–82.
63. Kasper LH, Pfefferkorn ER. Hydroxyurea inhibition of growth and DNA synthesis in *Toxoplasma gondii*: characterization of a resistant mutant. Mol Biochem Parasitol, 1982; 6: 141–50.
64. Melo EJT, Beiral HJ. Effect of hydroxyurea on the intracellular multiplication of *Toxoplasma gondii*, *Leishmania amazonensis* and *Trypanosoma cruzi*. Braz J Med Biol Res, 2003; 36: 65–69.
65. Nain S, Sharma A, Singh H, Paliwal S. Recent advances in use of semicarbazones as anticonvulsant agents: a review. J Biomed Therap Sci, 2015; 2: 1–7.
66. Beraldo H, Gambino D. The wide pharmacological versatility of semicarbazones, thiosemicarbazones and their metal complexes. Mini Rev Med Chem, 2004; 4: 31–9.
67. Casas JS, García-Tasende MS, Sordo J. Main group metal complexes of semicarbazones and thiosemicarbazones. A structural review. Coord Chem Rev, 2000; 209: 197–61.
68. Santos TA, Silva AC, Silva EB, Gomes BA, Espíndola JW, et al. Antitumor, and immonumodulatory activities of thiosemicarbazones and 1,3-Thiazole in Jurkat and HT-29 cells. Biomed Pharmacother, 2016; 82: 555-60.
69. French FA, Blanz EJ. The carcinostatic activity of alpha-(N) heterocyclic carboxaldehyde thiosemicarbazones I. Isoquinoline-1-carboxaldehyde thiosemicarbazone. Cancer Res, 1965; 25: 1454–58.
70. Tripathi AC, Gupta SJ, Fatima GN, Sonar PK, Verma A, Saraf SK. 4-Thiazolidinones: the advances continue. Eur J Med Chem, 2014; 72: 52–77.
71. Tenório RP, Carvalho CS, Pessanha CS, Lima JG, Faria AR, Alves AJ, Melo EJT, Góes AJS. Synthesis of thiosemicarbazone and 4-thiazolidinone derivatives and their *in vitro* anti-*Toxoplasma gondii* activity. Bioorg Med Chem Lett, 2005; 15: 2575–78.
72. Aquino TM, Liesen AP, Silva REA, Lima VT, et al. Synthesis, anti-*Toxoplasma gondii* and antimicrobial activities of benzaldehyde 4-phenyl-3-thiosemicarbazones and 2-[(phenylmethylene)hydrazono]-4-oxo-3-phenyl-5-thiazolidineacetic acids. Bioorg Med Chem, 2008; 16: 446–56.
73. Liesen AP, Aquino TM, Carvalho CS, Lima VT, et al. Synthesis and evaluation of anti-*Toxoplasma gondii* and antimicrobial activities of thiosemicarbazides, 4-thiazolidinones and 1,3,4-thiadiazoles. Eur J Med Chem, 2010; 45: 3685–91.
74. Carvalho CS, Melo EJT, Tenório RP, Góes AJS. Anti-parasitic action and elimination of intracellular *Toxoplasma gondii* in the presence of novel thiosemicarbazone and its 4-

thiazolidinone derivatives. Braz J Med Biol Res, 2010; 43: 139–49.

75. Aquino TM, Nascimento AAPL, Spacov ICG, Carvalho CS, et al. Synthesis, anti-T*oxoplasma gondii* and antimicrobial activities of 2-hydrazolyl-3-phenyl-5-(4-nitrobenzylidene)-4-thiazolidinone substituted derivatives. Lat Am J Pharm, 2011; 30: 1567–73.
76. Gomes MAGB, Carvalho LP, Rocha BS, Oliveira RR, Melo EJT, Maria EJ. Evaluating anti-*Toxoplasma gondii* activity of new serie of phenylsemicarbazone and phenylthiosemicarbazones *in vitro*. Med Chem Res, 2012; 22: 3574-80.
77. Dzitko K, Paneth A, Plech T, et al. 1,4-Disubstituted thiosemicarbazide derivatives are potent inhibitoes of *Toxoplasma gondii* proliferation. Mol, 2014; 19: 9926-46.
78. D'Ascenzio M, Bizzarri B, De Monte C, et al. Design, synthesis and biological characterization of thiazolidin-4-one derivatives as promising inhibitors of *Toxoplasma gondii*. Eur J Med Chem, 2014; 86: 17-30.
79. Du X, Guo C, Hansell E, et al. Synthesis and structure-activity relationship study of potent trypanocidal thiosemicarbazone inhibitors of the trypanosomal cysteine protease cruzain. J Med Chem, 2002; 45: 2695–07.
80. Pizzo C, Saiz C, Talevi A, et al. Synthesis of 2-Hydrazolyl-4-Thiazolidinones based on multicomponent reactions and biological evaluation against *Trypanosoma cruzi*. Chem Biol Drug Des, 2011; 77: 166-72.
81. Caputto ME, Fabian LE, Benítez D, et al. Thiosemicarbazones derived from 1-indanones as new anti-T*rypanosoma cruz*i agents. Bioorg Med Chem, 2011; 19: 6818–26.
82. Moreira DRM, Oliveira ADT, Gomes PATM, et al. Conformational restriction of aryl thiosemicarbazones produces potent and selective anti-*Trypanosoma cruzi* compounds which induce apoptotic parasite death. Eur J Med Chem, 2014; 75: 467-78.
83. Greenbaum DC, Mackey Z, Hansell E, et al. Synthesis and structure - activity relationships of parasiticidal thiosemicarbazone cysteine protease inhibitors against *Plasmodium falciparum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. J Med Chem, 2004; 47: 3212–19.
84. Merlino A, Benitez D, Chavez S, et al. Development of second generation amidinohydrazones, thio- and semicarbazones as *Trypanosoma cruzi* inhibitors bearing benzofuroxan and benzimidazole 1,3-dioxide core scaffolds. Med Chem Comm, 2010; 1: 216-28.
85. Soares ROA, Eschevarria A, Bellieny MS, Pinho RT, et al. Evaluation of thiosemicarbazones and semicarbazones as potential agents anti-*Trypanosoma cruzi*. Exp

Parasitol, 2011; 129: 381–87.

86. Carvalho LP, Gomes MAGB, Rocha BS, Oliveira RR, Maria EJ, Melo EJT. Anti-parasite effects of new thiosemicarbazones and their products thiazolidinone including cellular aspects of intracellular elimination of *Trypanosoma cruzi in vitro*. J. Dev. Drugs, 2014; 3.
87. Moreira DRM, Leite ACL, Cardoso MVO, et al. Structural design, synthesis and structure-activity relationships of thiazolidinones with enhanced anti-*Trypanosoma cruzi* activity. ChemMedChem, 2014; 9: 177–88.
88. Espíndola JWP, Cardoso MVO, Bezerra G, Filho GBO, et al. Synthesis and structure e activity relationship study of a new series of antiparasitic aryloxyl thiosemicarbazones inhibiting *Trypanosoma cruzi* cruzain. Eur J Med Chem, 2015; 101: 818–35.
89. Filho GBO, Cardoso MVO, Espíndola JWP, et al. Structural design, synthesis and pharmacological evaluation of 4-Thiazolidinones against *Trypanosoma cruzi*. Bioorg Med Chem, 2015; 23: 7478–86.
90. Britta EA, Silva AP, Ueda-Nakamura T, et al. Benzaldehyde thiosemicarbazone derived from limonene complexed with copper induced mitochondrial dysfunction in *Leishmania amazonensis*. PLoS One, 2012; 7: 1–12.
91. Britta EA, Scariot DB, Falzirolli H, et al. Cell death and ultrastructural alterations in *Leishmania amazonensis* caused by new compound 4-Nitrobenzaldehyde thiosemicarbazone derived from S-limonene. BMC Microbiol, 2014; 14: 236.
92. Melos JLR, Tores-Santos EC, Santos FV, et al. Novel 3,4-methylenedioxyde-6-X-benzaldehyde-thiosemicarbazones: synthesis and antileishmanial effects against *Leishmania amazonensis*. Eur J Med Chem, 2015; 103: 409–17.
93. Manzano JI, Cochet F, Boucherle B, et al. Arylthiosemicarbazones as antileishmanial agents. Eur J Med Chem, 2016; 123: 161–70.
94. Silva AC, Santos TAR, Silva IVB, et al. Aryl thiosemicarbazones: *in vitro* and immunomodulatory activities against *L. amazonensis*. Exp Parasitol, 2017; 177: 57–65.
95. Scariot DB, Britta EA, Moreira AL, Falzirolli H, Silva CC, Ueda-Nakamura T, Dias-Filho BP, Nakamura CV. Induction of early autophagic process on *Leishmania amazonensis* by synergistic effect of miltefosine and innovative semi-synthetic thiosemicarbazone. Front. Microbiol, 2017; 8: 1–16.
96. Schröder J, Noack S, Marhöfer RJ, et al. Thiosemicarbazones and triazine nitriles as inhibitors of *Leishmania mexicana* cysteine protease CPB. PLoS One, 2013; 8: 1–12.