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Original article

Evaluation of the leishmanicidal and cytotoxic effects of inhibitors for microorganism metabolic pathway enzymes

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ABSTRACT

Chemotherapy for leishmaniosis a neglected parasitic disease, is based on few drugs, which are toxic and present resistance issues. Efforts for the development of new therapies are essential for the control of leishmaniasis. Metabolic pathway enzymes are promising targets for new drugs against parasites. The search for effective drugs against key enzymes can take advantage of the similarities between metabolic pathways in different microorganisms trypanosomatids *Trypanosoma cruzi* and *Leishmania* and fungus *Saccharomyces cerevisiae*. In this report, leishmanicidal activity of the metabolic pathway enzymes inhibitors (IDs) of dihydroorotate dehydrogenase (DHODH), glyceraldehyde 3-phosphate dehydrogenase and cruzain-cysteine protease from *T. cruzi* and scitalona-desidratase, adenosine deaminase, succinate dehydrogenase complex II and hydroxynaphthalene reductase from *S. cerevisiae* was performed on *Leishmania amazonensis* extracellular promastigotes and amastigotes within macrophages. The most promising compound, ID195, which is a DHODH inhibitor was toxic against promastigotes and was selective for amastigotes over host cells.

1. Introduction

Leishmaniosis are a group of endemic diseases caused by the intramacrophage parasite *Leishmania*; promastigote is the lifecycle form found in the insect vector and amastigote lives in host macrophage parasitophorous vaculoles (PV) [1]. The severity of diseases varies, ranging from cutaneous or mucosal to visceral infection [2,3]. *Leishmania amazonensis* is transmitted mainly in the Amazon region and, is one of the species involved in localized and diffuse cutaneous leishmaniosis in Brazil [2,4]. The diseases are neglected by the pharmaceutical industry although chemotherapy remains the mainstream treatment for the leishmaniosis [3]. Currently there are only a few drugs for the treatment of cutaneous mucocutaneous and visceral leishmaniosis. Organic salt of pentavalent antimony has been the cornerstone for the treatment of all forms of leishmaniasis since the 1940s [5]. The compounds have to be given daily for at least three weeks and antimony therapy causes side effects such as weakness and myalgia, hepatotoxicity along with the most important one cardiopathy (Frezard et al.). Drug resistance is another reported problem [6]. Antimonial pentavalent is thought to act as a pro-drug that is reduced within the organism into more toxic and active SbIII, and the anti-*Leishmania* mechanism is probably related to its interaction with suphydryl containing biomolecules including thiols, peptides, proteins and enzymes [6]. Amphotericin B is a polyene antibiotic also used as a treatment for leishmaniosis since the 1960s [7] its side effects are fever chills, bone pain and renal toxicity [8]; this drug binds to ergosterol the predominant sterol in *Leishmania* but also recognizes cholesterol in human cells [9]. Aiming to decrease the adverse effects lipid formulations of amphotericin B are available, although the production is very expensive which makes their use difficult in poor countries [10]. More recently, miltefosine, an alkylphosphocholine has been used as an alternative drug for visceral leishmaniosis Nevertheless, there are disvantages to this treatment, including teratogenic effects and emergence of resistance [10,11]. In this scenario new and safe drugs are necessary to treat leishmaniosis. Yet, the discovery drug for the treatment of leishmaniosis is difficult due to several factors: the intracellular location of the parasite, the acidic pH of PV in host macrophage, and the multiple *Leishmania* species which infect humans. One strategy is to identify active low-molecular-mass ligands that modify the biological functions of

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Table 1
In vitro activity of synthetic inhibitors of metabolic enzymes against *L. amazonensis* promastigotes.

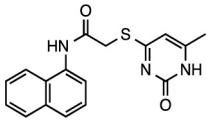
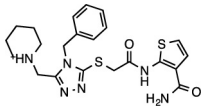
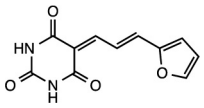
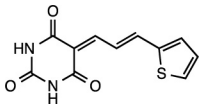
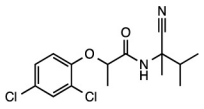
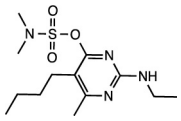
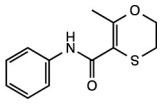
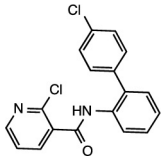
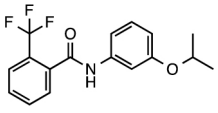
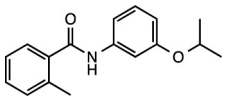
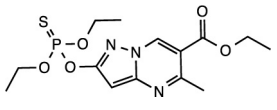
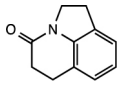
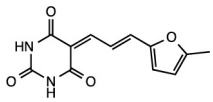
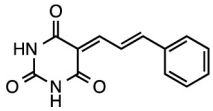
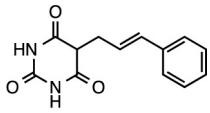
Compound	Chemical structure	Enzyme target (microorganism)	IC50 (μM)[a]
ID16		Glyceraldehyde-3-phosphate dehydrogenase (*T. cruzi*)	>100
ID42		Cruzain (*T. cruzi*)	>100
ID71		Dihydroorotate dehydrogenase (*T. cruzi*)	82.30 ± 0.34
ID130		Dihydroorotate dehydrogenase (*T. cruzi*)	85.90 ± 2.30
ID185		Scitalone dehydratase (*Saccharomyces cerevisiae*)	>200
ID186		Adenosine deaminase (*S. cerevisiae*)	>100
ID187		Succinate dehydrogenase complex II (*S. cerevisiae*)	>200
ID188		Adenosine deaminase (*S. cerevisiae*)	>200
ID189		Succinate dehydrogenase complex II (*S. cerevisiae*)	>200
ID190		Succinate dehydrogenase complex II (*S. cerevisiae*)	>200
ID192		Scitalone dehydratase (*S. cerevisiae*)	>200
ID193		Hydroxynaphthalene reductase (*S. cerevisiae*)	>200

Table 1 (*Continued*)

Compound	Chemical structure	Enzyme target (microorganism)	IC50 (μM)[a]
			
ID195		Dihydroorotate dehydrogenase (*T. cruzi*)	75.13 ± 2.70
ID393		Dihydroorotate dehydrogenase (*T. cruzi*)	>100
ID394		Dihydroorotate dehydrogenase (*T. cruzi*)	>100

[a] Drug concentration that inhibit 50% of the promastigote proliferation at 48 h incubation time.

selected targets (e.g., enzymes, membranes proteins and DNA) [12]. Metabolic pathway enzymes are promising candidates for new drugs against parasites because this pathway plays an essential role in their energy supply [13]. The reason behind the development of competitive inhibitors of metabolic pathway enzymes relies in the fact that such inhibitors can selectively bind parasite enzymes over the human enzyme [12]. Consequently, these enzymes have been targeted for the discover and design of competitive inhibitors and point out some structure activity relationship for similar inhibitors [14,15]. Recently synthesized compounds such as ribonucleosides inhibitors were investigated against trypanosomatids *Trypanosoma cruzi*, the Chagas disease agent and *Leishmania* [14].

The focus of key enzymes as candidates for the discovery of novel anti-trypanosomatids compounds can also take advantage on the similarities between correlated pathways in different organisms, as found in the discovery of activity of anti-*T. cruzi* activity of posaconazole and terbinafine, for instance [15,16]. These two known antifungal drugs that act on ergosterol biosynthesis by inhibiting the enzymes lanosterol 14α-demethylase and squalene epoxydase, respectively, are also able to kill *T. cruzi* by acting on the these enzymes [16,17]. This strategy may also be of help for the identification of enzymatic targets not yet explored in trypanosomatids and for the development of new chemotherapy treatments.

In this report, the leishmanicidal activity of the metabolic pathway enzymes inhibitors (IDs) listed in Table 1 was performed on *L. amazonensis* extracellular promastigotes and amastigotes within macrophages. In addition, the study of cytotoxicity for some IDs against macrophages was evaluated in this work.

2. Materials and methods

2.1. Assayed compounds

Compounds ID71 (CAS 343879-19-6), ID130 (CAS 1502813-63-9), ID393 (CAS 212375-00-3), ID394 (CAS 23450-62-6) are known TcDHODH (dihydroorotate dehydrogenase from *T. cruzi*) inhibitors with trypanocidal activity and were synthesized as previously described [18]; ID16 (CAS 905664-36-0) is a known TcGAPDH (glyceraldehyde-3-phosphate dehydrogenase from *T. cruzi*) inhibitor and was purchased from Chemdiv (www.chemdiv.com, cat. numb.: G856-8155); ID42 (CAS 930454-48-1) is a known cruzain inhibitor with trypanocidal activity (Wiggers et al.) and was purchased from Enanime (http://www.enamine.net, cat. numb.: T5772403); ID195 (CAS 343360-12-3) is a known TcDHODH inhibitor and was purchased from Vitas-M Laboratory (www.vitasmlab.com, cat. numb.: STK980406). The remaining compounds are all known antifungal agents with agrochemical application and known mechanisms of action (see Table 1). These were purchased from Sigma–Aldrich: ID185 (Fenoxanil, CAS 115852-48-7, cat. numb.: cat. numb.: 33842), ID186 (Bupirimate, CAS 41483-43-6, cat. numb.: 31510), ID187 (Carboxine, CAS 5234-68-4, cat. numb.: 45371), ID188 (Boscalid, CAS 188425-85-6, cat. numb.: 33875), ID189 (Flutolanil, CAS 66332-96-5, cat. numb.: PS2057), ID190 (Mepronil, CAS 55814-41-0, cat. numb.: 33361), ID192 (Pyrazophos, CAS 13457-18-6, cat. numb.: 45648), ID193 (Pyroquilon, CAS 57369-32-1, cat. numb.: 45650). All compounds were dissolved in a solution of culture medium containing 0.15% (v/v) DMSO (dimethyl sulfoxide) and then diluted serially using the same solution to achieve the target concentration for the evaluation. The final concentration of DMSO in cell cultures was below 0.02% (v/v) and did not affect the parasites and macrophage viability.

2.2. Parasite

L. amazonensis (MHOM/BR/75/Josefa strain) isolated from a patient with cutaneous leishmaniosis in Brazil were transfected with green fluorescent protein (gfp) as previously described [19]. Promastigotes were cultured at 26 °C in Earle 199 medium with 10% fetal calf serum [19]. All the experimental protocols were approved by the Ethics Committee for Research of the Biology Institute, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

2.3. Assessment of IDs effects on L. amazonensis promastigotes

Promastigotes cultured in 96-well plates at 26 °C (10×10^6 promastigotes/well) were treated with different doses of IDs

during 2, 24, 48 h. Their numbers were determined using a Neubauer hemocytometer [20] and photographed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon). Additionally, the relative intensity of fluorescence was analyzed in gfp-promastigotes incubated on black microplates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) as previously described [19]. The IC50 describes the drug concentrations that inhibit 50% of the promastigote proliferation and, was calculated using a curve fitting program (GraphPad Prism 6 software).

2.4. Assessment of IDs effects on L. amazonensis amastigotes

Primary mouse macrophages were obtained from BALB/c mice by peritoneal lavage, cultured on 24-well plates (5×10^5 macrophages/well) containing 13-mm diameter glass coverslips as described previously [21,22] and infected with 15×10^5 amastigotes for 1 h. The cell cultures were then washed to remove extracellular parasites. Fresh medium and IDs were added to the plates maintained at 37 °C in 5% CO_2, 5% O_2, and balanced N_2 for 24 h. For the evaluation of the infection index (percentage of infected macrophages × number of amastigotes per macrophage), cells on coverslips were stained with Giemsa and examined microscopically at 1000 magnification as described previously [21,22]. The IC50 describes the drug concentrations that inhibit 50% of infection index and, was calculated using a curve fitting program (GraphPad Prism 6 software).

2.5. Cell viability

Cell cultures were stained with Giemsa and the number of adherent macrophages counted in 20 random fields as described previously [20]. The IC50 describes the drug concentrations that inhibit 50% macrophage viability and, was calculated using a curve fitting program (GraphPad Prism 6 software).

2.6. Statistical evaluation

All experiments were repeated at least three times and the results are expressed as the mean ± SD. Data obtained under different conditions were analyzed statistically using the Student *t* test for independent samples ($p \leq 0.05$).

3. Results

3.1. IDs effects on L. amazonensis promastigotes

Initially, experiments were undertaken to evaluate the viability of promastigotes treated with IDs (Table 1). The most of IDs treatments were evaluated at 24 h and 48 h incubation time and revealed no toxicity for promastigotes i.e., the number of parasites cultured with ID16, ID42s, ID185, ID187, ID189, ID190, ID92, ID193, ID393 and ID394 is similar to that of parasites cultured without IDs. The ID71, ID130, and ID195 treatments reduced the number of promastigotes (IC50, 82.30 ± 0.34, 85.90 ± 2.30 and 75.13 ± 2.70 μM, respectively) (Table 1). The data obtained with another method for quantifying promastigotes, the gfp-*Leishmania* based assay is in accordance with the results obtained by microscopy cell counting (data not shown). The IC50 value of a standard anti-leishmanial drug amphotericin B was 3.44 ± 0.20 μM; however, a complete reduction of promastigotes was not obtained, similar to the pattern observed in parasites treated with ID71, ID130 and ID195 (data not shown).

Table 2
In vitro activity of synthetic inhibitors of metabolic enzymes against *L. amazonensis* amastigotes and macrophages.

Compound	IC50 (μM)		Index[c]
	Amastigotes[a]	Macrophages[b]	
ID71	100 ± 2.25	97.1 ± 8.10	<1
ID130	106 ± 3.52	99.0 ± 2.23	<1
ID195	48.01 ± 3.20	119 ± 4.12	>2

[a] Drug concentration that inhibit 50% of the infection index at 24 h incubation time.
[b] Drug concentration that inhibit 50% of the macrophage viability at 24 h incubation time.
[c] IC50 towards macrophages divided by IC50 towards infection index.

3.2. IDs effects on L. amazonensis amastigotes within macrophages

Next, IDs preparations that are toxic for promastigotes (ID71, ID130, and ID195) were tested against macrophages and amastigotes infecting macrophages. Leishmanicidal effects and cytotoxicity of IDs were estimated by counting and morphological analyses of macrophages (Table 2,Fig. 1). The macrophage cultures that were untreated with compounds had infection index values around 300, i.e., 60–70% of infected macrophages and 4–5 amastigotes per macrophage. The macrophages infected with *L. amazonensis* and treated for 24 h with ID70 or ID130 showed reduction in the infection index (IC50, 100 ± 2.25 and 106 ± 3.52 μM, respectively) (Table 2). In general viability of untreated infected macrophages was maintained at 90–95% during cell culture period, but decay to viability was observed in macrophages treated with ID71 and ID130 (IC50, 97.1 ± 8.10 and 99.0 ± 2.23 μM, respectively) (Table 2). Fig. 1 shows representative images of macrophage cultures monolayer on glass cover slips; untreated and uninfected cell macrophages adhered and spread on glass, untreated infected macrophages contained large vacuoles that harbor amastigotes and, the ID70- and ID130-treated cell cultures contained small and more elongated macrophages together with cellular debris (Figs. 1A-1D). There was a dose-dependent reduction in the infection index of *L. amazonensis* infected macrophages treated with ID195 (IC50, 48.01 ± 3.20 μM) (Table 2) and the compound proved to be less toxic to macrophages (IC50, 48.01 ± 3.20 μM); cells treated with ID195 presented a healthy morphology and vacuoles containing few parasites or no parasites (Fig. 1E). The IC50 value for amphotericin B, which was

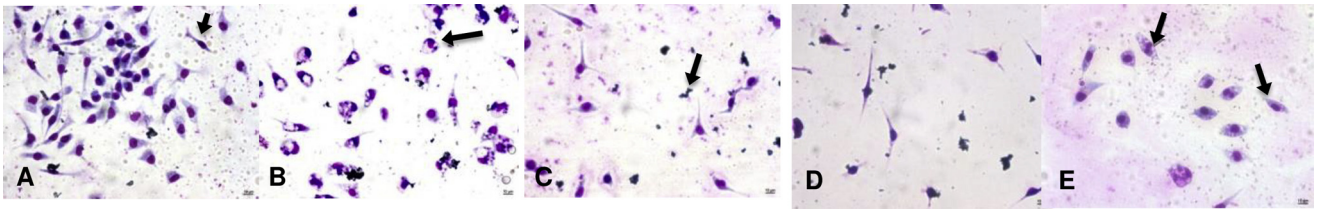


Fig. 1. Effect of enzyme synthetic inhibitors treatment on *L. amazonensis* amastigotes and macrophages. Mouse peritoneal macrophages were cultured for 24 h. Giemsa stained images of uninfected macrophages (A), the arrow indicates a macrophage with typical spread morphology; DMSO treated and infected macrophages (B), the arrow indicates a macrophage with vacuole containing amastigotes. ID70 treated and infected macrophages (C), the arrow indicates cellular debris; ID130 treated and infected macrophages (D); ID195 treated and infected macrophages (E), the arrows indicate macrophage without vacuoles and amastigotes. 1000×.

used as a positive control, was 0.03 ± 0.0001 μM, but a complete reduction of infection index was not obtained (data not shown).

4. Discussion

The metabolic pathway enzymes from trypanosomatides (*Leishmania*, *T. cruzi* and *Trypanosoma brucei*) have been proposed as targets for the rational search of new anti-parasite drugs [23,24]. Recently, some small synthetic molecules developed by virtual screening and isothermal titrating calorimetry have been identified as potent inhibitors of *T. cruzi* GAPDH and toxic for *S. cerevisae* and *T. cruzi* trypomastigotes [14,15]. In addition, the active site of DHODH to map structure differences between human, *T. cruzi* and *Leishmania* was studied [25]. The metabolic pathways enzymatic system similarity between trypanosomatides [25–27] prompted us to assay 17 compounds that are inhibitors designed of different metabolic enzymes with different scaffold and catalytic mechanism against *L. amazonensis*. Three out of the 17 compounds tested in this study exhibited toxicity against promastigotes. Some synthetic inhibitors of DHODH, glyceraldehyde 3-phosphate dehydrogenase and cruzain-cysteine protease (*T. cruzi*), scitalona-desidratase adenosine deaminase, succinate dehydrogenase complex II, and hydroxynaphthalene reductase (*S. cerevisiae*) did not have any effect on promastigotes. The reasons for the behavior of compounds are not clear but they could be related to their low affinity with the target enzymes. The compounds ID71, ID130, and ID195, which are inhibitors of DHODH from *T. cruzi* were toxic against *L. amazonensis* promastigotes. The DHODH is a dimeric enzyme that catalyzes the fourth step in the de novo pyrimidine biosynthetic pathway, with one flavin mononucleotide bound to each subunit as a prosthetic group [25]. This is also the fourth and rate-limiting step in the *de novo* pyrimidine pathway [28]. Despite the fact that the enzymatic function of DHODH is conserved in all organisms, the enzyme protein structure is quite different in prokaryotic and eukaryotic organisms. The predictive enzyme from *Leishmania major* has been cloned expressed in *Escherichia coli* crystallized and the structure solved by molecular replacement technique [29,30].

Leishmania two life cycles forms promastigotes found in insect vector and amastigotes adapted for life within macrophages differ in biochemical and antigenic characteristics and energy metabolism [31]. Thus the three compounds ID130, ID71 and ID195 were also tested against amastigotes within macrophage cultures. The compounds were toxic to amastigotes since they decrease the infection index. However ID71 and ID130 were not selective for parasite over the host since they affect macrophage survival. Similar results, i.e., enzyme inhibitors displaying toxicity toward macrophages were reported, for example, with inhibitors of cyclin depedent kinase [32] and compound inhibitors of *N*-myristoyl transferase [33]. The most promising compound ID195 was toxic against promastigotes and intracellular amastigotes; but when compared with amphotericin B ID195 was less toxic against the parasite. The ID195 decreased the infection index of macrophages infected with *L. amazonensis* (IC50, 48.01 ± 3.20 μM) at concentrations that did not significantly affect the survival of macrophages (IC50, 119 ± 4.12 μM). In addition, we have found that a significant decrease in the macrophage infection rate is obtained with concentrations of ID195 (IC50, 48.01 ± 3.20 μM) that are lower than those needed to inhibit promastigote viability (IC50, 75.13 ± 2.70 μM), suggesting that ID195 is transported across the macrophage plasma membrane reaching the PV and acting in amastigotes. To our knowledge, no studies addressing the effect of DHODH in *L. amazonensis* have been performed earlier. The data presented here suggest that ID195 may serve as chemical starting for other DHODH inhibitors; further studies will be requested to improve the selectivity and effectiveness of the enzyme synthetic inhibitors.

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