Bioorg. Med. Chem. Lett. 32 (2021) 127723

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design, synthesis, and evaluation of substrate – analogue inhibitors of *Trypanosoma cruzi* ribose 5-phosphate isomerase type B

Soledad Natalia Gonzalez [a,f], Jonathan J. Mills [b,f], Dante Maugeri [a], Christopher Olaya [b], Breana L. Laguera [c], Jeffrey R. Enders [d], Julian Sherman [e], Ana Rodriguez [e], Joshua G. Pierce [b], Juan José Cazzulo [a], Edward L. D'Antonio [c,*]

[a] *Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín – CONICET (IIBio-UNSAM), Avenida 25 de Mayo y Francia CP (1650), San Martín (Buenos Aires), Argentina*
[b] *Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Box 8204, Raleigh, NC 27695, USA*
[c] *Department of Natural Sciences, University of South Carolina Beaufort, 1 University Boulevard, Bluffton, SC 29909, USA*
[d] *Molecular Education, Technology and Research Innovation Center (METRIC), North Carolina State University, Raleigh, NC 27695, USA*
[e] *Department of Microbiology, New York University School of Medicine, 430 East 29th Street, New York, NY 10016, USA*

ARTICLE INFO

Keywords:
Neglected tropical diseases
Chagas' disease
Trypanosoma cruzi
Amastigote
Trypomastigote
Isomerase
Substrate-analogue inhibitors
Competitive inhibition
Trypsin digestion LC-MS/MS

ABSTRACT

Ribose 5-phosphate isomerase type B (RPI-B) is a key enzyme of the pentose phosphate pathway that catalyzes the isomerization of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P). *Trypanosoma cruzi* RPI-B (*Tc*RPI-B) appears to be a suitable drug-target mainly due to: (i) its essentiality (as previously shown in other trypanosomatids), (ii) it does not present a homologue in mammalian genomes sequenced thus far, and (iii) it participates in the production of NADPH and nucleotide/nucleic acid synthesis that are critical for parasite cell survival. In this survey, we report on the competitive inhibition of *Tc*RPI-B by a substrate – analogue inhibitor, Compound **B** ($K_i = 5.5 \pm 0.1$ μM), by the Dixon method. This compound has an iodoacetamide moiety that is susceptible to nucleophilic attack, particularly by the cysteine thiol group. Compound **B** was conceived to specifically target Cys-69, an important active site residue. By incubating *Tc*RPI-B with Compound **B**, a trypsin digestion LC-MS/MS analysis revealed the identification of Compound **B** covalently bound to Cys-69. This inhibitor also exhibited notable *in vitro* trypanocidal activity against *T. cruzi* infective life-stages co-cultured in NIH-3T3 murine host cells ($IC_{50} = 17.40 \pm 1.055$ μM). The study of Compound **B** served as a proof-of-concept so that next generation inhibitors can potentially be developed with a focus on using a prodrug group in replacement of the iodoacetamide moiety, thus representing an attractive starting point for the future treatment of Chagas' disease.

Chagas' disease is a vector borne disease of mammals caused by the parasitic protozoan *Trypanosoma cruzi* and constitutes a major health problem that is estimated to affect 6 to 7 million people worldwide.[1] Despite the fact that Chagas' disease was first described more than a century ago, chemotherapy is presently based only on two drugs, nifurtimox and benznidazole. These drugs are effective for acute phase infections, congenital infections, reactivated infections, and early chronic disease.[2] However, their efficacy during the chronic phase in adult patients is controversial.[3] These treatments are also prolonged and result in serious side effects that compromise the treatment continuity,[4,5] additionally, parasite resistance has been reported due to variations in the susceptibility of *T. cruzi* strains [6,7]. Another anti-infective that is effective at countering *T. cruzi* parasite growth is Amphotericin B. This compound is mainly used as an anti-Leishmanial agent,[1] but it has had a restricted clinical use for Chagas' disease. Therefore, the development of new chemotherapeutic agents against Chagas' disease has become an urgent need.

The pentose phosphate pathway (PPP) is a metabolic route that starts

Abbreviations: G6P, glucose 6-phosphate; PPP, pentose phosphate pathway; R5P, ribose 5-phosphate; RPI, ribose 5-phosphate isomerase; Ru5P, ribulose 5-phosphate; *Tc*RPI-B, *Trypanosoma cruzi,* ribose 5-phosphate isomerase type B.

* Corresponding author.
E-mail address: edantonio@uscb.edu (E.L. D'Antonio).
[f] Authors contributed equally to this work.

https://doi.org/10.1016/j.bmcl.2020.127723
Received 22 September 2020; Received in revised form 17 November 2020; Accepted 23 November 2020
Available online 26 November 2020

with glucose 6-phosphate (G6P), the first glycolytic intermediate, and is classically divided into two branches: an oxidative branch from G6P to ribulose 5-phosphate (Ru5P), with the reduction of two molecules of $NADP^+$; and a non-oxidative branch, which ultimately, when functioning as a cycle, leads back to glycolytic intermediates. The PPP plays two major roles in *T. cruzi* metabolism: (a) it provides NADPH, a coenzyme necessary for biosynthetic reactions and a key molecule for parasite protection against oxidative stress[8] and (b) it provides ribose 5-phosphate (R5P), which is used as a precursor for nucleotide biosynthesis.[9] Ribose 5-phosphate isomerase (RPI, EC 5.3.1.6) is an enzyme of the non-oxidative branch of the PPP and it catalyzes the reversible aldose – ketose isomerization between R5P and Ru5P **(**Fig. 1**)**. At the present time, there are two isoenzymes capable of catalyzing this reaction and they are known as RPI type A (RPI-A) and RPI type B (RPI-B). These enzymes do not share a common ancestor and they exhibit differences amongst their 1° – 3° protein structures.[10] The RPI-As are the most widely distributed in the three kingdoms of life, including most eukaryotic organisms, fungi, and some bacteria. Most RPI-Bs are found in prokaryotic organisms with a few exceptions in lower eukaryotes, such as the trypanosomatids, some fungi, and in the insect *Anopheles gambiae*.[11]

The essentiality of the RPIs was already shown for some organisms like *Escherichia coli*, *Saccharomyces cerevisiae*, and *Leishmania infantum*.[12–15] Moreover, although the essentiality was not completely proven in *Trypanosoma brucei*, silencing of the RPI-B gene led to the reduction of parasite growth and lower parasitemia of infected mice.[16] *T. cruzi* only expresses an RPI type B (*Tc*RPI-B), that was cloned, expressed, and characterized showing that residue Cys-69 was essential for the isomerization, and that His-102 was required for the opening of the furanose ring of R5P.[17] *Tc*RPI-B performs a vital role in all phases of the *T. cruzi* life cycle[8] and is absent from all mammalian genomes sequenced thus far. Interestingly, mammalian organisms have a structurally unrelated RPI-A. Since *Tc*RPI-B and *Homo sapiens* RPI-A (*Hs*RPI-A) are considered analogous enzymes and their active sites are completely different,[10] the design of highly selective inhibitors should be possible. Such inhibitors would serve the long-term goal in drug discovery by reducing adverse side effects in humans and by having a reference point to design newer inhibitors with enhanced anti-*T. cruzi* effectiveness.[18,19] Taken together, the crucial role of *Tc*RPI-B for the *T. cruzi* cell cycle, its essentiality (previously shown in other trypanosomatids), and the fact that it is non-homologous in comparison to other host mammalian RPIs, makes *Tc*RPI-B appear to be a suitable drug-target for the development of new chemotherapeutic agents against the parasite. This is particularly important since there have only been a limited number of inhibitors for RPIs that typically achieved inhibition constants (K_i) observed in the low millimolar range.[20,21]

The substrate of *Tc*RPI-B is R5P (Fig. 1) and previous designs of *Tc*RPI-B competitive inhibitors have been identified, such as D-allose 6-phosphate[22] and 4-phospho-D-erythronohydroxamic acid[17] (Fig. 2**a**). Moreover, a high-resolution X-ray crystal structure of the *Tc*RPI-B – R5P complex was previously solved and R5P is shown bound in the *Tc*RPI-B active site (PDB entry 3K7S; Fig. 3**a**).[22] Stern and colleagues determined that Cys-69 was essential for catalysis by site-directed mutagenic

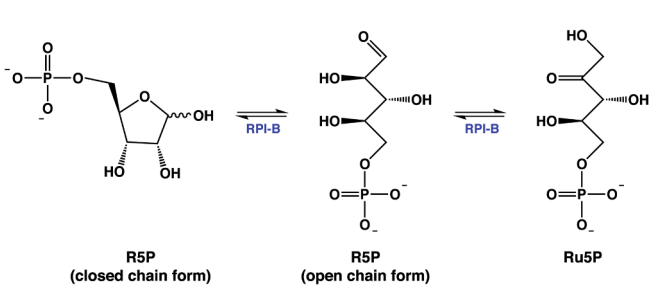

Fig. 1. Isomerization reaction between R5P and Ru5P as catalyzed by *Tc*RPI-B.

studies, and from this, we reasoned that substrate – analogue inhibitors could be used to target Cys-69 (Fig. 2**b**).[17] Interestingly, Cys-69 is positioned well into the active site cavity and since iodoacetamide groups are well known to react with cysteine sulfhydryl groups from peptide mapping experiments (Fig. 3**b**), we proposed that compounds containing a halogen group, such as compounds **B – E** (Fig. 2**b**) would irreversibly bind in the active site of *Tc*RPI-B through the same reaction by cysteine. A model for the inhibitor was constructed in reference to the known crystal structure of the *Tc*RPI-B – R5P complex using the program *WinCoot 0.8.9*. Compounds **B – E** were predicted to act as potent inhibitors and Fig. 3**c** shows a predicted 3-dimensional model for any of the "reacted" compounds **B – E** that would have lost their corresponding halogen atoms.

In the present study, a series of R5P (open-chain form) substrate – analogue derivatives termed Compounds **A** – **E** were conceived, and we set out to determine if those compounds were competitive inhibitors of *Tc*RPI-B. We determined that Compound **B** was the most potent inhibitor yet tested against *Tc*RPI-B ($K_i = 5.5 \pm 0.1$ μM), and it exhibited a notable *in vitro* trypanocidal activity (IC_{50} of 17.40 ± 1.055 μM). Thus, Compound **B** might represent an attractive starting point for the development of novel drugs for future treatment options of Chagas' disease, either alone or in combination with other inhibitors.

Compounds **A** – **E** (Fig. 2**b**) were tested for the possible inhibition of *Tc*RPI-B. Although Compound **A** was not part of the haloacetamide series, we included it as a derivative to explore if it could possibly inhibit the enzyme as a simple test. Compounds **B** – **E** are indeed haloacetamides that differ from each other just by having a different halogen X group. As a first step at inhibition testing, it was necessary to check if the compounds inhibited the enzymes used in the coupled assay employed to determine RPI activity. To this end, the activities of both *Tc*RPI-B and the first coupled enzyme, *T. cruzi* ribulose 5-phosphate epimerase (*Tc*RPE-1), were assayed as described (see Supplementary Information). Testing the *Tc*RPE-1 activity was enough, since an inhibitory effect on any of the other coupled enzymes would also be detected. The inhibition attained with four concentrations of the compounds (0.2, 0.5, 1.0, and 2.0 mM) on the activities of *Tc*RPI-B and *Tc*RPE-1 are shown in Tables 1 and 2, respectively. Compounds **A** and **D** had a similar low inhibitory effect on both enzymes at all of the concentrations assayed, indicating that *Tc*RPI-B was not inhibited. Compound **C** inhibited both *Tc*RPI-B (82%) and *Tc*RPE-1 (35%) at 2.0 mM. Since the inhibition of *Tc*RPI-B was not high, considering the inhibitor concentrations tested, and the inhibition of *Tc*RPE-1 (or any other of the coupled enzymes) was certainly not negligible, it would be very difficult to perform a kinetic study of this effect. Surprisingly, Compound **E** showed an unexpected behavior, since the activity of *Tc*RPI-B, but not that of *Tc*RPE-1, was very much increased (32-fold at 1.0 mM).

Compound **B** caused complete inhibition of *Tc*RPI-B (98.8%) at 1.0 mM, and at this concentration *Tc*RPE-1 was not inhibited. Therefore, Compound **B** was chosen for further kinetic studies. We determined the value of K_i for this compound, by performing a Dixon plot of 1/V as a function of [Compound **B**] **(**Fig. 4**a)** at three concentrations of R5P (0.4, 0.8, and 2 mM). The value calculated from the extrapolation of the intersection of the three lines with the X-axis was 5.5 ± 0.1 μM (mean of four independent experiments). The plot of [R5P]/V *vs.* [Compound **B**], at the same substrate concentrations assayed for the K_i determination is shown in Fig. 4**b**; since the three lines were parallel, the inhibition was determined to be competitive, as shown by the Cornish-Bowden method.[23]

Fig. 3 shows the substrate or inhibitor interactions in the active site of *Tc*RPI-B. To test the possibility of a covalent bond being formed between the inhibitor and Cys-69, *Tc*RPI-B was incubated with Compound **B** in the absence of substrate, and was subjected to trypsin digestion afterwards. The resultant enzymatic digestion was analyzed by LC-MS/MS. Protein segment [V66-R86] (Fig. 5) showed an $MS1^{3+}$ mass spectrum, which included the inhibitor, Compound **B**, covalently bound to Cys-69 (color-coded red). The portion of the inhibitor that linked onto

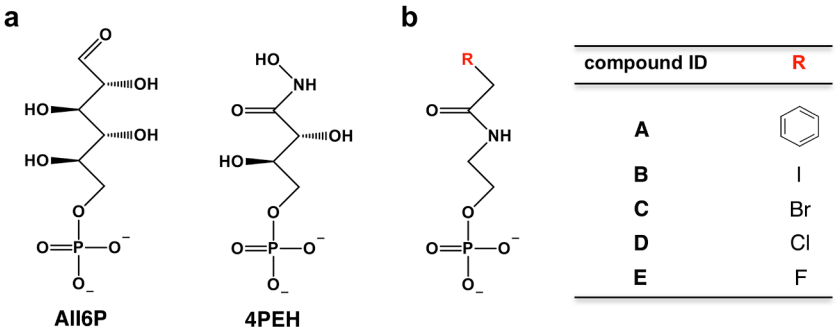

Fig. 2. *Tc*RPI-B inhibitors. (a) Open-chain forms of inhibitors D-allose 6-phosphate (All6P, K_i = 15 mM) and 4-phospho-D-erythronohydroxamic acid (4PEH, K_i = 1.2 mM). (b) Compounds **A** – **E** as substrate – analogue inhibitors conceived by using an inhibitor model closely related to R5P/Ru5P.

Cys-69 had a molecular mass of 178.9984 g/mol; additionally, that portion of the inhibitor lacked the iodine atom. The $MS1^{3+}$ peak appeared in the mass spectrum at 727.0285, which included residue segment V66-R86 and the bound inhibitor portion. Other protein segments appeared in the mass spectrum analysis that did not include the bound inhibitor, such as the y_4^+ [protein segment: P83-R86], y_5^+ [protein segment: V82-R86], y_9^+ [protein segment: A78-R86], and y_{11}^+ [protein segment: S76-R86]. The following masses were detected: 428.2621 for y_4^+, 527.3288 for y_5^+, 911.5163 for y_9^+, and 1111.6832 for y_{11}^+.

In order to evaluate the trypanocidal activity of the compounds, a trypomastigote mammalian host cell infection assay was carried out using a transgenic *T. cruzi* strain expressing the reporter enzyme β-galactosidase (see Supplementary Information). Through these assays, β-galactosidase activity was considered proportional to *T. cruzi* amastigote – trypomastigote viability. Results illustrated in Fig. 6**a** show that Compound **B** was found to be selectively active against *T. cruzi* infective life-stages co-cultured in NIH-3T3 murine host cells, displaying a moderate micromolar inhibition of *T. cruzi in vitro*, with an IC_{50} of 17.40 ± 1.055 μM. The reason why Fig. 6**a** does not show a plateau in its IC_{50} plot is due to not screening to higher concentrations of Compound **B** beyond 50 μM. The other compounds, Compounds **A** and **C** – **E**, were ineffective in the dose range tested (up to 50 μM). None of the compounds tested were toxic to 3T3 cells before significantly affecting the growth of parasites. Under these assay conditions, the IC_{50} of amphotericin B used as a positive control was found to be 0.23 ± 0.02 μM **(**Fig. 6**b)**, comparable with the value of 0.125 μM reported by Horvath and Zierdt.[24]

Currently available drugs for Chagas' disease treatment are effective for acute phase infections, congenital infections, reactivated infections, and the early chronic phase disease,[2] but have a controversial efficacy during the chronic phase in adult patients.[3] The chronic phase is a category where a majority of people are currently infected with *T. cruzi*. Furthermore, these drugs also seem to be inadequate for their toxicity, leading to discontinuation of treatment for about 30% of patients[4,5] and for the resistance developed by some strains of the parasite.[6,7] As such, there is a need for the development of new therapeutic agents against Chagas' disease capable of targeting all stages of the disease in a safe and efficacious manner. For all the reasons stated in the Introduction, *Tc*RPI-B appears to be a suitable drug-target. We tested five potential *Tc*RPI-B substrate – analogue inhibitors that gave rise to a series of R5P (open chain form) derivatives (Compounds **A – E**), most of them predicted to react with the sulfhydryl group of a cysteine sidechain in the active site of the enzyme. Although haloacetamides are quite reactive compounds and are probably not the most practical types of inhibitors to be used as drugs because of cross reactivity, our study investigated on a proof-of-concept purpose to ascertain the anti-*T. cruzi* effect and the effectiveness of these compounds as substrate – analogue inhibitors against *Tc*RPI-B. From all of the compounds tested, it was only Compound **B** that caused complete inhibition of *Tc*RPI-B (98.8%) at 1.0 mM concentration (Table 1), at which *Tc*RPE-1 was not inhibited (Table 2). Kinetic studies showed that Compound **B** acted as a competitive inhibitor of *Tc*RPI-B, competing with its natural substrate R5P with a K_i of 5.5 ± 0.1 μM (Fig. 4**a**). To the best of our knowledge, Compound **B** is the most potent inhibitor tested against *Tc*RPI-B. This finding is very interesting since only a limited number of inhibitors of RPIs have been reported in the literature.[20,21] All of the reported inhibitors are analogues of the intermediate *cis*-enediolate (5-phospho-D-ribonohydroxamic acid) of the isomerization reaction, with K_i values of 1.2 mM or higher.[25] Moreover, for the majority of these compounds, only the IC_{50} values were determined.[17] From the above mentioned series of inhibitors, the more promising one, 4PEH, which achieved a K_i in the low millimolar range (K_i of 1.2 mM), was also described to be a potent competitive inhibitor (K_i of 0.029 ± 0.003 mM) of the spinach RPI-A[21] enhancing the occurrence of an off-target effect over human RPI-A if it were employed as part of a Chagas' disease chemotherapy. Surprisingly, we observed that *Tc*RPI-B in the presence of Compound **E** revealed a substantial increase in activity with substrate R5P (Table 1), functioning as an enzyme activator instead of an inhibitor, despite its similarity with Compounds **B – D**. This striking behavior resulted in a very interesting observation and we will certainly go further into this matter in future surveys, since it was not the purpose of the present work.

To further study the enzyme – inhibitor interaction, we first attempted to obtain the X-ray crystal structure of the *Tc*RPI-B – Compound **B** complex, that would have included a more thorough structural understanding of this interaction. However, it was not possible at the present time to obtain good X-ray diffraction quality protein crystals. In the inhibitor soaking experiments of inhibitorless *Tc*RPI-B crystals with Compounds **A** – **E** dissolved in mother liquor, the method seemed to be quite harsh to yield crystals having a suitable integrity for X-ray diffraction. Even after several attempts of crystal soaking, or even co-crystallization trials, inhibitorless *Tc*RPI-B crystals would substantially degrade or they simply would not afford crystal growth, respectively. In order to study this interaction through another experimental method, we incubated intact *Tc*RPI-B with Compound **B** for 30 min followed by a trypsin digestion. The sample was then tested by LC-MS/MS to see if the sulfhydryl sulfur-atom of Cys-69 would make a covalent bond with a carbon-atom of the iodoacetamide portion of the inhibitor. Fig. 5 displays a convincing $MS1^{3+}$ peak with the added molecular mass of 178.9984 added to protein segment VLA**C**GSGIGMSIAANKVPGVR (bolded Cys-69) totaling a mass of 727.0285 with the inhibitor bound after covalent attachment. This result showcases that Compound **B** reacted with *Tc*RPI-B at site Cys-69 with the mass of the iodine atom not being present, as expected. More specifically, a carbon from the iodoacetamide moiety of the inhibitor underwent a nucleophilic attack from the sulfhydryl group of Cys-69 at the enzyme substrate binding site. In summary, we demonstrated a competitive inhibition mediated by Compound **B**, in the presence of R5P, while in its absence, the results of our study indicated a mechanism – based inactivation. The difference observed in both approaches could be due to the protective effect of the substrate on the active center.

For the biological evaluation of all compounds, we tested for

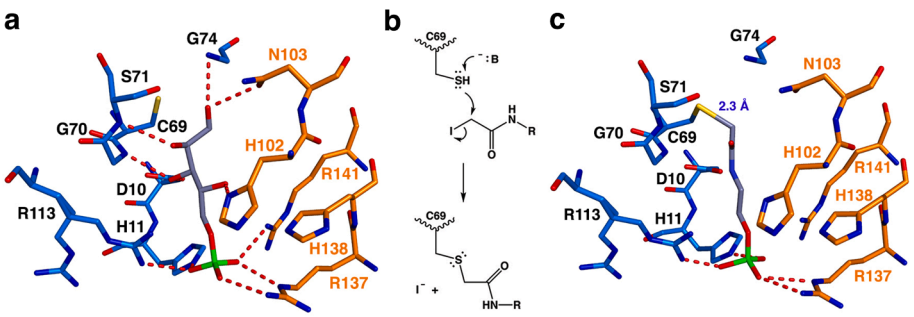

Fig. 3. Substrate or inhibitor interactions in the active site of *Tc*RPI-B. (a) X-ray crystal structure of the *Tc*RPI-B – R5P complex (PDB entry 3K7S) in the active site. Carbon atoms color-coded blue and orange represent different subunits. (b) Proposed reaction mechanism for the sulfur – carbon bond formation between the sulfhydryl group of *Tc*RPI-B (Cys-69) and an iodoacetamide moiety from Compound **B**. (c) Proposed irreversible complex of Compound **B** bound in the active site of *Tc*RPI-B.

Table 1
Enzymatic inhibition of *Tc*RPI-B by Compounds **A – E**.[a]

Inhibitor (mM)	% of *Tc*RPI-B activity				
	A	**B**	**C**	**D**	**E**
0.2	98.1 ± 3.2	25.2 ± 3.5	78.5 ± 4.8	107 ± 9	994 ± 107
0.5	97.4 ± 0.5	7.5 ± 3.9	62.7 ± 5.9	105 ± 7	2042 ± 686
1.0	95.3 ± 4.2	1.2 ± 0.3	41.3 ± 2.7	98.5 ± 2.1	3194 ± 921
2.0	89.2 ± 3.5	0.0 ± 0.0	18.3 ± 2.5	83.7 ± 8.9	2718 ± 269

NOTE: [a] Effect of compounds over *Tc*RPI-B activity. Inhibition attained with four concentrations (0.2, 0.5, 1.0, and 2.0 mM) of Compounds **A** – **E** on the activity of *Tc*RPI-B. The percentages of activity shown are the mean of three independent determinations ± SEM.

Table 2
Enzymatic inhibition of *Tc*RPE-1 by Compounds **A – E**.[a.]

Inhibitor (mM)	% of *Tc*RPE-1 activity				
	A	**B**	**C**	**D**	**E**
0.2	100 ± 5	89.5 ± 4	91.1 ± 5.1	100 ± 6	111 ± 10
0.5	91.9 ± 4.3	89.5 ± 4	85.8 ± 4.4	103 ± 5	103 ± 7
1.0	87.4 ± 3.1	103 ± 5	77.3 ± 7.5	95.5 ± 7.9	93.2 ± 11.1
2.0	85.9 ± 4.4	57.4 ± 0.4	65.1 ± 5.2	82.6 ± 12	83.5 ± 9.7

NOTE: [a]Effect of compounds over *Tc*RPE-1 activity. Inhibition attained with four concentrations (0.2, 0.5, 1.0, and 2.0 mM) of Compounds **A** – **E** on the activity of *Tc*RPE-1. The percentages of activity shown are the mean of three independent determinations ± SEM.

trypanocidal activity against *T. cruzi* infecting mammalian cells. Since *T. cruzi* trypomastigotes are incubated with the host cells at the same time as the test compounds, the assay determines the activity of the compounds against trypomastigote infection of host cells and/or amastigote proliferation after host cell invasion. The use of the trypomastigote form and the amastigote form is relevant for drug discovery studies since they are the relevant parasite stages of mammalian host infection.[26,27] Additionally, the amastigote form is responsible for the chronic phase of Chagas' disease, where the available treatments are only partially beneficial.[28] Through this approach, we found that while none of the compounds tested were toxic to 3T3 cells before significantly affecting the growth of parasites, only Compound **B** was capable of exerting a moderate trypanocidal activity *in vitro* (Fig. 6) with an anti-*T. cruzi* effect, which was very close to that of benznidazole.[29] However, while the use of nitroimidazoles draws in concern over cytotoxicity, mutagenicity, and genotoxicity apparently related to DNA damage by the bioreduction products of the nitro group,[30] a substrate – analogue inhibitor such as Compound **B** might avoid these types of issues related to adverse effects during treatment.

The IC_{50} observed for Compound **B** *vs*. *T. cruzi* infective life-stages was 17.40 ± 1.055 μM and is moderately potent. In comparison of this assay to the *Tc*RPI-B enzyme inhibition assay, where Compound **B** was determined to have a K_i of 5.5 ± 0.1 μM (Fig. 4), the IC_{50} value is predicted to be 11 μM (e.g. $IC_{50} = 2K_i$ for the case of competitive inhibition and when $[S] = K_M$.[31,32] Due to the high reactivity of the iodoacetamide moiety with sulfhydryl functional groups, it was surprising to observe such close IC_{50} values between assays, which most likely correlates to low off-target effects. However, we expect Compound **B** to have a higher degree of cross-reactivity if introduced into *in vivo* assays. Treatment of a parasitic disease has the challenge where a drug requires good biological stability, in particular, being able to not react with anything in the bloodstream of the mammalian host. As such, it would be strategic to re-design Compound **B** into a prodrug where it would have a masking group on the iodoacetamide moiety. Once the prodrug makes entry into the parasite cell by passive diffusion and activates by either enzymatic or chemical cleavage, Compound **B** could quickly react with Cys-69 in the active site of *Tc*RPI-B. Ruda and co-workers have examined six prodrug designs that have mostly been successful in *T. brucei* parasites where they created a masking group for the phosphate component of their inhibitors of 6-phosphogluconate dehydrogenase.[33,34] In creating prodrug designs for Compound **B** that in turn target *Tc*RPI-B, could, in the long-term lead to novel therapeutic agents that may well be employed as an alternative treatment for the chronic phase of Chagas' disease either alone or in combination with other inhibitors. Although this path is still very long, the identification of novel promising lead compounds for drug development in Chagas' disease is an important first step towards this.

The work described herein was centered on the design and synthesis of substrate – analogue inhibitors of *Tc*RPI-B, a promising drug-target in the search for a new alternative to the current treatment of Chagas' disease. The inhibitors developed (Compounds **A** – **E**) have haloacetamide functional groups (principally) for the purpose of forming a

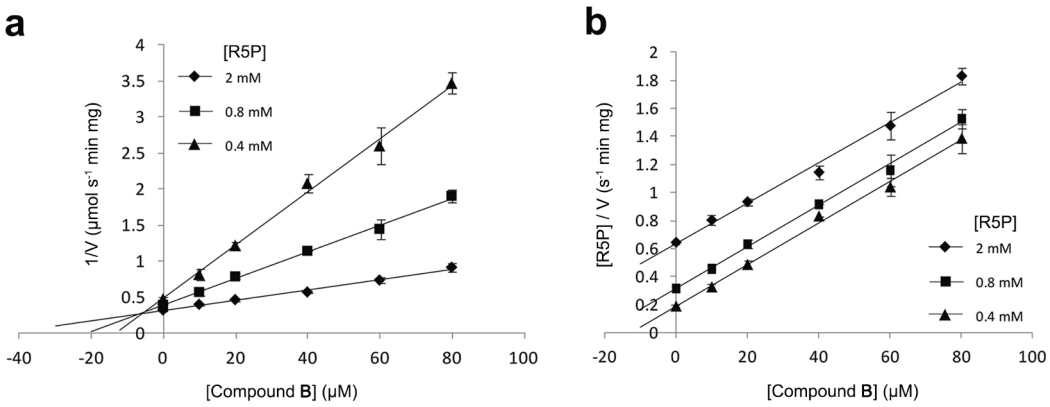

Fig. 4. Kinetics of inhibition: *Tc*RPI-B and Compound **B**. (a) Dixon plots of 1/V as a function of [Compound **B**] to determine the value of K_i for Compound **B**. The value calculated from the extrapolation of the intersection of the three lines with the X-axis was 5.5 ± 0.1 µM (mean of four independent experiments). (b) Plot of [R5P]/V *vs.* [Compound **B**], to determine the type of inhibition for Compound **B**. Since the three lines were observed to be parallel, the inhibition was concluded as competitive inhibition. The inhibitor concentrations assayed were 0, 10, 20, 40, 60, and 80 µM, at three concentrations of R5P (0.4, 0.8, and 2 mM). In panels (a) and (b), one of four independent experiments are shown as an example. The experimental points shown are the mean of two determinations.

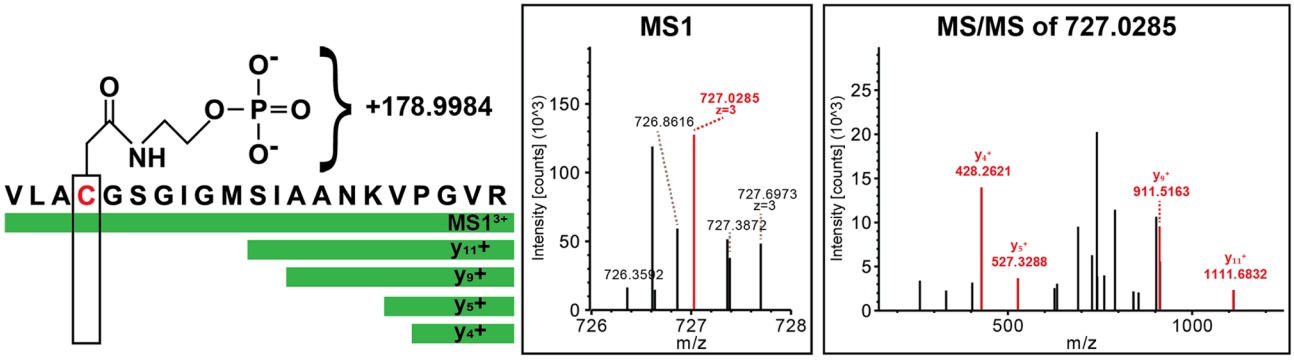

Fig. 5. The tandem mass spectrum of the peptide from *Tc*RPI-B (residues V66-R86) that is labeled to Cys-69 by Compound **B** (left panel). The MS1-ion that is labeled by the inhibitor lacking the iodine atom (+178.9984 Da) is colored-coded in red with mass shown (middle panel). The unlabeled y-ions are colored-coded in red and masses are shown (right panel). All unlabeled y-ions have a + 1 charge and the MS1-ion has a + 3 charge.

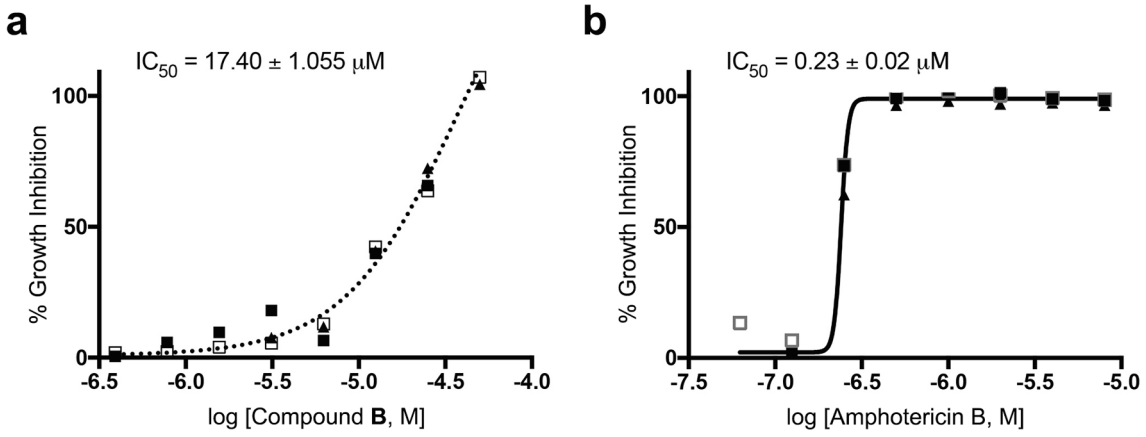

Fig. 6. Dose-response curves expressed as the percentage of parasite growth inhibition as a function of inhibitor concentration of (a) the *Tc*RPI-B competitive inhibitor, Compound **B**, activity and (b) amphotericin B activity on *T. cruzi* (Tulahuen strain) intracellular amastigote growth inhibition in NIH-3T3 fibroblasts. The IC_{50} determinations were made through *GraphPad Prism*. All IC_{50} measurements were performed in triplicate.

covalent bond with an internal cysteine thiol group located in the active site. The study followed by means of a biochemical and biological evaluation that revealed Compound **B** to bind as a competitive inhibitor in the active site of *Tc*RPI-B with a K_i of 5.5 ± 0.1 µM and that the sulfhydryl sulfur atom of Cys-69 forms a covalent attachment with a carbon-atom of the iodoacetamide portion of Compound **B** less the iodine atom. Furthermore, Compound **B** revealed substantial trypanocidal activity with an IC_{50} of 17.40 ± 1.055 µM. Although the compounds were designed as a proof-of-concept, in which a substrate – analogue inhibitor class could be demonstrated for *Tc*RPI-B, the

iodoacetamide moiety is very susceptible to nucleophilic attack from thiol groups, moreover, in order to further improve the design of Compound **B** to create a more effective compound for an *in vivo* scenario, altering the iodoacetamide moiety to include a temporary inert prodrug form as a replacement seems attractive.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The work was supported by an Advanced Support for Innovative Research Excellence: Track I (ASPIRE-I) grant to E.L.D. and an undergraduate research grant through the Magellan program to B.L.L. that were provided by the University of South Carolina, Office of the Vice President for Research. This work was additionally supported by the National Agency for Promotion of Scientific and Technological Research to J.J.C. from the Argentinian Ministry of Science and Technology (ANPCyT, MinCyT), grant PICT 2013-0969. We thank Zachary J. Taylor for technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127723.

References

1 World Health Organization, Media Centre Fact Sheets Homepage. https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis). Updated 2019 (accessed Jan, 2019).
2 Boscardin SB, Torrecilhas ACT, Manarin R, et al. Chagas' disease: an update on immune mechanisms and therapeutic strategies. *J Cell Mol Med*. 2010;14:1373–1384.
3 Duschak VG, Couto AS. An insight on targets and patented drugs for chemotherapy of Chagas disease. *Recent Pat Anti-Infect Drug Discovery*. 2007;2:19–51.
4 Castro JA, de Mecca MM, Bartel LC. Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Hum Exp Toxicol*. 2006;25:471–479.
5 Viotti R, Vigliano C, Lococo B, et al. Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities, *Expert Rev. Anti-Infect Ther*. 2009;7:157–163.
6 Murta SMF, Gazzinelli RT, Brener Z, Romanha AJ. Molecular characterization of susceptible and naturally resistant strains of *Trypanosoma cruzi* to benznidazole and nifurtimox. *Mol Biochem Parasitol*. 1998;93:203–214.
7 Wilkinson SR, Taylor MC, Horn D, Kelly JM, Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc Natl Acad Sci USA*. 2008;105:5022–5027.
8 Barrett MP. The pentose phosphate pathway and parasitic protozoa. *Parasitol. Today*. 1997;13:11–16.
9 Maugeri DA, Cazzulo JJ. The pentose phosphate pathway in *Trypanosoma cruzi*, *FEMS Microbiol. Lett.* 2004;234:117–123.
10 Capriles PV, Guimarães AC, Otto TD, Miranda AB, Dardenne LE, Degrave WM. Structural modelling and comparative analysis of homologous, analogous and specific proteins from *Trypanosoma cruzi* versus *Homo sapiens*: putative drug targets for Chagas' disease treatment. *BMC Genomics*. 2010;11:610.
11 Morrison HG, McArthur AG, Gillin FD, et al. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science*. 2007;317:1921–1926.
12 Skinner AJ, Cooper RA. The regulation of ribose-5-phosphate isomerisation in *Escherichia coli* K12. *FEBS Lett*. 1971;12:293–296.
13 Sørensen KI, Hove-Jensen B. Ribose catabolism of *Escherichia coli*: characterization of the rpiB gene encoding ribose phosphate isomerase B and of the rpiR gene, which is involved in regulation of rpiB expression. *J Bacteriol*. 1996;178:1003–1011.
14 Miosga T, Zimmermann FK. Cloning and characterization of the first two genes of the non-oxidative part of the *Saccharomyces cerevisiae* pentose-phosphate pathway. *Curr Genet*. 1996;30:404–409.
15 Faria J, Loureiro I, Santarém N, et al. Disclosing the essentiality of ribose-5-phosphate isomerase B in trypanosomatids. *Sci Rep*. 2016;6:1–16.
16 Loureiro I, Faria J, Clayton C, et al. Ribose 5-phosphate isomerase B knockdown compromises *Trypanosoma brucei* bloodstream form infectivity. *PLoS Neglected Trop. Dis.* 2015;9, e3430.
17 Stern AL, Burgos E, Salmon L, Cazzulo JJ. Ribose 5-phosphate isomerase type B from *Trypanosoma cruzi*: kinetic properties and site-directed mutagenesis reveal information about the reaction mechanism. *Biochem J*. 2007;401:279–285.
18 Adams CP, Brantner VV. Estimating the cost of new drug development: is it really 802 million dollars? *Health Aff (Millwood)*. 2006;25:420–428.
19 Cavasotto CN, Phatak SS. Homology modeling in drug discovery: current trends and applications. *Drug Discovery Today*. 2009;14:676–683.
20 Woodruff W, Wolfenden R. Inhibition of ribose-5-phosphate 4-phosphoerythronate isomerase. *J Biol Chem*. 1979;254:5866–5867.
21 Burgos E, Salmon L. Synthesis and evaluation of new 4-phospho-D-erythronic acid derivatives as competitive inhibitors of spinach ribose-5-phosphate isomerase. *Tetrahedron Lett*. 2004;45:753–756.
22 Stern AL, Naworyta A, Cazzulo JJ, Mowbray SL. Structures of type B ribose 5-phosphate isomerase from *Trypanosoma cruzi* shed light on the determinants of sugar specificity in the structural family. *FEBS J*. 2011;278:793–808.
23 Cornish-Bowden A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem J*. 1974;137:143–144.
24 Horvath AE, Zierdt CH. The effect of amphotericin B on *Trypanosoma cruzi in vitro* and *in vivo*. *J. Trop. Med. Hyg.* 1974;77:144–149.
25 Sinatti, V. d. V. C., Baptista, L. P. R., Alves-Ferreira, M., Dardenne, L., Silva, J. H. M., and Guimarães, A. C. (2017) In silico identification of inhibitors of ribose 5-phosphate isomerase from Trypanosoma cruzi using ligand and structure based approaches, J. Mol. Graphics Modell. 77, 168-180.
26 Soeiro MNC, Werbovetz K, Boykin DW, Wilson WD, Wang MZ, Hemphill A. Novel amidines and analogues as promising agents against intracellular parasites: a systematic review. *Parasitology*. 2013;140:929–951.
27 Zingales B, Miles MA, Moares CB, et al. Drug discovery for Chagas disease should consider *Trypanosoma cruzi* strain diversity. *Mem. Inst. Oswaldo Cruz*. 2014;109:828–833.
28 Buschini AM, Ferrarini L, Franzoni S, et al. Genotoxicity revaluation of three commercial nitroheterocyclic drugs: nifurtimox, benznidazole, and metronidazole. *J. Parasitol. Res.* 2009;2009:1–11.
29 Von Trompowsky ACM, Conde TR, Lemos RC, et al. *In vitro* genotoxicity of nitroimidazoles as a tool in the search of new trypanocidal agents. *Mem Inst Oswaldo Cruz*. 2019;114(e190017):1–10.
30 Boechat N, Carvalho AS, Salomão K, et al. Studies of genotoxicity and mutagenicity of nitroimidazoles: demystifying this critical relationship with the nitro group. *Mem Inst Oswaldo Cruz*. 2015;110:492–499.
31 Cer RZ, Mudunuri U, Stephens R, Lebeda FJ. IC_{50}-to-K_i: a web-based tool for converting IC_{50} to K_i values for inhibitors of enzyme activity and ligand binding. *Nucleic Acids Res*. 2009;37:W441–W445.
32 Haupt LJ, Kazmi F, Ogilvie BW, et al. The reliability of estimating K_i values for direct, reversible inhibition of cytochrome P450 enzymes from corresponding IC_{50} values: a retrospective analysis of 343 experiments. *Drug Metab Dispos*. 2015;43:1744–1750.
33 Ruda GF, Alibu VP, Mitsos C, et al. Synthesis and biological evaluation of phosphate prodrugs of 4-phospho-D-erythronohydroxamic acid, an inhibitor of 6-phosphogluconate dehydrogenase. *ChemMedChem*. 2007;2:1169–1180.
34 Ruda GF, Wong PE, Alibu VP, et al. Aryl phosphoramidates of 5-phospho erythronohydroxamic acid, a new class of potent trypanocidal compounds. *J Med Chem*. 2010;53:6071–6078.