Disulfiram as a novel inactivator of *Giardia lamblia* triosephosphate isomerase with antiangiardial potential

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**A B S T R A C T**

Giardiasis, the infestation of the intestinal tract by *Giardia lamblia*, is one of the most prevalent parasitosis worldwide. Even though effective therapies exist for it, the problems associated with its use indicate that new therapeutic options are needed. It has been shown that disulfiram eradicates trophozoites *in vitro* and is effective *in vivo* in a murine model of giardiasis; disulfiram inactivation of carbamate kinase by chemical modification of an active site cysteine has been proposed as the drug mechanism of action. The triosephosphate isomerase from *G. lamblia* (GTIM) has been proposed as a plausible target for the development of novel antiangiardial pharma-therapies, and chemical modification of its cysteine 222 (C222) by thiol-reactive compounds is evidenced to inactivate the enzyme. Since disulfiram is a cysteine modifying agent and GTIM can be inactivated by modification of C222, in this work we tested the effect of disulfiram over the recombinant and trophozoite-en-dogenous GTIM. The results show that disulfiram inactivates GTIM by modification of its C222. The inactivation is species-specific since disulfiram does not affect the human homologue enzyme. Disulfiram inactivation induces only minor conformational changes in the enzyme, but substantially decreases its stability. Recombinant and endogenous GTIM inactivates similarly, indicating that the recombinant protein resembles the natural enzyme. Disulfiram induces loss of trophozoites viability and inactivation of intracellular GTIM at similar rates, suggesting that both processes may be related. It is plausible that the giardicidal effect of disulfiram involves the inactivation of more than a single enzyme, thus increasing its potential for repurposing it as an antiangiardial drug.

1. Introduction

*Giardia lamblia* (Syn. *Giardia duodenalis, Giardia intestinalis*) is a flagellated, bi-nucleate protozoan parasite of the Diplomonadida order that colonizes the human upper small intestine causing giardiasis. The clinical picture of the acute disease is characterized by diarrhea, abdominal pain, nausea and vomit, but chronic infections can progress to malabsorption syndrome, malnutrition and growth retardation in the pediatric population. Giardiasis is one of the main parasitosis in the world with a global estimate of 280 million symptomatic infections per year (Lane and Lloyd, 2002). First-line pharmacotherapies against *G. lamblia* include nitroimidazoles (metronidazole and tinidazole) and benzimidazoles (albendazole or mebendazole), although nitazoxanide, furazolidone, quinacrine, chloroquine and paramomycin can also be effective (Busatti et al., 2009; Watkins and Eckmann, 2014). Even though current therapies have proven to be useful, many have unpleasant side effects, which reduce treatment compliance. Additionally, recurrence rates are high and first-line treatment failures are not uncommon (Busatti et al., 2009; Watkins and Eckmann, 2014). Furthermore, pharmacological resistance, both *in vivo* and *in vitro*, has been documented (reviewed in Ansell et al., 2015; Leitsch, 2015). Together, the available data indicates that new therapeutic alternatives against *G. lamblia* are required.

In the quest of new therapeutic options for giardiasis, diverse approaches have been proposed, including for example, the use of natural products, the generation of vaccines and the synthesis of new chemical...
compounds (García-Torres et al., 2012). In this regard, drug repurposing stands out as an attractive option to find alternative anti-giardiasis options. Drug repurposing refers to the development of new clinical indications for existing, approved drugs (Oprea et al., 2011; Martorana et al., 2016; Sbaraglini et al., 2016). The reasoning behind this idea is that the de novo development of a drug is a long and expensive process, while repositioning drugs that have already passed preclinical and clinical stages can substantially reduce the risks and costs of production (Oprea et al., 2011; Martorana et al., 2016; Sbaraglini et al., 2016). On this view, the drug disulfiram has emerged as an interesting option for repurposing.

Disulfiram (Fig. 1A) was the first FDA approved drug to treat alcohol dependence; it causes aversion to alcohol by an acute toxic reaction when both substances are combined. Disulfiram reacts covalently with sulfhydryl groups of free cysteine residues forming a dithio diethylcarbamoyl adduct (Fig. 1B). This drug inactivates human aldehyde dehydrogenase by chemical modification of an active site cysteine residue, hindering the metabolism of acetaldehyde and causing its accumulation in the bloodstream. The accumulation of acetaldehyde causes unpleasant effects such as headache, sweating, flushing, dizziness, palpitations, nausea and vomiting. Owing to its toxicity and associated non-compliance, disulfiram is no longer considered in the first line of pharmacological options for the treatment of alcoholism (Crowley, 2015). In addition to its primary indication for alcohol addiction, several alternative uses for disulfiram have been proposed.

Disulfiram blocks invasion and angiogenesis (Shian et al., 2003), inhibits the activity of cancer multidrug resistance proteins (Sauna et al., 2004), and the ubiquitin-proteasome system (Kona et al., 2011); therefore, it has been considered as a potential adjuvant medication for cancer. By its effect inactivating the betaine aldehyde dehydrogenase of Pseudomonas aeruginosa, disulfiram has been proposed as a plausible antimicrobial agent (Velasco-García et al., 2006); and by its deleterious effects on the hepatitis C and respiratory syncytial viruses as a novel antiviral drug (Lee et al., 2016; Boukhvalova et al., 2010). Interestingly, disulfiram has proven to be effective against G. lamblia trophozoites in vitro and in a murine model of giardiasis (Nash and Rice, 1998; Galkin et al., 2014). It has been proposed that the anti-giardial activity of disulfiram can be related to the inactivation of the G. lamblia carbamate kinase (Galkin et al., 2014) or to an unidentified Zn-finger protein (Nash and Rice, 1998). Therefore, in the face of the broad repertoire of proteins targeted by disulfiram, we hypothesized that the anti-giardial effect of this drug could involve more than one molecular target. The idea is attractive because a multi-target drug can be more efficient by acting synergistically at diverse levels.

G. lamblia relies exclusively on fermentative metabolism for ATP generation (Adam, 2001); therefore, glycolytic enzymes have been suggested as molecular targets for anti-giardial drug design (Hilpold et al., 1999; López-Velázquez et al., 2004; Galkin et al., 2007). In this connection, the triosephosphate isomerase from G. lamblia (GITIM) has been previously proposed as a feasible target against which to develop new pharmacotherapies (López-Velázquez et al., 2004). The plausibility of GITIM as a pharmacological target is supported by RNA-interference experiments showing that the decrease of the GITIM expression is incompatible with trophozoite survival (Marcial-Quino et al., manuscript in preparation).

Previous work further showed that chemical modification of C222 by thiol-reactive compounds inactivated GITIM at micromolar concentrations with minor effects on the activity of human triosephosphate isomerase (HsTIM) (Enriquez-Flores et al., 2008; Enriquez-Flores et al., 2011). By analogy with its mechanism of action, proton pump inhibitors (PPIs) were suggested as GITIM inactivators (Reyes-Vivas et al., 2014; García-Torres et al., 2016). PPIs (omeprazole and its derivatives, lansoprazole, pantoprazole, esomeprazole and rabeprazole) acts on acid-peptic disease by inactivating the gastric H+ -ATPase by chemical modification of an essential cysteine residue on the enzyme. PPIs inactivated GITIM in the micromolar range by covalent modification of C222 without affecting HsTIM (Reyes-Vivas et al., 2014; García-Torres et al., 2016). In addition, omeprazole showed to be effective against wild type and drug-resistant G. lamblia strains showing potency similar to first-line drugs used for giardiasis (Reyes-Vivas et al., 2014). The cytotoxic effect of omeprazole was concomitant with the inactivation of GITIM on trophozoites, suggesting that both processes are related (Reyes-Vivas et al., 2014).

Since disulfiram is a recognized cysteine modifer agent and GITIM can be inactivated by modification of its C222, in this work we explored the effect of disulfiram on GITIM. The results indicate that disulfiram efficiently and selectively inactivates recombinant GITIM by chemical modification of C222. The inactivation of GITIM entails minor conformational changes and concurs with decreased stability of the protein. Recombinant and endogenous GITIM are inactivated similarly by disulfiram, indicating that the recombinant protein resembles the endogenous enzyme. Disulfiram induces loss of trophozoites viability and inactivation of intracellular GITIM at similar rates, suggesting that both processes may be related. Therefore, it is plausible that the anti-giardial effect of disulfiram involves more than a molecular target.

2. Material and methods

2.1. General materials and procedures

Analytical grade salts and buffers were acquired from Sigma-Aldrich. Glycerol-3-phosphate dehydrogenase (GDH) and NADH were purchased from Roche. Bacterial culture mediums and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Amresco. Trophozoites culture reagents were acquired from BD and Sigma-Aldrich. Profinity nickel-resin was from BIO-RAD. Protein concentration was determined by the bicinchoninic acid method or by absorbance at 280 nm for purified proteins ($E_{280} = 26,600 \text{ M}^{-1} \text{ cm}^{-1}$ for GITIM and $E_{280} = 32,595 \text{ M}^{-1} \text{ cm}^{-1}$ for HsTIM). Protein purity was checked by SDS-PAGE according to Laemmli (1970). TIM activity was determined by following the oxidation of NADH at 340 nm in a coupled assay (Oepser and Meyerhof, 1950). The standard reaction mixture consisted of 100 mM triethanolamine/10 mM EDTA pH 7.4 (TE buffer), 1 mM GAP, 0.2 mM NADH and 0.9 units of GDH; the reaction was
datasets were and following the inactivation temporal courses; individual inactivation to obtain the inactivation rate constants (λ) was determined. The effect of disulfiram on dGlTIM-C222A and HsTIM was assayed under the same experimental conditions, except that disulfiram concentrations ranged from 50 to 500 μM.

2.2. Expression and purification of recombinant proteins

Recombinant proteins used in this work (GITIM C202A, GITIM C202A/C222A and WT HsTIM) have been previously expressed, purified and characterized (Reyes-Vivas et al., 2007; Hernández-Alcántara et al., 2013; de la Mora-de la Mora et al., 2015). In brief, individual genes from GITIM and HsTIM cloned into the pET-HisTEVP vector were expressed in the *Escherichia coli* BL21(DE3)pLysS or BL21-CodonPlus (DE3)-RIL strains as His-Tagged proteins and purified by immobilized-metal affinity chromatography (Hernández-Alcántara et al., 2013; de la Mora-de la Mora et al., 2015). The histidine tag was removed with tobacco etch virus protease as reported previously (Hernández-Alcántara et al., 2013; de la Mora-de la Mora et al., 2015).

In *G. lambia*, TIM might exist in dimeric, tetrameric, and higher oligomeric states, both in vitro and in vivo (López-Velázquez et al., 2004; Reyes-Vivas et al., 2007). Oligomerization beyond dimers is due to the formation of intermolecular disulfide bonds between C202 of dimeric GITIMs (Reyes-Vivas et al., 2007). Therefore, in order to have a homogeneous dimeric sample, experiments were conducted over the C202A mutant; it has been shown that mutation C202A affects neither the enzyme activity nor the inactivation kinetics of GITIM by chemical modification of C222 (Enríquez-Flores et al., 2011). The double mutant C202A/C222A was used to test whether C222 is the molecular target of GITIM inactivation by disulfiram. Hereafter, for simplicity, the dimeric-stables GITIM C202A and C202A/C222A are respectively named dGITIM and dGITIM-C222A.

2.3. Kinetic characterization of dGITIM inactivation by disulfiram

The concentration of disulfiram that decreases the activity of dGITIM by 50% (IC50) was calculated by incubating dGITIM (0.2 mg/ml) at drug concentrations ranging from 0.01 to 30 μM by 2 h at 25 °C in TE buffer; afterwards, the residual activity under standard conditions was determined. The effect of disulfiram on dGITIM-C222A and HsTIM was assayed under the same experimental conditions, except that disulfiram concentrations ranged from 50 to 500 μM.

The second-order inactivation rate constant for disulfiram was obtained essentially as reported for other cysteine-modifying compounds (Enríquez-Flores et al., 2011). In brief, pseudo-first order inactivation rate constants were determined by incubating dGITIM (0.2 mg/ml, 25 °C, TE buffer) at disulfiram concentrations of 5, 40, 75 and 110 μM and following the inactivation temporal courses; individual inactivation datasets were fitted to a mono-exponential decay model (Nt = N0e−λt) to obtain the inactivation rate constants (λ) at each disulfiram concentration. Individual rate constants were plotted against the disulfiram concentration and fitted to a lineal equation model (y = m * x + b), where the slope represents the second-order inactivation rate constant.

2.4. Structural characterization of disulfiram-modified GITIM

dGITIM was completely inactivated (> 95%) by incubation of the enzyme (0.2 mg/ml, 25 °C, TE buffer) with disulfiram 110 μM by 30 min; at this time, free disulfiram was removed by five successive tenfold dilution/concentration steps in 30 kDa Amicon Ultra Centrifugal Filter Units. The same procedure was performed on the dGITIM-C222A mutant, albeit enzyme inactivation was not observed (see below). Residual activity and protein concentration of the disulfiram-modified dGITIM and dGITIM-C222A was determined as indicated in Section 2.1. Free cysteine quantification before and after disulfiram modification of dGITIM and dGITIM-C222A was determined by the use of Ellman’s reagent (5,5-dithio-bis-(2-nitrobenzoic acid) as previously reported (Reyes-Vivas et al., 2007). The structural features of dGITIM with or without modification by disulfiram were explored by circular dichroism (CD), fluorescence spectroscopy and molecular exclusion chromatography. Ultra violet circular dichroism (UV-CD) spectra from 190 to 280 nm (dGITIM 0.1 mg/ml in phosphate buffer 50 mM pH 7.4, 25 °C) were recorded in a Jasco J-810 spectropolarimeter. Protein intrinsic fluorescence spectra from 300 to 450 nm (dGITIM 0.1 mg/ml in phosphate buffer 50 mM pH 7.4, 25 °C) after excitation at 280 nm were obtained in a Perkin-Elmer LS-55 spectrophuorimeter. In both, CD and fluorescence experiments, blanks (buffer without protein) were subtracted from experimental samples. Molecular exclusion chromatography was performed in a Superose 6 10/300 GL column coupled to an Äkta FPLC system. The mobile phase was Tris-HCl 50 mM pH 8.0 plus NaCl 150 mM at 0.2 mL/min; protein elution was monitored by following the absorbance at 280 nm. Column calibration was performed with Bio-Rad molecular weight standards.

2.5. Stability study of disulfiram-modified GITIM

The effect of disulfiram on the structural stability of inactivated dGITIM (0.1 mg/ml in phosphate buffer 50 mM pH 7.4) was investigated by following the decrease of its CD signal at 222 nm in response to the increase in temperature; scans from 25 to 75 °C were conducted at a rate of 20 °C/h. The fraction of unfolded protein and melting temperature (Tm) values were calculated as previously reported (Enríquez-Flores et al., 2008).

2.6. Disulfiram effects over *G. lambia* trophozoites and endogenous GITIM

*G. lambia* WB trophozoites (ATCC 30957) were seeded at 7.5 × 10⁶ cells in 9 mL screw-capped borosilicate tubes and grown in TYI-S-33 medium supplemented with bile and fetal calf serum at 37 °C for 72 h. Cultures in log phase were chilled for 15 min in an ice bath to harvest the *Giardia* trophozoites. Trophozoites were washed with PBS pH 7.2 and the size of the *Giardia* population determined in a hemocytometer. 2 × 10⁸ trophozoites per well were treated with disulfiram concentrations ranging from 0.053 to 3.37 μM in a 96 well microplate and incubated for 24 h at 37 °C in a closed wet chamber. Next, the plate was chilled in an ice bath to detach the cells and a sample of 50 μL from every well were re-cultured in fresh medium for 24 h at 37 °C. Afterwards, the trophozoites were washed and incubated with XTT-tetrazolium salt for 4 h at 37 °C and the concentration of synthesized formazan measured in a MultiSkan Go spectrophotometer (ThermoScientific) at 490 nm (Ponce-Macotela et al., 1994). Controls were: 1) TYI-S-33 medium without parasites, 2) trophozoites without disulfiram growing in TYI-S-33 medium, 3) parasites exposed to DMSO at 0.5% v/v, and 4) trophozoites incubated with metronidazole as standard anti diarrhal drug. The experiments were performed six times. The mortality was calculated with the formula: % Mortality = [(ODC−ODM)/(ODC−ODA)] × 100, where OD0, OD and OD0 are the optical densities of control trophozoites, experimental points and culture medium, respectively. The LD25, LD50 and LD75 were calculated by linear regression with the JMP 9.0 software.

The effect of disulfiram on endogenous GITIM was tested as follows: 6 × 10⁶ trophozoites were exposed at LD25, LD50 and LD75 concentrations for 24 h at 37 °C. Tubes with trophozoites were chilled, cells harvested, washed twice with PBS buffer and resuspended in 500 μL of the same buffer supplemented with Complete protease inhibitor cocktail (Roche). Trophozoites were broken by sonication (3 cycles of 15 s at the lowest potency with 45 s intervals in an ice-water bath) and centrifuged for 15 min at 15,000 × g and 4 °C. Protein concentration in the supernatant was determined and diluted to 0.2 mg/mL in TE Buffer. This sample was exposed to disulfiram 110 μM and the residual activity of GITIM measured in a temporal course under standard conditions. In a parallel experiment, the inactivation of recombinant dGITIM was performed under the same procedure, except that 0.2 mg/mL of pure recombinant protein was used. The activity of endogenous GITIM in the cell lysate was completely dependent on the presence of the TIM start.
substrate (GAP) and the coupling enzyme (GDH), indicating that activity measurements on the total extract are specific for GITIM.

The correlation between the inactivation of endogenous GITIM and the antigiardial effect of disulfiram was tested on trophozoites exposed to disulfiram concentrations necessary to attain approximately 75, 50 and 25% of surviving cells. Trophozoites were then counted and treated as indicated in the preceding paragraph to determine the GITIM activity of each sample. Controls (100% of surviving cells) did not include disulfiram.

3. Results

It has been previously demonstrated that chemical modification of C222 disrupts the catalytic activity of GITIM (Enríquez-Flores et al., 2011; Reyes-Vivas et al., 2014). In this connection, it was feasible that disulfiram, a known cysteine chemical modifier, could disturb the GITIM activity. In order to test this hypothesis, the effect of disulfiram on the functional and structural properties of GITIM was assayed.

3.1. Disulfiram inactivates effectively GITIM

The effect of disulfiram on the catalytic activity of GITIM was firstly tested. Disulfiram inactivated GITIM in a concentration-dependent manner with an IC50 value of 6.6 μM (Fig. 2). The inactivation of GITIM by disulfiram obeys pseudo-first order decay kinetics at concentrations ranging from 5 to 110 μM (Fig. 3A); from the pseudo-first order inactivation rate constants at each disulfiram concentration (Fig. 3B), the second-order inactivation rate constant was calculated as 35 M−1 s−1. The results indicate that disulfiram efficiently inactivates GITIM in a concentration-dependent manner.

3.2. The target of disulfiram inactivation is the C222 of GITIM

Mutagenesis data of all cysteine residues on GITIM have indicated that C222 is the molecular target of inactivation by cysteine chemical modifiers (Enríquez-Flores et al., 2011, Reyes-Vivas et al., 2014), and indeed this is the case for disulfiram too. In the absence of C222, i.e. in the GITIM-C222A mutant, disulfiram has only a slight effect on the activity of the enzyme at concentrations almost 100-fold higher than the IC50 of GITIM (Fig. 4). Free cysteine quantification indicates that more than one cysteine residue was modified in the disulfiram-inactivated GITIM, but only C222 is responsible for the inactivation of the enzyme. The WT GITIM has five cysteine residues but in GITIM there are four and in GITIM-C222A only three (see Material and Methods section 2.2). Before reacting with disulfiram, four and three free cysteine residues were found in GITIM and GITIM-C222A, respectively. However, after total inactivation with disulfiram, two free cysteine residues were found in GITIM and, at equivalent time, two in GITIM-C222A. The result indicates that disulfiram modifies two cysteine residues in GITIM and only one in GITIM-C222A. Together, the results indicate that disulfiram reacts with more than one cysteine residue, but inactivation of GITIM is only related to the modification of C222.

3.3. The inactivation of GITIM by disulfiram is species-specific

The effect of disulfiram on the human orthologous TIM was assayed to test the specificity of its effect in G. lamblia. Disulfiram has negligible

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Fig. 2. Inactivation of GITIM by disulfiram. GITIM (0.2 mg/ml) was incubated by 2 h at 25 °C with the indicated concentrations of disulfiram; at that time, the activity under standard conditions was measured. The inset shows the concentration of disulfiram that reduce the activity of GITIM at a half. The values are means ± s.d. of three independent experiments.
effects on the human enzyme activity at the highest concentrations of the drug that were tested (Fig. 4). HsTIM possesses a cysteine residue (C217) equivalent to GlTIM C222, but it's still not affected by disulfiram.

3.4. The inactivation of GlTIM by disulfiram involves minor structural changes

In order to gain insights on its mechanism of inactivation, the structural effects of chemical modification by disulfiram over GlTIM were studied. Chemical modification by disulfiram does not induce alterations of the secondary structure, as indicated by the similarity of the circular dichroism spectra of non-inactivated and inactivated GlTIM (Fig. 5). In contrast, disulfiram induces a 40% quenching on the intrinsic fluorescence of the protein (190 vs 120 a.u. for the unmodified and modified GlTIM, respectively) and provokes a 4 nm red-shift on the maximum emission fluorescence wavelength (331 nm for the unmodified protein vs 335 nm for the disulfiram-modified enzyme) (Fig. 6); the result indicates the solvent exposure of the protein fluorophores after modification by disulfiram. The inactivation by disulfiram does not induce changes in the profile of molecular exclusion chromatography (Fig. 7); in both samples (modified or unmodified), the elution time is compatible with a protein molecular mass of ∼55 kDa. The result indicates that modification of GlTIM by disulfiram does not alter the dimeric structure of the enzyme. Altogether, the results indicate that inactivation of GlTIM by disulfiram causes minor structural changes to the protein.
3.5. The modification by disulfiram decreases the stability of \( \text{dGITIM} \)

The effect of disulfiram on the stability of \( \text{dGITIM} \) was studied; the thermostability of the enzyme was monitored by following the decrease in the circular dichroism signal at 222 nm as function of temperature (Fig. 8). The results indicate that disulfiram induce a remarkable decrease on the global stability of the protein, as indicated by the diminution of 7.8 °C on the \( T_{m} \) of inactivated \( \text{dGITIM} \) (57.2 °C vs 49.4 °C for the unmodified and modified \( \text{dGITIM} \), respectively).

3.6. Disulfiram affects the viability of \( G. \ lamblia \) trophozoites and endogenous \( \text{GITIM} \) activity

The previously reported giardicidal effect of disulfiram (Nash and Rice, 1998; Galkin et al., 2014) was confirmed. Disulfiram decreases the viability of cultured \( G. \ lamblia \) trophozoites in a dose-dependent manner with a calculated LD50 value of 2.3 \( \mu \)M.

In order to test if the results obtained with the recombinant protein can be extrapolated to the naturally occurring GITIM, a total protein extract obtained from \( G. \ lamblia \) trophozoites was exposed to disulfiram. Under analogous experimental conditions the effect of disulfiram on the GITIM activity is very similar (Fig. 9), independently of the enzyme source (endogenous or recombinant).

Finally, the relationship between the inactivation of GITIM and the giardicidal effect of disulfiram was examined (Fig. 10). The results indicate that inactivation of endogenous GITIM correlates with the decreasing viability of trophozoites exposed to disulfiram, suggesting that both processes may be related.

4. Discussion

Giardiasis is one of the main intestinal parasitosis in the world affecting mostly developing countries (Feng and Xiao, 2011). Given its impact in impoverished regions, giardiasis was included as part of the WHO Neglected Disease Initiative in 2004 (Savioli et al., 2006). As previously stressed, the pharmaceutical and biotechnology companies have little interest in infectious diseases of poverty, primarily because investment cannot guarantee financial return (Trouiller et al., 2002; Pedrique et al., 2013). Therefore, it is hard to expect that efforts to develop alternative therapeutics against this group of diseases can come from environments other than academic and public research initiatives. As new drug development can consume large amounts of resources, low-cost alternatives are necessary. In this regard, repurposing existing drugs can be an efficient way to develop new therapeutic alternatives against neglected diseases (Sbaraglini et al., 2016).

Drug repurposing of disulfiram as an antigiardial agent has been proposed, and inactivation of \( G. \ lamblia \) carbamate kinase has been proposed as its antigiardial mechanism of action (Galkin et al., 2014). However, based on the wide array of molecules targeted by disulfiram (Kona et al., 2011; Sauna et al., 2004; Shian et al., 2003; Velasco-García et al., 2006; Lee et al., 2016; Boukhvalova et al., 2010), we hypothesized that disulfiram can act on more than one enzyme of \( G. \ lamblia \).

In this work we demonstrated the effectiveness of disulfiram as inactivator of GITIM, an enzyme previously proposed as a plausible target to develop pharmacological therapies against giardiasis (López-
Table 1

| Compound    | Second-order inactivation rate constant $M^{-1} s^{-1}$ | Ref.                                      |
|-------------|--------------------------------------------------------|-------------------------------------------|
| Disulfram   | 35.0                                                   | This work                                 |
| Pantoprazole| 0.29                                                   | (Garcia-Torres et al., 2016)              |
| Omeprazole  | 0.60                                                   | (Garcia-Torres et al., 2016)              |
| Rabeprazole | 2.66                                                   | (Garcia-Torres et al., 2016)              |

Disuliram is an efficient inactivator of dGlTIM as indicated by the value of the IC$_{50}$ (6.6 μM) and the second-order inactivation rate constant (35 M$^{-1} s^{-1}$) obtained for this drug (Figs. 2 and 3). These values can be contrasted with the recently reported properties of PPIs as inactivators of GITIM (Garcia-Torres et al., 2016). The IC$_{50}$ value of disuliram is 5–50-fold lower than the IC$_{50}$ values for PPIs, which ranged from ~30 to ~300 μM (Garcia-Torres et al., 2016). Further, the second-order inactivation rate constant for disuliram was 120, 58 and 13-fold higher than for pantoprazole, omeprazole, and rabeprazole, respectively (Table 1). It is important to highlight that, in concordance with previous data on the inactivation of GITIM by chemical modification of C222 (Enriquez-Flores et al., 2008; Enriquez-Flores et al., 2011), inactivation experiments with disuliram were performed at 25 °C whereas for PPIs these were conducted at 37 °C. It can be assumed that under equal experimental conditions, the preponderance of disuliram over PPIs (as measured by the IC$_{50}$ and the second-order inactivation rate constant) will increase appreciably. In fact, the IC$_{50}$ of disuliram at 37 °C is ~2 μM.

The C222 residue is the molecular target of inactivation of dGlTIM by disuliram (Fig. 4), as previously demonstrated for other sulfhydryl reactive compounds (Enriquez-Flores et al., 2011; Reyes-Vivas et al., 2014; Garcia-Torres et al., 2016). This inactivation is species-specific, as indicated by the lack of inhibition of HsTIM (Fig. 4). This behavior is consistent with previous data showing that HsTIM is barely a second-order inactivation rate constant) will increase appreciably. In

Finally, the parallel between the giardicidal effect of disuliram and the inactivation of intracellular GITIM (Fig. 10), suggests that both processes may be correlated.

In sum, the results in this work demonstrate that disuliram is an effective species-specific inactivator of GITIM with higher potency than previously tested drugs. The chemical modification of C222 by disuliram involves the complete inactivation of the enzyme and the destabilization of the protein structure, thus acting at both functional and structural levels. Disuliram acts not only in the recombinant protein but also inhibits the endogenous GITIM activity. Is therefore feasible that inactivation of GITIM by disuliram contributes to its giardicidal effect.

In addition to inhibiting the G. lamblia carbamate kinase (Galkin et al., 2014), here we have shown that disuliram inhibits GITIM, both in vitro and likely in vivo. Therefore, it is possible that disuliram may exert its anti-parasitic effect at more than one molecular target (at least two enzymes from two different metabolic pathways, at this time). The data are encouraging because a multi-target drug acting on non-related targets could circumvent current drug-resistance mechanisms. Indeed, disuliram is evidenced to kill the metronidazole-resistant G. lamblia strain 713M3 (Galkin et al., 2014).

5. Conclusion

Giardiasis is one of the main parasitosis in the world, but affecting mainly the underdeveloped world. As a neglected disease, there is a need to develop low-cost therapeutic alternatives against it. Drug re-purposing can be an affordable alternative to develop new pharmacotherapies for giardiasis, and disuliram can be a promissory lead to direct these efforts.

Conflicts of interest

None.

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In the in vitro experiments with trophozoites, the antiparasitic effect of disuliram was confirmed; the obtained LD$_{50}$ value (2.3 μM) is in the same range that the minimum lethal concentration previously reported for Galkin et al., 2014 (3.1 μM) and Nash and Rice., 1998 (1.23 μM). The similarity of the inactivation curves of the recombinant and the trophozoite-extracted GITIM by disuliram (Fig. 9) indicates that both enzymes behaves almost identically; therefore, it can be presumed that recombinant GITIM is a good surrogate model of the natural enzyme. Finally, the parallel between the giardicidal effect of disuliram and the inactivation of intracellular GITIM (Fig. 10), suggests that both processes may be correlated.

In sum, the results in this work demonstrate that disuliram is an effective species-specific inactivator of GITIM with higher potency than previously tested drugs. The chemical modification of C222 by disuliram involves the complete inactivation of the enzyme and the destabilization of the protein structure, thus acting at both functional and structural levels. Disuliram acts not only in the recombinant protein but also inhibits the endogenous GITIM activity. Is therefore feasible that inactivation of GITIM by disuliram contributes to its giardicidal effect.

In addition to inhibiting the G. lamblia carbamate kinase (Galkin et al., 2014), here we have shown that disuliram inhibits GITIM, both in vitro and likely in vivo. Therefore, it is possible that disuliram may exert its anti-parasitic effect at more than one molecular target (at least two enzymes from two different metabolic pathways, at this time). The data are encouraging because a multi-target drug acting on non-related targets could circumvent current drug-resistance mechanisms. Indeed, disuliram is evidenced to kill the metronidazole-resistant G. lamblia strain 713M3 (Galkin et al., 2014).

5. Conclusion

Giardiasis is one of the main parasitosis in the world, but affecting mainly the underdeveloped world. As a neglected disease, there is a need to develop low-cost therapeutic alternatives against it. Drug re-purposing can be an affordable alternative to develop new pharmacotherapies for giardiasis, and disuliram can be a promissory lead to direct these efforts.

Conflicts of interest

None.

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