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

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ABSTRACT

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* (GlTIM) has been proposed as a plausible target for the development of novel antigiardial pharmacotherapies, 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 GlTIM can be inactivated by modification of C222, in this work we tested the effect of disulfiram over the recombinant and trophozoite-endogenous GlTIM. The results show that disulfiram inactivates GlTIM 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 GlTIM inactivates similarly, indicating that the recombinant protein resembles the natural enzyme. Disulfiram induces loss of trophozoites viability and inactivation of intracellular GlTIM 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 antigiardial 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

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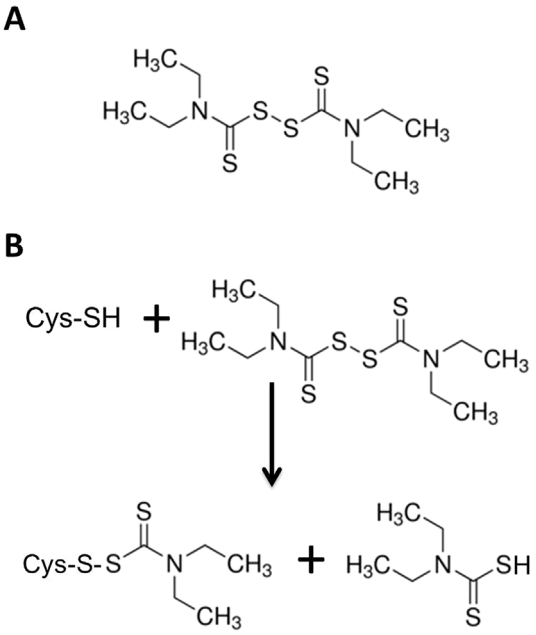

Fig. 1. Structure and reaction mechanism of disulfiram. (A) Chemical structure of disulfiram (1,1′,1″,1‴-[disulfanediylbis(carbonothioylnitrilo)]tetraethane) (B) Disulfiram reacts with sulfhydryl groups of free (reduced) cysteine residues forming a mixed disulfide.

compounds (García-Torres et al., 2012). In this regard, drug repurposing stands out as an attractive option to find alternative antigiardiasis 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 dithiodiethylcarbamoyl 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, dyspnea, 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 antigiardial 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 antigiardial 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 antigiardial drug design (Hiltpold et al., 1999; López-Velázquez et al., 2004; Galkin et al., 2007). In this connection, the triosephosphate isomerase from *G. lamblia* (GlTIM) has been previously proposed as a feasible target against which to develop new pharmacotherapies (López-Velázquez et al., 2004). The plausibility of GlTIM as a pharmacological target is supported by RNA-interference experiments showing that the decrease of the GlTIM 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 GlTIM at micromolar concentrations with minor effects on the activity of human triosephosphate isomerase (HsTIM) (Enriquez-Flores et al., 2008; Enríquez-Flores et al., 2011). By analogy with its mechanism of action, proton pump inhibitors (PPIs) were suggested as GlTIM 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 GlTIM 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 GlTIM on trophozoites, suggesting that both processes are related (Reyes-Vivas et al., 2014).

Since disulfiram is a recognized cysteine modifier agent and GlTIM can be inactivated by modification of its C222, in this work we explored the effect of disulfiram on GlTIM. The results indicate that disulfiram efficiently and selectively inactivates recombinant GlTIM by chemical modification of C222. The inactivation of GlTIM entails minor conformational changes and concurs with decreased stability of the protein. Recombinant and endogenous GlTIM are inactivated similarly by disulfiram, indicating that the recombinant protein resembles the endogenous enzyme. Disulfiram induces loss of trophozoites viability and inactivation of intracellular GlTIM at similar rates, suggesting that both processes may be related. Therefore, it is plausible that the antigiardial 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 (ε_{280} = 26,600 M^{-1} cm^{-1} for GlTIM and ε_{280} = 32,595 M^{-1} 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 (Oesper 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