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RESEARCH PAPER

Quinolines-1,2,3-triazolylcarboxamides exhibits antiparasitic activity in *Trichomonas vaginalis*

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Abstract Increased prevalence of metronidazole-resistant infections has resulted in a search for alternative drugs for the treatment of trichomoniasis. In the present study, we report the evaluation of *in vitro* activity of three quinolines-1,2,3-triazolylcarboxamides (QTCA-1, QTCA-2 and QTCA-3) against *Trichomonas vaginalis*, evaluation of cytotoxicity in CHO cells and expression of genes related to hydrogenosome by real time PCR. Nine concentrations of these compounds were analyzed for *in vitro* activity against ATCC 30236 isolate of *T. vaginalis*. QTCA-2 reported a cytotoxic effect against 100% of *T. vaginalis* trophozoites at a final concentration of 80 μM with an IC_{50} of 50 μM. The kinetic growth curve of trophozoites indicated that QTCA-2 reduced the growth by 70% at a concentration of 80 μM after an exposure of 12 h, and induced complete parasite death at 24 h. QTCA-2 induced less than 30% of cytotoxicity in CHO-K1 cells at 80 μM and data showed this concentration and lower ones had no significant cytotoxic effect when compared to the control. There was no significant difference in gene expression (pyruvate-ferredoxin oxidoreductase A and B; Malic enzyme D; Hydrogenase; β-tubulin) when compared to control and MTZ. Further *in silico* analysis showed that QTCA-2 had significant binding free energy with *T. vaginalis* lactate dehydrogenase (−9.3 kcal/mol), purine nucleoside phosphorylase (−9.1 kcal/mol) and triosephosphate isomerase (−7.3 kcal/mol). The present study offers new perspectives for exploring the potential of this class of molecules as an additional option for the treatment of trichomoniasis.

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Introduction

Trichomonas vaginalis is a protozoan infecting genitourinary tract and causing the most frequent non-viral, sexually transmitted infection (STI) in the world. According to World Health Organization (WHO, 2012), an estimated 276 million cases of trichomoniasis have been reported worldwide, most of them occurring in women.

Drugs of choice for treating trichomoniasis are derived from 5-nitroimidazoles, more specifically metronidazole and tinidazole, which are approved by Food and Drug Administration (FDA-EUA). The 5-nitroimidazoles, especially metronidazole, are the most widely used antimicrobial agents for the treatment of trichomoniasis. However, metronidazole resistance is an increasingly recognized problem in treating patients infected with trichomoniasis (Lumsden, Robertson, Heyworth, & Harrison, 1988; Vieira, Brandelli, Veríssimo, & Tasca, 2012). Metronidazole enters the parasite by passive diffusion as a nonlethal prodrug. Once inside the cell, metronidazole is reduced, resulting in the generation of nitro radicals, which lead to DNA damage and cell death. *T. vaginalis* cells use pyruvate-ferredoxin oxidoreductase (PFOR) and ferredoxin-linked enzymes to metabolize pyruvate to acetate *via* acetyl coenzyme A to gain ATP. When a nitroimidazole is present, its nitro groups capture electrons from ferredoxin, and the nitro radicals are generated (Kulda, 1999). Although cure rates are significant, there are factors that are associated with the increase of resistant *T. vaginalis*. It has been reported that treatment failures, which are attributed mainly to non-adherence to treatment or reinfection, and factors such as low drug absorption or insufficient bioavailability, could be related to resistance (Lumsden et al., 1988).

For this, an intensive search for new molecules as potential drugs with antiparasitic activity for the treatment of trichomoniasis is required. Here, we highlight the organic compounds of quinolines as possible drugs for the treatment of trichomoniasis. Pharmacological uses of these organic compounds have been studied for different biological properties such as antitumor (Begnini et al., 2017), antioxidants (Dorey, Lockhart, Lestage, & Casara, 2000), anti-tuberculosis (Candéa et al., 2009), anti-fungal (Musiol et al., 2006), antibacterial (Ferretti, Neto, Morel, Kaufman, & Larghi, 2014), anti-inflammatory and antinociceptive (Wilhelm et al., 2014) and antiprotozoal (Di Pietro et al., 2015). Thus, the aim of this study was to evaluate the *in vitro* anti-*T. vaginalis* activity of three quinolines-1,2,3-triazolylcarboxamides (QTCA) derivatives.

Materials and methods

Chemicals

The activity of quinolines-1,2,3-triazolylcarboxamides (QTCA) diluted in dimethyl sulfoxide (DMSO) was analyzed in *T. vaginalis*. Initially, we selected three compounds: QTCA-1, QTCA-2, and QTCA-3. QTCA molecules were synthetized by organocatalytic synthesis as described in a previous study (Begnini et al., 2017). Briefly, the compounds were synthesized in high yields by the reaction of 4-azido-7-chloroquinoline with a range of β-oxoamides in the presence of a catalytic amount of pyrrolidine using DMSO as solvent at room temperature. The following quinoline derivative compounds were evaluated in this study: 1-(7-chloroquinolin-4-yl)-5-methyl-N-phenyl-1H-1,2,3-triazole-4-carboxamide (QTCA-1); 1-(7-chloroquinolin-4-yl)-N,5-diphenyl-1H-1,2,3-triazole-4-carboxamide (QTCA-2) and 1-(7-chloroquinolin-4-yl)-N-phenyl-5-(trifluoromethyl)-1H-1,2,3-triazole-4-carboxamide (QTCA-3).

Parasite culture conditions

In this study, *T. vaginalis* 30236 isolate from American Type Culture Collection (ATCC) was used. Trophozoites were axenically cultured in trypticase-yeast extract-maltose (TYM) medium without agar (pH 6.0), supplemented with 10% sterile bovine serum (inactivated at 56 °C), and incubated at 37 °C (Diamond, 1957). The culture with 95% viability, confirmed through observation of motility, morphology, and trypan blue exclusion (0.4%) assay under the light microscope at 400× magnification was considered alive and utilized for the evaluation of activity of quinolines-1,2,3-triazolylcarboxamides (QTCA).

Anti-*Trichomonas vaginalis* assay

The three QTCA derivatives (QTCA-1, QTCA-2, and QTCA-3) were tested for *in vitro* activity against *T. vaginalis*, according to Sena-Lopes et al. (2017). For screening, QTCA-1, QTCA-2, and QTCA-3 were used at a final concentration of 100 μM. All assays were performed in 96-well microtiter plates (TPP). The parasites were seeded at an initial density of 2.6×10^5 trophozoites/mL of TYM and incubated with QTCA drugs. Three controls were carried out: trophozoites only, vehicle for solubilization of the derivatives (0.6% DMSO), and MTZ 100 μM (as positive control, metronidazole – Sigma-Aldrich). The microculture plates were incubated at 37 °C with 5% CO_2 for 24 h. After that, a preparation containing trophozoites and trypan blue (0.4%) at a ratio of 1:1 was counted in a Neubauer chamber and cultures with 95% viability were utilized for assays.

Only the compound that reduced the viability of parasites to 100% was used to determine the minimum inhibitory concentration (MIC) by considering different concentrations such as 6.25, 12.5, 25, 50, 60, 70, 80, 90, and 100 μM. The parasite pellets used to establish MIC and the concentrations below and above as well as controls were inoculated in fresh TYM medium at 37 °C. Parasites were counted in a Neubauer chamber with trypan blue every 24 h for 96 h to confirm MIC. The viability of trophozoites was assessed by exclusion of trypan blue dye, motility, and morphology. The IC_{50} was determined (half the maximum inhibitory concentration) at concentrations of 6.25, 12.5, 25, 50, 60, 70, 80, 90, and 100 μM as described in the MIC method.

A kinetic growth curve was constructed to obtain a more comparable activity profile of QTCA compounds against *T. vaginalis*. The viability of trophozoites was observed under a light microscope for 96 h after incubation at respective MIC. Growth analysis was performed at the following times: 1, 6, 12, 24, 48, 72, and 96 h by trypan blue (0.4%) exclusion method and by determining the motility and morphology. All the assays were performed independently at least three

times in triplicate and the results were expressed as the percentage of viable trophozoites in comparison with untreated parasites.

Cytotoxicity assay

Chinese Hamster Ovary (CHO-K1) cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil) and cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) (Vitrocell Embriolife), supplemented with 10% fetal bovine serum (FBS) (Vitrocell Embriolife), 1% L-glutamine, and 1% penicillin/streptomycin. The cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2.

The viability of CHO-K1 cell line after treatment with QTCA-2 was determined by measuring the reduction of soluble 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to water-insoluble formazan. Cells were seeded at a density of 2×10^4 cells per well in a volume of 100 μL in 96-well plates and grown at 37 °C in 5% CO_2 atmosphere for 24 h before being used in the assay. The cells were then treated with 2.5, 5, 20, 40 and 80 μM of QTCA 02 compound for 24 h. DMSO (vehicle alone), metronidazole (MTZ) and no treated cells were used as vehicle (VC), positive, and negative controls, respectively. Following incubation, 20 μL of MTT (Sigma-Aldrich) in a concentration of 5 mg/mL were added to each well, and the cells were incubated for an additional 3 h at 37 °C. The differences in the total cellular metabolism were detected at a wavelength of 492 nm using a microplate reader. The inhibition (%) of cell proliferation was determined comparing treated cells and control cells as follows: inhibitory growth = $(1 - Abs_{492\ \text{treated cells}}/Abs_{492\ \text{control cells}}) \times 100\%$. The CC_{50} was calculated using GraphPad Prism 5.0 Software. All observations were validated by at least three independent experiments in triplicate.

Gene expression of *T. vaginalis*

Total RNA from *T. vaginalis* trophozoites (10^7 cells/sample) was isolated using Trizol Reagent kit (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 0.5 μg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) following the manufacturer's protocol. PCR mixtures (20 μL) containing 10 μL of SYBR Green PCR Master Mix (Applied Biosystems, UK), 300 nM of primers, 1 μL of cDNA, and RNase-DNase-free water were subjected to 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. A melting curve analysis was performed at 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s. Real time PCR reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using the primers described in Table 1 (pyruvate-ferredoxin oxidoreductase A (PFOR A) and pyruvate-ferredoxin oxidoreductase B (PFOR B); Malic enzyme D; Hydrogenase; β-tubulin) (Mead, Fernadez, Romagnoli, & Secor, 2006; Dos Santos, De Vargas Rigo, Frasson, Macedo, & Tasca, 2015). The primer sets were designed and optimized using the Primer 3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), based on predefined parameters. B-tubulin gene was used as normalizer in all analyses.

Molecular docking of QTCA-2 and *T. vaginalis* proteins

Considering the promising *in vitro* activity of QTCA-2 against *T. vaginalis*, we sought to explore other relevant biological targets for this compound using molecular docking simulation. QTCA2 was built and optimized in the software Avogadro 1.1.1 (Hanwell et al., 2012). The crystallographic structures of the following *T. vaginalis* enzymes were used as targets: lactate dehydrogenase (TvLDH; 5A1T), purine nucleoside phosphorylase (TvPNP; 1Z36) and triosephosphate isomerase (TvTPI; 3QST), which were retrieved from RCSC Protein Data Bank (PDB) (/www.pdb.org/). The CHIMERA 1.5.3 (Pettersen et al., 2004) software was used to remove ligands, ions, and water and to minimize the structure of proteins, using the Gasteiger charges with 500 steps of minimization in all molecular targets. The molecular docking simulation was performed using the software Autodock Vina 1.1.2 (Trott and Olson, 2010) and the binding sites were defined by the co-complexed ligands in the crystal structure and the grid box was set up according to the corresponding residues. The conformers with the lower binding free energy (ΔG) were analyzed in the Accelrys Discovery Studio 3.5 (Dassault Systèmes BIOVIA, 2016). The ΔG represents the spontaneity in which the protein–ligand interaction is going to occur and an arbitrary cut-off was used for the study: any position with a binding energy higher than −6.0 kcal/mol was excluded from the analysis with Autodock Vina 1.1.2.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) using a probability value of $p < 0.05$. Tukey's test was applied to identify significant differences between the means of different treatments (GraphPad Prism 5.0 Software).

Results

Chemicals

Chemical structure and yields for QTCA-1, QTCA-2 and QTCA-3, synthesized through the cycloaddition enamide azide between β-ketoamides and 4-azido-7-chloroquinoline can be seen in Fig. 1.

Anti-*Trichomonas vaginalis* assay

Analysis of data obtained from the screening of compounds reported that the compound QTCA-2 reduced the parasite viability by 100% when compared with the negative control (only trophozoites). The other two compounds, namely QTCA-1 and QTCA-3 reduced the parasite viability by 60% and 80%, respectively (Fig. 2). As expected, the negative controls (only trophozoites and DMSO) exhibited positive motility and negative staining with trypan blue (0.4%),

Table 1 Primers used in qRT-PCR.

Gene	Primer	Sequence (5′–3′)	GenBank access number	TVAG gene numbers	Reference
PFOR A	F R	CGGCTACGGTATGTTCAAGG TCCTTGTCCTGATCCCAAAC	U16822	TVAG_198110	Mead et al. (2006)
PFOR B	F R	CTGCAAGCTCCTTACACAGC AAGAGGGAGTTAGCCCAAGC	U16823	TVAG_230580	Mead et al. (2006)
Malic enzyme (D)	F R	CATCTGTTAGCCTCCCAGTCCACGAGCAGCTTGTTCATCCT	U16839	TVAG_412220	Mead et al. (2006)
Hydrogenase	F R	TGCACACGAAAGAAGGATGA TCGCATGGTGTATCTGGTAA	U19897	N.A.[a]	Mead et al. (2006)
β-Tubulin (tub2)	F R	TACTCCATCGTCCCATCTCC CCGGACATAACCATGGAAAC	L05469	TVAG_062880	Dos Santos et al. (2015)

[a] N.A, not applicable, number not available.

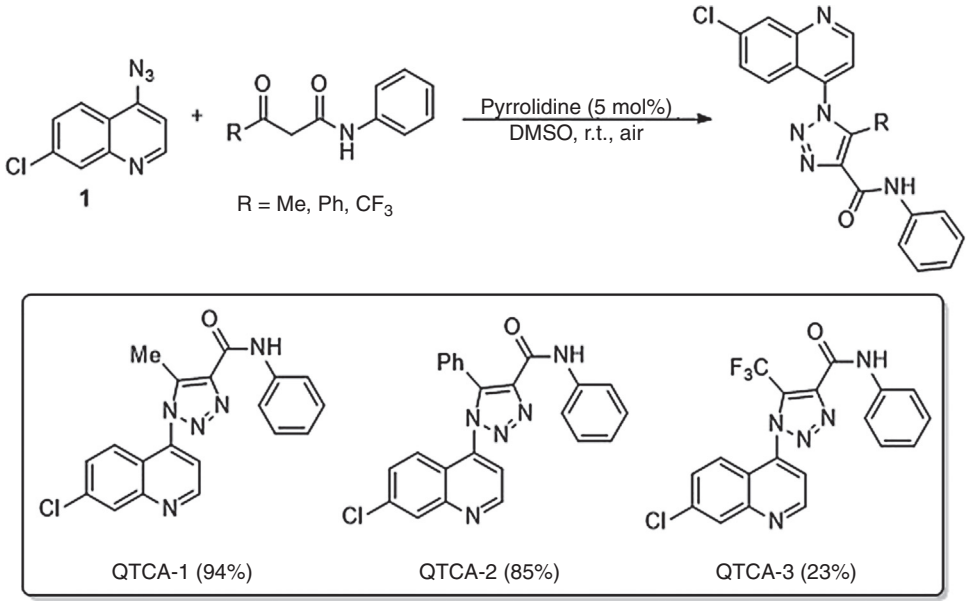

Figure 1 Chemical structure and yields for QTCA-1, QTCA-2 and QTCA-3, synthesized through the cycloaddition enamide azide between β-ketoamides and 4-azido-7-chloroquinoline.

whereas the positive control MTZ completely reduced the parasite viability. The positive control depicted negative motility and stained blue in all the assays performed after 24 h exposure. Since a 100% reduction in parasite viability was observed with QTCA-2, the compound was selected to perform other tests. QTCA-2 demonstrated an optimal anti-*T. vaginalis* activity at a concentration of 80 μM, which was confirmed by MIC and IC_{50} of 50 μM, both at 24 h exposure (Fig. 3).

Kinetic growth analysis

The analysis of the kinetic growth curve of *T. vaginalis* trophozoites treated with QTCA-2 displayed a reduction in the growth of trophozoites by 70% at a concentration of 80 μM after 12 h exposure. In addition, a complete reduction of proliferation of parasite was observed at 24 h (Fig. 4).

Cytotoxic assay

The MTT assay showed that QTCA-2 has no cytotoxic activity when exposed to CHO-K1 cell lines for 24 h. None of five doses tested were able to decrease viability more than 30% and no statistical differences were observed compared to negative, VC and MTZ controls (Fig. 5). Metronidazole (100 μM), reported a cell growth inhibition of 8.5% and QTCA-2 (80 μM) reported a cell growth inhibition of 29.7%. These results suggested that QTCA-2 is no more cytotoxic than the commercially available drugs for trichomoniasis.

Gene expression of *T. vaginalis*

The expression level of PFOR A, PFOR B, hydrogenase and malic enzyme genes after treatment with 80 μM of QTCA-2 were evaluated by qRT-PCR. No significant difference was

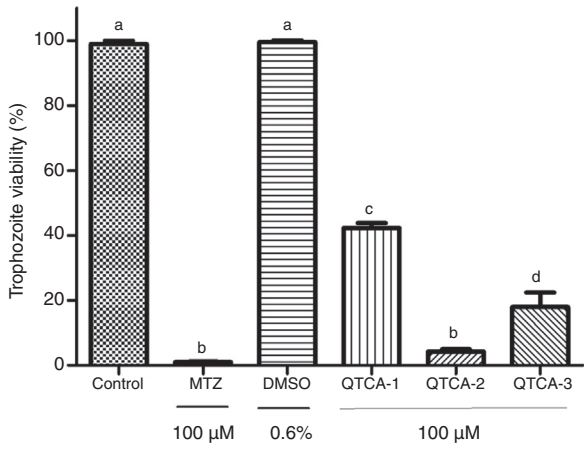

Figure 2 Antiparasitic activity of QTCA-1, QTCA-2 and QTCA-3 at 100 μM concentration against *Trichomonas vaginalis* 30236 isolate by trypan blue assay (0.4%) after 24 h of exposure. The trophozoite growth was completely inhibited by QTCA-2 in 24 h of incubation. Vehicle for solubilization (0.6% DMSO), metronidazole 100 μM (MTZ), control (untreated trophozoites). Data represent means ± standard deviation of at least three experiments in triplicate. Different letters (a, b, c and d) indicate difference between treatments. The differences were considered significant when $p < 0.05$.

observed in the expression of these genes when compared to negative, positive and vehicle controls (Fig. 6).

Molecular docking of QTCA2 and *T. vaginalis* proteins

The potential *in vitro* activity of QTCA-2 prompted us to investigate other possible targets that could contribute to the anti-*T. vaginalis* activity of this compound.

In silico analysis demonstrated that best docking position of QTCA-2 in TvLDH (5A1T) had a binding free energy (ΔG_{bind}) of -9.3 kcal/mol^{-1} (Fig. 7A). QTCA-2 interacts with ARG48 (distance of 2.36 Å) and THR239 (distance of 2.64 Å) through hydrogen bonds, and with ILE16 and ILE128 through hydrophobic interactions. QTCA-2 is also in close proximity to GLY11, GLY14, GLN15, ASP41, VAL86, ALA87, SER88, GLY129, ASN130 and PHE238.

QTCA-2 also had high docking scores for TvPNP (1Z36), with a ΔG_{bind} of -9.1 kcal/mol^{-1} (Fig. 7B). Additional analysis showed that QTCA-2 forms a carbon hydrogen bond with THR164 (at a distance of 3.75 Å) and a π-donor hydrogen bond with ASN161 (3.72 Å), and it is also involved in hydrophobic interactions with PHE159, LEU166, ALA167 and ILE206. Van der Waals forces between QTCA-2 and CYS91, GLY92, ASN160, GLN163, MET170, VAL178, GLU179 and ASP204 may also contribute to the protein–ligand interaction.

Regarding TvTPI (3QST), the docking score with QTCA-2 was -7.3 kcal/mol^{-1} (Fig. 7C). Of note, a conventional hydrogen bond is formed between QTCA-2 and PHE44 (at a distance of 3.21 Å), while the residues GLU63 and ARG97 are involved in electrostatic interactions with QTCA-2. LYS11, ALA12, HIS94 and ARG97 interact with QTCA-2 through hydrophobic interactions. Van der Waals forces between QTCA-2 and ASN9, PRO43, VAL45, GLU96, ILE91, ILE100 and LEU101 may also contribute to the protein–ligand interaction.

Discussion

In this study, we evaluated the *in vitro* anti-*T. vaginalis* activity of three Quinolines-1,2,3-triazolylcarboxamides (QTCA) (Fig. 1). We observed that only QTCA-2, which has an extra radical phenyl in its structure, reduced the viability of trophozoites by 100%. The low selective toxicity against *T. vaginalis* is attributed to the fact that many quinolines compounds have an antioxidant activity and *T. vaginalis* lacks an antioxidant defense. The exact mechanisms of action of quinoline drugs should still be better elucidated specifically for each group of derivatives, but it is believed that in the case of the antimalarial activity this mechanism is based on the inhibition of hemozoin biocrystallization, which leads to the release of the heme group, which is toxic to the parasite because of its ability to generate reactive oxygen species (Muraleedharan & Avery, 2007).

The same compounds evaluated in this study, were tested against breast cancer cells (MCF-7 and MDA-MB-231). Results showed a significant decrease of viability in a dose dependent manner after treatment with 7-chloroquinoline derivatives QTCA-1, QTCA-2, and QTCA-3. Of the three compounds, QTCA-1 was the most effective as described by the authors. QTCA-1 displayed the highest cytotoxic activity and apoptosis induction was also significantly higher in the hormonal-independent breast cancer cells (MDA-MB-231) (Begnini et al., 2017). However, in our study QTCA-1 did not present anti-*T. vaginalis* activity. Furthermore, QTCA-3 did not prove effective in either breast cancer cells or against *T. vaginalis*.

Synthetic molecules containing 1,2,3-triazole units have already been described as having various biological activities, including antituberculosis, antibacterial, anticancer and anti-Leishmania (Agalave, Maujan, & Pore, 2011; Masood et al., 2017; Wilhelm et al., 2014).

A study evaluated the *in vitro* activity of twenty one compounds derived from tricyclic heterofusioned quinolones against *T. brucei*, *T. cruzi*, and *Leishmania infantum*. All the novel heterofused quinoline derivatives featuring a protonatable aminomethylphenyl group at the B-ring were more potent against *T. brucei* than the standard drug and also demonstrated leishmanicidal activity. Most of the compounds tested also showed to be active against *T. cruzi* following the same profile found for *T. brucei*. The pyranquinoline was selected as the compound with the best anti-protozoal potential for the three parasites (Di Pietro et al., 2015).

The quinoline class composes a wide range of antimalarial agents, which are widely employed, and inhibit the formation of β-hematin (malaria pigment), which is lethal to the parasite. More specifically, the 4-aminoquinoline derivatives represent potential sources of antimalarials, such as the example of chloroquine, the most widely used antimalarial in the world. To evaluate antimalarial activity, twelve drugs derived from 4-aminoquinoline were obtained and some of these derivatives were used to obtain platinum (II). These compounds were tested *in vivo* in murine model and showed

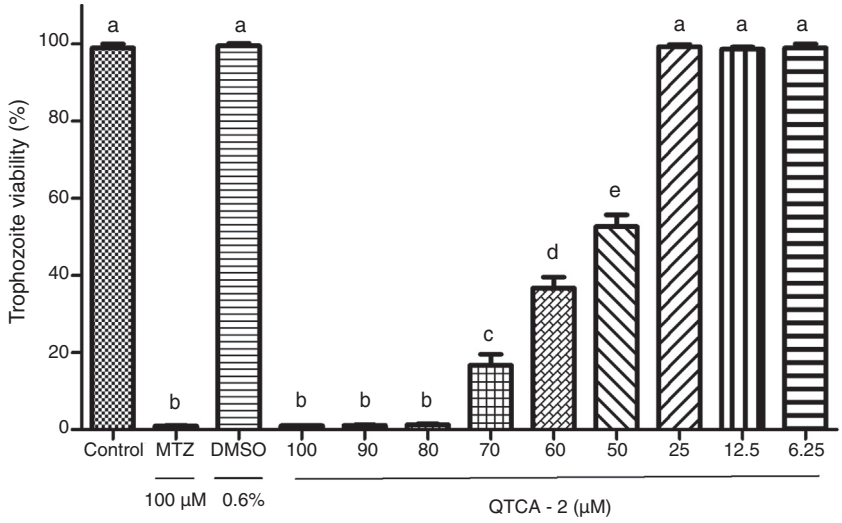

Figure 3 Determination of MIC and IC_{50} of *Trichomonas vaginalis* 30236 isolate after treatment with QTCA-2 at 6.25, 12.5, 25, 50, 60, 70, 80, 90 and 100 μM concentrations after 24 h of exposure. Trophozoites growth was completely inhibited by QTCA-2 in 24 h at 80 μM as MIC and 50 μM determined as IC_{50}. Vehicle for solubilization (0.6% DMSO), metronidazole 100 μM (MTZ), control (untreated trophozoites). Data represent means ± standard deviation of at least three experiments in triplicate. Different letters (a, b, c and d) indicate difference between treatments. The differences were considered significant when $p < 0.05$.

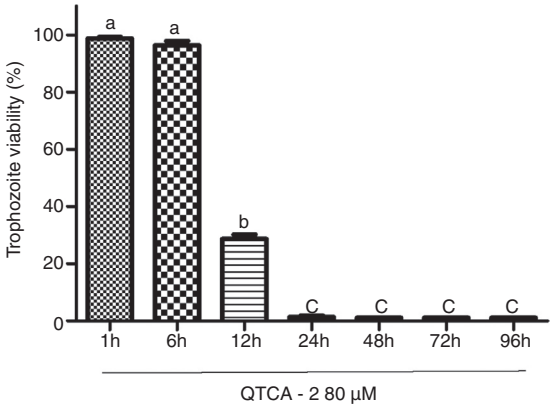

Figure 4 Kinetic growth curve of *Trichomonas vaginalis* 30236 isolate after treatment with QTCA-2 at 80 μM for a period of 1, 6, 12, 24, 48, 72, and 96 h. Trophozoites growth was completely inhibited by QTCA-2 in 24 h of incubation. Data represent means ± standard deviation of at least three experiments all in triplicate. Different letters (a, b, c and d) indicate difference between treatments. The differences were considered significant when $p < 0.05$.

remarkable inhibition of the parasite multiplication values, most of which ranged from 50 to 80%. In addition, they were not cytotoxic. Thus, they may be the subject of further research for new antiparasitic agents (De Souza et al., 2011).

Resistance to nitroimidazoles has been observed in *in vitro*-derived isolates (Lumsden et al., 1988; Upcroft & Upcroft, 2001). Anaerobic resistance is associated with a decrease in PFOR and hydrogenase activity, thus resulting in less activation of metronidazole (Conrad, Bradic, Warring, Gorman, & Carlton, 2013; Kulda, 1999). In contrast, aerobic resistance is thought to be due to impaired oxygen scavenging possibly because of decreased oxidase activity (Leitsch, 2016; Menezes, Frasson, & Tasca, 2016).

Considering parasite resistance to nitroimidazoles, *T. vaginalis* has been shown to have homologs of bacterial nitroreductases and nitroimidazole reductases that are absent in most eukaryotes and are related to the reduced susceptibility to MTZ in *Helicobacter pylori* and Bacteroides (Pal et al., 2009). It is still unclear whether these genes are associated with sensitivity to nitroimidazole in the parasite, but it has been shown that these enzymes can activate MTZ in the cytosol and in the hydrogenosome, opposing previous reports of activation that occur exclusively by the hydrogenosome enzymes such as pyruvate-ferredoxin oxidoreductase and hydrogenase (Kulda, 1999; Menezes et al., 2016).

Here, we investigated the gene expression of enzymes associated with anaerobic resistance and the mechanism of action to MTZ (PFOR A, PFOR B, Malic enzyme D; Hydrogenase; β-tubulin) in *T. vaginalis* 30236 cultured with QTCA-2. No significant differences were observed in the mRNA levels of the enzymes by resistance or susceptibility to metronidazole or QTCA-2. The lack of correlation between gene expression and resistance or susceptibility may be the result of differences in expression at the protein level or because the mechanism of action of QTA-2 involves other biochemical pathways or genes.

The negative regulation or even absence of flavin reductase and alcohol dehydrogenase activity in strains of *T. vaginalis* with high levels of MTZ resistance, as well as thioredoxin activity, has been shown to be the same in all strains evaluated (Leitsch, Kolarich, & Duchêne, 2010; Leitsch et al., 2009). In addition, the clinical resistance to MTZ in *T. vaginalis* is fundamentally different from the resistance to high level MTZ induced in the laboratory (Conrad et al., 2013). Anaerobic resistance is induced in the absence of oxygen and is a consequence of a loss of the

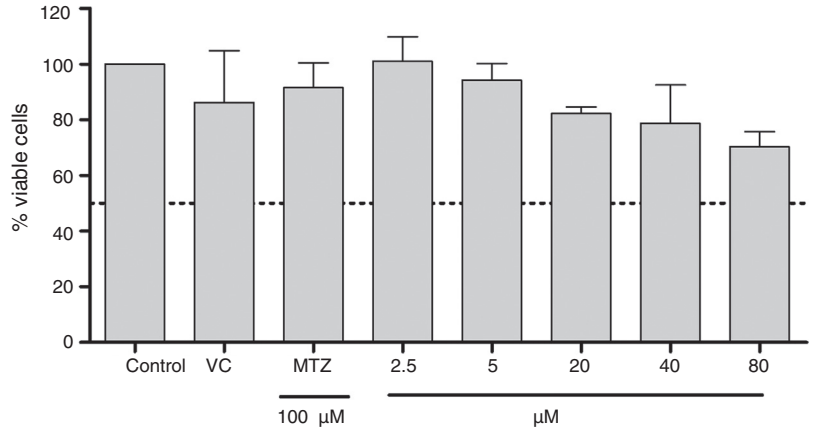

Figure 5 Cytotoxicity effect of QTCA-2 in CHO-K1 cells. Cell proliferation in CHO-K1 was investigated by MTT assay. Data are expressed as means ± SEM from three independent experiments. Different letters (a, b, c and d) indicate difference between treatments. The differences were considered significant when $p < 0.05$.

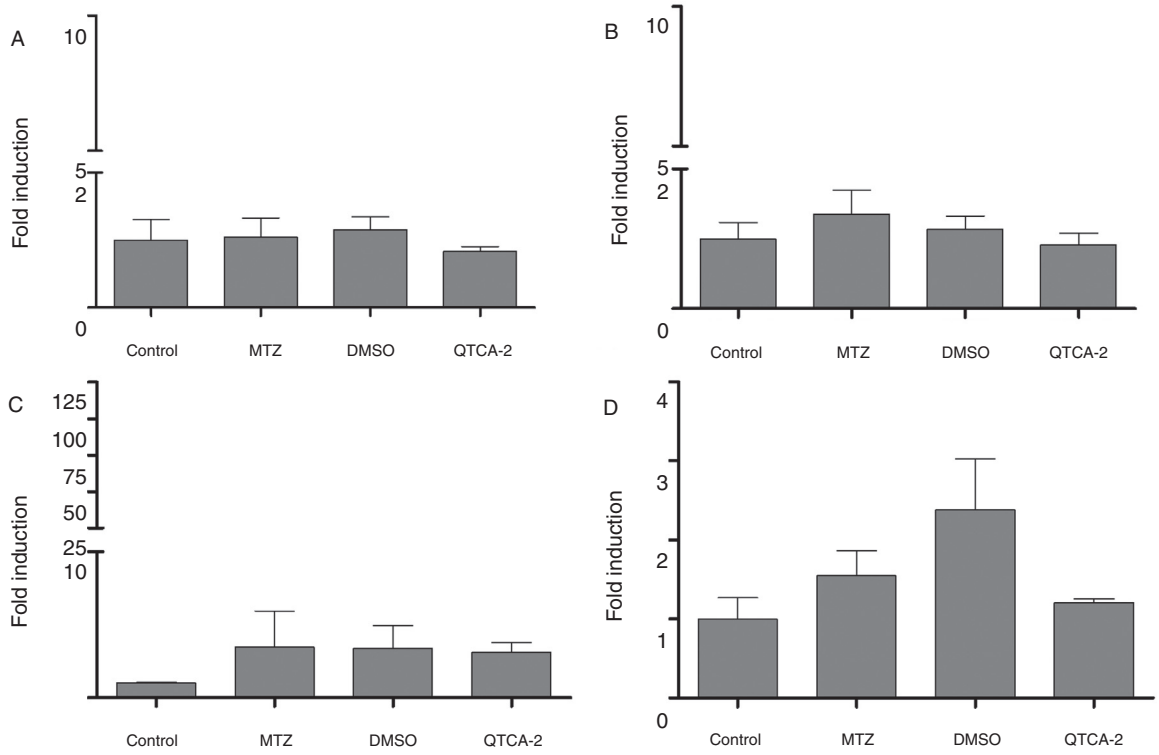

Figure 6 QTCA-2 treatment does not alter the gene expression profile in *Trichomonas vaginalis*. The gene expression profile was determined by qRT-PCR. PFOR A (A); PFOR B (B); Hydrogenase (C) and Malic enzyme (D). Data are expressed as means ± SEM from three independent experiments. VC, vehicle control; MTZ, metronidazole.

drug-activating enzymatic pathways that are responsible for the reduction of the MTZ prodrug in toxic intermediates (Kulda, 1999). On the other hand, the aerobic resistance to MTZ seems to be related to high concentrations of intracellular oxygen as a consequence of the decrease of the oxygen elimination capacity that interferes with the activation of the nitroimidazoles (Leitsch, 2016; Menezes et al., 2016).

In an attempt to propose the mechanism of action of QTCA-2 against *T. vaginalis*, we performed an *in silico* investigation of possible enzymes that could be modulated by the quinoline derivative. The cytosolic enzyme lactate dehydrogenase (LDH) is required for *T. vaginalis* survival since it is a key enzyme in glycolysis, therefore, the high docking score of QTCA-2 in the active site of TvLDH (−9.3 kcal/mol) may favor an antiparasitic activity of this compound. It is worth mentioning that QTCA-2 interacts through hydrogen bonds with THR239 and ARG48, which are also involved in the binding of NADH to TvLDH (Steindel, Chen, Wirth, & Theobald, 2016). Additionally, THR239 and ASN130, which bind with the LDH inhibitor oxamate, are also part of the

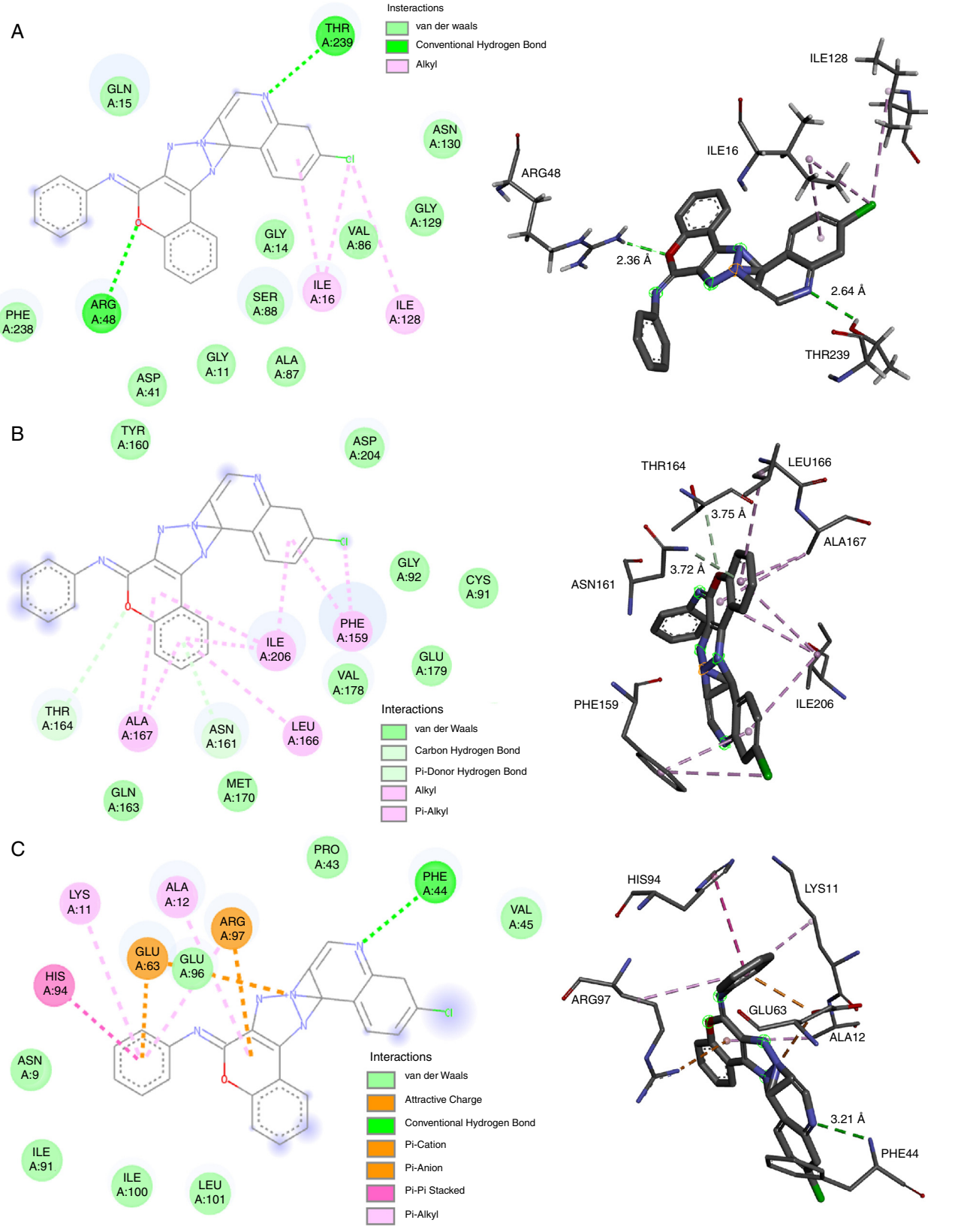

Figure 7 *In silico* analysis of the binding position of QTCA-2 with *T. vaginalis* proteins. 2D and 3D representation of the binding position of QTCA-2 to TvLHD (A), TvPNP (B) and TvTPI (C).

binding mode of QTCA-2 to TvLDH. Overall, it is possible to note that the best docking pose of QTCA-2 in TvLDH involves several residues that also interact to NADH, and this binding mode could account for the high docking score of QTCA-2.

Purine salvage is an essential function for all obligate parasitic protozoa, and for *T. vaginalis*, it exclusively depends on the functions of a nucleoside phosphorylase (PNP) and a nucleoside kinase, and therefore, the purine auxotrophy of *T. vaginalis* makes PNP a key target for antiparasitic chemotherapy (Rinaldo-matthis et al., 2007). Here, we showed that QTCA-2 interacts (−9.1 kcal/mol) with PHE159 and ILE206 and is in close proximity of CYS91, GLY92, VAL178, GLU179 and ASP204. It is known that the purine-binding site of TvPNP consists of four hydrophobic residues, PHE159, VAL178, MET180, and ILE206 (Zang, Wang, Wu, Ealick, & Wang, 2005), therefore, we may assume that modulation of the purine salvage may be one target for the antiparasitic activity of the quinoline derivative. Interestingly, QTCA-2 interacts with some of the same residues that are responsible for the activity of formycin A, a known TvPNP inhibitor, namely CYS91, GLY92, ASP159, VAL178, GLU179 and ASP204 (Zang et al., 2005).

Triosephosphate isomerase (TPI) is a cytoplasmic glycolytic enzyme that plays essential roles in glycolysis, gluconeogenesis and pentose phosphate pathway (Wierenga et al., 2010). Recent data have suggested that TvTPI may have alternative functions when localized at the parasite surface and could be an additional ''moonlighting'' metabolic enzyme in *T. vaginalis* (Miranda-ozuna et al., 2016), and as consequence, a promising target for antiparasitic chemotherapy. Here, we reported that QTCA-2 has a docking score of −7.3 kcal/mol with TvTPI, forming hydrophobic interactions with the catalytic residues LYS11 and HIS94 (Figueroa-Angulo et al., 2012). Additionally, a hydrogen bond with PHE44 may strength the protein–ligand interaction and contribute to the anti-*T. vaginalis* activity evaluated *in vitro*.

Moreover, although it was not possible to test QTCA-2 in MTZ resistant *T. vaginalis* isolates, which authors consider to be a next step that should be taken toward better understanding this molecule's potential, if the abovementioned docking and qRT-PCR are taken into account it's possible to hypothesize that QTCA-2 might present a certain antiparasitic activity against MTZ resistant *T. vaginalis* isolates. That is, because QTCA-2 seems to interact with other molecules, such as TvPNP and TvLDH, besides the ones that are modulated in resistant strains, as for instance PFOR and flavin reductase. However, *in vitro* studies are still needed to confirm or deny this hypothesis.

Conclusions

The compound QTCA-2 exhibited high antiparasitic activity with little cytotoxicity. Moreover, *in silico* analysis showed that QTCA-2 interacts with different proteins that are relevant for *T. vaginalis* survival, and therefore, this modulation may account for the antiparasitic activity of this compound; however, studies that are more refined are needed in order to detect possible alterations in the parasite morphology and in the amount and localization of these proteins in the parasite, which will validate the putative mechanism of action of QTCA-2 proposed by the *in silico* analysis. The characterization of biochemical and molecular targets of the parasite is a potential strategy for new therapies. More studies should be conducted to better identify the mechanism of action of new drugs and the effectiveness of the quinoline compounds in the present study offers new perspectives for exploring the potential of this class of molecules as an additional option for the treatment of trichomoniasis.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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