Structural analysis and molecular docking of trypanocidal aryloxy-quinones in trypanothione and glutathione reductases: a comparison with biochemical data

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A set of aryloxy-quinones, previously synthesized and evaluated against \textit{Trypanosoma cruzi} epimastigotes cultures, were found more potent and selective than nifurtimox. One of the possible mechanisms of the trypanocidal activity of these quinones could be inhibition of trypanothione reductase (TR). Considering that glutathione reductase (GR) is the equivalent of TR in humans, biochemical, kinetic, and molecular docking studies in TR and GR were envisaged and compared with the trypanocidal and cytotoxic data of a set of aryloxy-quinones. Biochemical assays indicated that three naphthoquinones (Nq-h, Nq-g, and Nq-d) selectively inhibit TR and the TR kinetic analyses indicated that \textit{Nq-h} inhibit TR in a noncompetitive mechanism. Molecular dockings were performed in TR and GR in the following three putative binding sites: the catalytic site, the dimer interface, and the nicotinamide adenine dinucleotide phosphate-binding site. In TR and GR, the aryloxy-quinones were found to exhibit high affinity for a site near it cognate-binding site in a place in which the noncompetitive kinetics could be justified. Taking as examples the three compounds with TR specificity (TRS) (Nq-h, Nq-g, and Nq-d), the presence of a network of contacts with the quinonic ring sustained by the triad of Lys62, Met400', Ser464' residues, seems to contribute hardly to the TRS. Compound Nq-b, a naphthoquinone with nitrophenoxyl substituent, proved to be the best scaffold for the design of trypanocidal compounds with low toxicity. However, the compound displayed only a poor and non-selective effect toward TR indicating that TR inhibition is not the main reason for the antiparasitic activity of the aryloxy-quinones.

Keywords: molecular docking; aryloxy-quinones; trypanothione reductase; glutathione reductase; Chagas disease

1. Introduction

Parasitic diseases are a major obstacle to human health and economic development in many parts of the world and cause high rates of mortality and morbidity (WHO, 2011). Current therapies against these diseases are unsatisfactory, with treatment failure being common due to widespread resistance and severe side effects (Bern, 2011; Bern et al., 2007; Rassi, Russi, & Marin-Neto, 2010; Viotti et al., 2006; Yun et al., 2009). Thus, there is a need for the development of new, efficient, and safe drug (Carraro, Iriarne, & Paulino, 2016; Hoelz et al., 2015; Manoel-Caetano Fda & Silva, 2007; Molina et al., 2014; Woodcock & Woosley, 2008). In recent years, naphthoquinones and heterocyclic derivatives are considered privileged structures in the development of novel drugs against parasitic diseases such as trypanosomiasis (Prati et al., 2015; Salas, Faundez, Morello, Maya, & Tapia, 2011).

There are several proposed mechanisms of action for the trypanocidal activity of quinone derivatives. One of them is the well-known ability of quinones for generating reactive oxygen species (ROS) through redox cycling with molecular oxygen and consequently oxidative stress and cell death (Benites et al., 2010; Henry & Wallace, 1996; Kovacic, 2007; O’Brien, 1991; Paulino et al., 2008).

The metabolic differences between the parasite and host cells to avoid the damage mediated by ROS are considerable (Gutteridge & Halliwell, 2000; Salas et al., 2011). Trypanothione reductase (TR) constitutes a major component of the general oxidative-stress defence in \textit{Trypanosoma cruzi} (Turrens, 2004) and is absent in mammals. The main function of TR is to maintain a reducing intracellular environment by keeping trypanothione in the dithiol state (Gonzalez-Chavez, Olin-Sandoval, Rodriguez-Zavala, Moreno-Sanchez, & Saavedra, 2015). TR is

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essential in all trypanosomatids. Parasites with lowered TR activity display an increased sensitivity toward hydrogen peroxide (Fairlamb, Blackburn, Ulrich, Chait, & Cerami, 1985; Gonzalez-Chavez et al., 2015). The counterpart of the parasite TR in the mammalian host is glutathione reductase (GR). A comparison with the available crystal structures of GR (Berkholz, Faber, Savvides, & Karplus, 2008; Karplus & Schulz, 1987, 1989; Pai, Karplus, & Schulz, 1988; Pai & Schulz, 1983) reveals that the overall structure of the two enzymes is highly conserved. There is an overall 40% sequence identity and both enzymes are homodimers. TR may also replace thioredoxin reductase, although trypanosomatids do possess a conventional thioredoxin.

The key feature of TR and GR is the mutually exclusive specificity for their cognate disulfide substrate. Thus, in principle, it should be possible to inhibit selectively the parasite enzyme without affecting the mammalian one. A detailed analysis of the enzymes (Iribarne, Paulino, Aguilera, Murphy, & Tapia 2002) indicates that in both TR and GR, several alternative sites exist in addition to the binding sites for the disulfide substrate and nicotinamide adenine dinucleotide phosphate (NADPH) and the dimer interface.

For a set of aryloxy-quinones previously synthetized and tested for their trypanocidal and cytotoxic activities (Vazquez et al., 2015), it was proposed that their selective antiparasitic activities may correlate with the inhibition of TR. To prove this hypothesis, here, we report on the biochemical and kinetic analysis of recombinant T. cruzi TR and human GR inhibition. In parallel, in silico molecular docking studies of TR/GR complexes with aryloxy-quinones in all putative binding sites aimed at a deeper understanding of the atomic interactions between these compounds and TR and GR, respectively. As a result, quinone derivatives with good and selective TR inhibitor capacity and with low GR affinity can be proposed as candidates to develop more effective drugs against Chagas and other diseases caused by trypanosomatids.

2. Methodology

A set of 28 aryloxy-quinones (Figure 1) were previously synthetized and tested (Vazquez et al., 2015). The set of quinones was divided in three subsets based on three main scaffolds as naphthoquinones, quinolinequinones, and furanoquinones. Moreover, each main scaffold has different aryloxy substituents. To understand the manner in which these molecules function in the parasite, as well as in the couple TR/GR trypanosomatids and mammalian enzymes, in a first step of this work twenty of these molecules were selected to make TR/GR inhibition and kinetics studies. In a second step an in silico strategy was applied to the entire set of quinones.

2.1. Biochemical studies

TcTR was prepared following a published procedure (Walsl, Bradley, & Nadeau, 1991). Trypanothione disulfide (TS2) was generated enzymatically as described previously (Comini, Dirdjaja, Kaschel, & Krauth-Siegel, 2009).

2.1.1. TR assays

The activity of TR was measured in 40 mM Hepes, 1 mM EDTA, pH 7.5 at 25 °C. The assay mixture contained, in a total volume of 1 mL, 100 μM NADPH, 5–10 μM of TcTR, and 50 μL DMSO as control or the same volume with the tested compound dissolved at a final concentration of 100 μM when the quinone inhibition was tested. The reaction was started by the addition of 100 μM of TS2 and NADPH consumption was measured by following the absorbance decrease at 340 nm at 25 °C (ε = 6.22 mM\(^{-1}\) cm\(^{-1}\)) (Persch et al., 2014). From these data, the percentage of inhibition was calculated. Many compounds proved to be insoluble under the assay conditions and thus could only be measured at rather low concentrations. One representative compound of each subset of compounds (Nq-h, Fq-c, and Qq-b) was subjected to a detailed kinetic analysis.

2.1.2. GR assays

GR was purified from human erythrocytes by the method previously reported (Worthington & Rosemeyer, 1974). The enzyme was stored at a concentration of 5 mg/mL in potassium phosphate buffer, pH 7.0, I .1 M, containing .2 M KCl, 1 mM EDTA, and .1% (v/v) 2-mercaptoethanol.

The activity of human GR was measured in 200 mM KCl, 60 mM K2HPO4, and 1 mM EDTA, pH 6.9 at 25 °C. The assay mixture contained in a total volume of 1 mL, 100 μM NADPH, and 5–20 μM of hGR and 5 mM of inhibitor on DMSO.

After checking for nonspecific NADPH oxidation for two minutes, 1.1 mM of GSSG was added to start the enzymatic reaction. The decrease in NADPH concentration was monitored spectrophotometrically. Residual enzyme activity in the presence of inhibitor was determined relative to controls containing DMSO according to Nordhoff et al. (Fernandez-Blanco, Font, & Ruiz, 2016; Maran, Fernandez, Barbieri, Font, & Ruiz, 2009; Nordhoff, Bucheler, Werner, & Schirmer, 1993).

2.2. In silico assays

All calculations were done running the Molecular Operating Environment (MOE 2015.10, Chemical Computing Group Inc., http://www.chemcomp.com) suite on Linux, on a workstation with a quad-core processor hyperthread equipped.
A molecular docking of 28 carbo and heterocyclic derivatives of aryloxy-quinones was done to study the interactions with the putative enzymatic receptors TR and GR. This procedure implies a ligand and a receptor preparation, the development of a validated docking procedure and the molecular docking itself.

2.2.1. Ligand preparation: modeling and conformational analysis

The three-dimensional (3D) structures of the 28 molecules under study (Vazquez et al., 2015) (Figure 1) were submitted to energy minimization by means of MMFF94x force field (Halgren, 1996a, 1996b) and the program suite MOE. To use all flexibility capacity of the program, a conformational analysis was performed for all naphthoquinones, quinolinequinones, and furanoquinones using the low-mode molecular dynamics (LowModeMD) approach (Labute, 2010) to search minimum energy conformers. The resulting conformations are saved to the output database provided they meet the energetic and geometric criteria.

An energy minimization was done and terminated when the root mean square (RMS) gradient test fall below .005 kcal/mol. The maximum number of energy minimization iterations was selected as 500. Two conformations are judged equal if the optimal heavy atom RMS superposition distance is less than the specified tolerance of .25 Å. All conformations with an energy greater than the sum of the global minimum energy plus a cutoff of 7 kcal/mol were discarded.

2.2.2. Crystal structure of the putative targets

2.2.2.1. Trypanothione reductase. The catalytic function of TR is the reduction of its cognate substrate TS2 to the

Figure 1. Chemical structures of aryloxy-quinones with trypanocidal interesting activities.
dithiol form T(SH)₂. TR is active as a homodimer with a subunit mass of about 52 kDa. The two identical catalytic sites are composed of residues of both subunits and comprise at least two regions, namely the NADPH site and the disulfide substrate site, which are separated by the flavin ring (Paulino et al., 2005).

The crystal structure of *T. cruzi* TR complexed with its physiological substrate TS₂ was obtained from the Protein Data Base (PDB 1BZL), 2.4 Å resolution (Bond et al., 1999). The homodimeric protein is composed of two peptide chains each containing 486 amino acids, two molecules of the cofactor flavin adenine dinucleotide (FAD) and two molecules of ligand, the *bis*(gamma-glutamyl-cysteinyl–glycinyl) spermidine (TS₂). Both TR cocrystallized ligands are posed each one in both catalytic sites.

### 2.2.3.1. Catalytic-binding site and neighborhoods.

Cys53 and Cys58 form the catalytic redox-active site dithiol/disulfide of TR and are located at the bottom of the cleft near the isoalloxazine ring of FAD (Bond et al., 1999). In GR, the respective residues are Cys58 and Cys63 (Janes & Schulz, 1990; Karplus, Pai, & Schulz, 1989; Pai & Schulz, 1983; Pai et al., 1988).

When the TS₂-binding site in TR is described based on a list of contacting amino acids at a 4.5 Å distance of TS₂, it could observed that amino acids from both A and B chains participate in the binding (Bond et al., 1999).

Research from our groups gave detailed information about these structural characteristics (Hikichi, Paulino, Hansz, & Tapia, 1995; Iribarne, Paulino, Aguier, & Tapia, 2009; Iribarne et al. 2002; Paulino et al., 2005). The overall structure of the active site of GR is similar to that of TR. However, in contrast to the negatively charged TS₂ binding site in TR, the GSSG-binding site in GR displays an overall positive charge.

A hydrophobic region named “Z-site” was defined in 1991 by el-Waer, Douglas, Smith, and Fairlamb (1991). They suggested the Z-site as a relevant binding pocket for TR inhibitors although Glu466 and Glu467, the residues mainly responsible for the electrostatic interactions with the inhibitors, are conserved in GR and TR (de Paula da Silva, Bernardes, da Silva, Zani, & Carvalho, 2012). Thus, it is difficult to assume a specific binding of TR inhibitors at this site. Indeed, although docking simulations yielded the Z-site as favored binding site, the crystal structure revealed the inhibitor at the hydrophobic wall lining the TS₂-binding site (Persch et al., 2014). Here we will demonstrate that for aryloxyquinones, a mixture of both catalytic and Z-site residues, in its neighborhood, could confer selectivity.

### 2.2.3.2. Alternative putative binding sites.

In each active site of the homodimer, the cofactor FAD is found, together with the dithiol/disulfide bridge, essential for catalysis. During catalysis, reducing equivalents flow from NADPH to the disulfide substrate (e.g. trypanothione) with FAD and the disulfide bridge as intermediates. Two proton relays, one at each site, modulate the transfer. This complexity justifies the proposal of the NADPH site as a putative-binding site, in the same way by which the endogenous substrate binding site was proposed before.

At the subunit interface, both enzymes have a cavity that was marked in the Site Finder strategy, as a likely binding site. This is in accordance with previous crystal structures of GR inhibitor complexes showing ligand binding at this site (e.g. Bilzer et al., 1984; Savvides & Karplus, 1996). In comparison to GR, the cavity of TR is more extended and narrow, contacting both catalytic sites in neighborhood of the above-described Z-site (Figure 2). This topology like a bridge between both catalytic sites caused us to think that this site may bind molecules. For this reason, in the docking approaches, we compared this region with other sites.

### 2.2.4. Molecular docking

Separate configurational databases in which each one of naphthoquinones, quinolinequinones, and furanoquinones...
configurations were used. As ligand, it was used a configurational database of molecules.

Four sites are assayed separately. The catalytic binding sites were defined from a 4.5 Å sphere around the co-crystallized ligand of TR and GR, respectively. The interface site definition was made through the Site Finder procedure above described. In the case of NADPH, a 4.5 Å sphere around the FAD adenine moiety defined the site.

Docking validation tests were performed with three set of conformational databases for naphthoquinones, quinolinequinones, and furanoquinones and the Catalytic Site 1. Two placement methods: Triangle Matcher and Alpha PMI (Udatha, Sugaya, Olsson, & Panagiotou, 2012) were assayed. Affinity dG (AdG) (Halgren, 1996a, 1996b) and London dG were assayed for scoring and re-scoring. Finally, MMFF94x force field was used to energy minimize the resulting structures.

2.2.5 Protein–ligand interactionfingerprints (PLIF)

The PLIF descriptors implemented in the MOE were used as a benchmark with respect to interaction fingerprints. Interactions are classified as hydrogen bonds,
ionic interactions, and surface contacts according to the residues. The PLIF descriptors for all protein-bound ligands were generated with the default parameter set in MOE and presented in Population Display and the Barcode display.

The Population Display is a histogram showing the number of ligands (Y-axis) with which each residue (plotted in the X-axis) interacts. In the Barcode representation of binding interactions, they are horizontal lines (rows) that correspond to each of the studied compounds.

Additionally, two other analysis of PLIF results were performed and PLIF-ContactMap and PLIF-HeatMap were generated.

2.2.6. H-Bond–ligand interactions
The ligand interactions application provides a tool to visualize an active site of a complex in diagrammatic form. A selection of interacting entities, which includes hydrogen-bonded residues, close but nonbonded residues, solvent molecules and ions are drawn about the ligand, their positions in 2D being chosen to be representative of the observed 3D distances, as well as taking into account aesthetic considerations.

3. Results and discussion

3.1. Enzymatic inhibition and kinetics

3.1.1. TR and GR inhibition screening
Table 1 summarizes the experimental data obtained for all aryloxy-quinones investigated in this study. The trypanocidal activity toward T. cruzi, cytotoxicity vs. J-774 cells (Vazquez et al., 2015), as well as the in vitro TR/GR inhibitor activities are given.

Considering the three subsets of aryloxy-quinones studied, a naphthoquinone (Nq-h) proved to be the most active inhibitor of TR. However, no correlation between TR inhibition and the antiparasitic activity efficiency was observed. Whereas compound Nq-b showed the highest trypanocidal activity, it was a comparably weak inhibitor of TR and was even more active against GR. Thus, the compound is a good example that other mechanisms are responsible for the cellular mode of action of the quinones.

Nq-b is a naphthoquinone with a nitro substituent in its aryloxy moiety which may be the reason for its excellent trypanocidal activity (IC_{50} T. cruzi epimastigote = 0.2 μM). It is even more potent than nifurtimox (IC_{50} T. cruzi epimastigote = 7 μM) and possibly acts by a similar mechanism.

Nq-h was the most effective and selective inhibitor of TR. Its trypanocidal activity was good; although ten fold lower than that of Nq-b. The halogen (Br) on the naphthoquinone and the O-naphthyl substituent suggest that the electronegativity as well as the planar aromaticity may confer selectivity for TR vs. GR inhibition.

3.1.2. TR kinetic assays
Nq-h, Qq-b, and Fq-c were selected as representatives for the naphthoquinone, quinolinquinone, and furan-quinone subsets and subjected to a detailed kinetic analysis. The type of inhibition was derived from Line-weaver–Burk plots (Supplementary Material Figure I). The inhibitor constants K_i were calculated from direct plots. The data obtained are shown in Table 2. All three compounds inhibited TR with a noncompetitive type of inhibition indicating that their binding site does not coincide with that for TS2. With a K_i value of 1.1 μM, Nq-h was clearly the best TR inhibitor.

3.2. The 3D structure of the assayed quinones
database and it Structure–Activity Relationships (SAR)
Table 3 summarizes the main structural characteristics and results to allow a SAR analysis of the trypanocidal and TR inhibitory activities.

3.2.1. SAR with respect to the trypanocidal activity
Being that the aim of this search for a trypanocidal non-toxic drug is to improve the design of nifurtimox (better growth inhibition together with a lesser toxicity), it was been considered that a quinone with a good trypanocidal activity must inhibit T. cruzi growth in a concentration under .17 μM, a medium activity will correspond to concentrations between .17 and 7 μM, and quinones with inhibitory concentrations over 7 μM (that of nifurtimox) will be considered with low activity.

As we concluded in a previous paper (Vazquez et al., 2015), when comparing the naphthoquinones, furanoquinones, and quinolinequinones, the naphthoquinone moiety proved to be the best substructure for trypanocidal activity and lower toxicity. This is because the naphthoquinones were specially analyzed here. Inside the naphthoquinone sample, we could observe many variants including the addition of nitro, bromine, phenyl, or naphthyl moieties.

The presence of two putative redox active moieties: the quinonic ring and the nitrophenyloxy group (Nq-b) is an example of a very good improvement giving to the molecule very good (the best) activity in T. cruzi. With respect to the toxicity (J-774 cells), a comparison with nifurtimox seems put in evidence that the presence of the quinonic planar structure together with a phenoxy group, gave to the molecule selectivity in favor to the
parasite inhibition (low toxicity than nifurtimox). Finally, the addition of a halogen when a nitro moiety is present (like in Nq-e) seems to lower the T. cruzi selective growth inhibition.

The presence of a halogen or a dimethyl-phenyloxy (comparing Nq-a with Nq-d or Nq-a with Nq-e) did not help to improve the T. cruzi growth inhibition either to lower the toxicity.

The results of molecules with an alpha or beta naphtyloxy moieties, with or without a bromine atom in the structure, seems indicate that in which refers the T. cruzi growth inhibition these moieties have quite similar impact. In some cases (comparing Nq-i with Nq-j), the bromination contributes to lower the toxicity when a beta naphtyloxy is present in the structure. The halogenation of an alpha naphtyloxy moiety as in the case of the Nq-h seems not to give to the molecule a special either selective activity (compared with Nq-g).

3.2.2. SAR with respect to the TR/GR inhibitory activity

The nitro moiety, so good in the design of a trypanocidal molecule, is not related with a good and selective TR inhibition. This is the case of Nq-b and Nq-e, which are not selective TR inhibitors even if they are nontoxic and trypanocidal molecules.

A naphthoquinone scaffold accompanied by a phenyl nitro compound like that of Nq-b is depicted as the ideal scaffold to reach higher T. cruzi inhibition together with very low toxicity, even if the action mechanism will not be the selective inhibition of TR.

The phenoxy group is good when it has a bromination in the naphthyl moiety (Nq-d). The alpha napthyloxy group as in Nq-h and Nq-g, seems to be related to a selective TR inhibition. When the alpha napthyloxy group has an halogen (Nq-h), the selectivity is as good than when it is absent (Nq-g).

They are no TR inhibition data to analyze the influence of a beta naphthyl (as it is the case of Nq-j) even if both have low activity in GR.

In the last columns of the Table 3, main questions referring these observations were YES/NO answered. In consequence, those molecules with both YES answers (T. cruzi and TR-selective inhibition): Nq-h, Nq-d, and Nq-g, were selected to further discussion.

Nq-h, Nq-d, and Nq-g are good and selective T. cruzi inhibitors and at the same time of TR. For these
Table 3. Summary of the structural characteristics for the assayed sets of naphtoquinones (Columns 2 to 6). Columns 7 and 8: color-coded *T. cruzi* inhibition growth and J-774 measurements (Vazquez et al., 2015): *T. cruzi* growth inhibition was considered TRYPANOCIDAL (GREEN) for compound concentrations below .17 μM; MEDIUM for concentrations between .17 and 7 μM and LOW (RED) those equal or higher than 7 μM (that of Nfx). Columns 9 and 10: color coded TR and GR inhibition results. TR inhibition was considered good (GREEN) when inhibitions of more than 43% were detected with concentrations below 5 μM. All other cases were considered low (RED); GR inhibition was considered low (RED) when a 50% inhibition was obtained for concentrations above 100 μM; all other cases were considered a low inhibition (GREEN). Column 11: Index of selectivity (IS) evaluated from data of Table 1 for the *T. cruzi* inhibition as the ratio between the J-774 by the *T. cruzi* growth inhibition concentrations. Values over 90 were considered as SELECTIVES (GREEN), between 45 and 90 MEDIUM SELECTIVITY (YELLOW) and BAD SELECTIVITY (RED) (<45). Column 12: The Index of Selectivity for the inhibition of TR (TRS) was calculated as the ratio between the values in the fourth by the fifth columns of the Table 1 and were classified as SELECTIVE TR INHIBITION (GREEN) when a value over 20 was obtained. Column 13: the ratio of free energies in TR by GR (data from Supplementary Material Table A). Selectivity is assigned to rates over 1.11 (GREEN). Last two columns: main questions are YES/NO answered.

| Molecule | Nitro | Br | Naphthoxy | Phenylx | Methylenylx | *T. cruzi* activity | Putative human | TR inhibition | GR inhibition | *T. cruzi* selectivity (IS) | TR selectivity (TRS) | In silico free energies TR/GR ratio | Inhibit *T. cruzi* with low toxicity? | Inhibit selectively TR? |
|----------|-------|----|-----------|---------|--------------|---------------------|--------------|--------------|--------------|-------------------------------|-------------------|--------------------------------|----------------------|-----------------|
| iQ-a     | x     |    | N         | x       |              | Highly tripanocidal | Nontoxic     | Inhibition   | Inhibition   | 250                          | 1.5               | 1.1                           | YES                  | NO              |
| iQ-b     | x     | x  | x         |         |              | Higher tripanocidal activity | Nontoxic     | Low Inhibition | Inhibition   | 1250                         | 10                | 0.05                          | YES                  | NO              |
| iQ-c     |       |    | x         |         |              | Tripanocidal | Medium toxicity | Inhibition   | Inhibition   | 74                           | 1.4               | 1.0                           | YES                  | NO              |
| iQ-d     | x     | x  | x         |         |              | Tripanocidal | Nontoxic     | Inhibition   | Low Inhibition | 164                          | 20                | 0.93                          | YES                  | YES             |
| iQ-e     | x     | x  | x         |         |              | Tripanocidal | Nontoxic     | Low Inhibition | Low Inhibition | 418                          | 10                | 0.99                          | YES                  | NO              |
| iQ-f     | x     | x  | x         |         |              | Tripanocidal | Nontoxic     | Low Inhibition | Low Inhibition | -                            | -                 | 1.0                           | YES                  | NO              |
| iQ-g     | x     | α  | x         |         |              | Tripanocidal | Medium toxicity | Inhibition   | Low Inhibition | 83                           | 40                | 1.11                          | YES                  | YES             |
| iQ-h     | x     | x  | α         |         |              | Tripanocidal | Medium toxicity | Inhibition   | Low Inhibition | 89                           | 20                | 1.2                           | YES                  | YES             |
| iQ-i     | x     | β  |           |         |              | Tripanocidal | Medium toxicity | ins         | Low Inhibition | 74                           | -                 | 1.05                          | YES                  | NO              |
| iQ-j     | x     | x  | β         |         |              | Tripanocidal | Non-toxic   | ins         | Low Inhibition | 112                          | -                 | 0.89                          | YES                  | NO              |
3.3. Molecular docking

The previous observations were limited by the lacking of all necessary experimental results. This is because in this work we had the proposal of doing all in silico evaluation of the complete set of the quinones under study. The in silico will be in this case, complementary to all the discussion envisaged in the Section 3.2.

3.3.1. Docking validation

The validation step aimed into obtaining the broader range of energies to assure a good conformation sampling. The full set of 10 naphthoquinones, nine quinolinequinones, and nine furanquinones was used to generate three conformational databases of 63, 59, and 64 conformers, respectively, and they were docked in the Catalytic Site 1 of the TR (1BZL).

The results are presented in the Table 4. The best results were obtained for the Alpha PMI and the London ⊗ G methods.

As the second criterion of validation, we look for a maximum overlap between the cocrystallyzed and docked TS2. The Alpha PMI and the London ⊗ G methods were used to dock the TS2 in the catalytic Site 1. The overlapping between the cocrystallyzed and docked TS2 was measured and resulted an root mean square deviation (RMSD) of 2.02 Å.

In consequence, the procedure was declared validated.

3.3.2. Docking in the catalytic sites of TR and GR

The three conformational databases were used as “ligand” in the docking.

As “Receptor,” they were settled all atoms of TR or GR. As “Site,” it was defined a sphere of 4.5 Å around the crystallographic ligand TS2 or RGS, respectively. After selection, TS2 (or RGS) molecules were deleted and FAD atoms remained as unselected atoms.

With the aim of comparing within the experimental results shown in the Table 1, in the Supplementary Material Table A, were detailed the ΔG of the best docked conformation for each set of aryloxy-quinones. All poses were classified in two ranges of high and low energies.

All high energetic poses were red-colored ant the other in yellow. The results are shown in the Figure 2 and in Supplementary Material Figures II and III.

As it could be observed inside the left bottom frame of the Figure 2(A), two populations of naphthoquinones are detected in TR. The red colored poses, that is, the more energetic bindings, are in a site in the borderline between the Z site and the catalytic site.

When the furane and quinoline quinones were docked against the catalytic site 1 of TR, similar results were obtained being the more energetic bindings near to the catalytic site in the borderline with the Z site (Supplementary Material Figures II(a) and III(a)).

To describe graphically, this new aryloxy-quinones site, an electrostatic map was obtained for TR and GR (Figure 2(A) and (B) left top frames). The Electrostatic Feature Map in MOE is an application of the Poisson–Boltzmann Equation to the prediction of electrostatically preferred locations of hydrophobic, H-bond acceptor and H-bond donor locations.

The best energies in the docking against GR were observed in a subsite of the catalytic site 1 similar to that detected for TR (left bottom frame in Figure 2(B) and Supplementary Material Figures II(b) and III(b)).

3.3.3. Docking in the alternative sites of TR and GR

The docking for the three conformational databases in the interface site of TR (1BZL) and y GR (4GR1) shown that all best scored poses were placed in the middle of the interface but with lesser free energies than in the other assayed sites (data not shown). As an example, in the Supplementary Material, Figure IV shows the naphthoquinones docked in the interface site of TR and GR.

The results of the docking in the NADPH sites in TR and GR are shown in the Supplementary Material Table A and Figure V for the case of naphthoquinones.

3.4. Analysis of docked complexes in TR and GR

3.4.1. Analysis of docking in the catalytic site by PLIF

A massive analysis of contacts between docked quinones in TR and GR was performed by means of PLIF. For data visualization, the Barcode and Population Display and PLIF-ContactMap and PLIF-HeatMap tools were used.

All Side chain H-donor or acceptor (ChDon/ChAcc), Backbone H-donor or acceptor (BkDon/BkAcc), Solvent H-donor and acceptor (O), Ionic attraction (I), and Surface contact (Surf) were analyzed.

3.4.1.1. Barcode and Population Display. The Figure 3(a) and (b) show the Barcode Display and Figure 4(a) and (b) show the Population Display
(or histogram) made for all 28 docked quinones in GR and TR, respectively.

Two list of contacts emerges and they are, in the case of TR is: Lys62, Thr66, Leu399′, Met400′, His401′, Lys402′, Asp432′, Asn433, His461′, Thr463′, and Ser464′.

In the case or GR, the list of contacts is: Lys67, Asn71, Tyr106, Ile113, Tyr114, Ile343, Arg347, Thr404′, Met406′, Leu438′, His467′, Thr469′, Ser470′, Glu473′, and Thr476′.

Those contacts that have counterpart in TR/GR are highlighted in bold: Lys62 in TR is Lys67 in GR; Thr66 in TR is Asn71 in GR; Leu399′ in TR is Met406′ in GR (a nonconservative change); His461′ in TR is the His467′ in GR; Thr463′ in TR is Thr469′ in GR and Ser464′ in TR is Ser470′ in GR.

### Table 4. All combinations of docking conditions assayed to docking validation. Binding energy values are in kcal/mol.

| Placement | Affinity dG | London dG | Affinity dG | London dG |
|-----------|-------------|-----------|-------------|-----------|
| Naphthoquinones | −6.72 to −5.6 | −13.6 to −6.9 | −6.96 to −1.74 | −7 to −1.65 |
| Furanequinones | −7.11 to −5.5 | −13.7 to −2.99 | −7 to −1.65 | −10.83 to −6 |
| Quinolinequinones | −6.88 to −5.69 | −13.7 to −2.29 | −6.65 to −1.7 | −10 to −5.85 |

Figure 3. Barcode display analysis of PLIF results.
Notes: The horizontal rods correspond to each of the studied compounds. A black rod indicates the presence of an interaction with residues shown in the X-axis at the bottom of each graph. 3(a) Barcode display in TR. 3(b) Barcode display in GR.
All other contacts of docked quinones in TR does not have a counterpart in GR and vice versa.

In the Figure 4(a) and (b), the Y-axis shows the relative counts for the bits, directly related with the number of contacts. For example, the Lys62 has the three higher bars and that means that this amino acid makes three kinds of contacts with the most of quinones. On the contrary, Asn433 has three bars with very low Y-value meaning that it forms three different and nonfrequent contacts. Lys62 is of most importance for the TR binding and is evidenced by the three columns in the Figure 3(a). In the case of GR, Lys67 forms hydrogen bonds with all the analyzed quinones evidenced by the two columns if the Figure 3(b). Even if the mammalian enzyme offers a more electrostatic region to binding and that the Lys62 is conserved in GR, some selectivity for TR could be evidenced by an increased number of contacts.

Leu399′ in TR is a contact detected for quinones that is a nonconserved residue in GR (Met406′). This contact seems to be important to the pose of quinones in the TR. However, Met406′ makes more contacts in GR than Leu399′ in TR, allowing to conclude that both residues are of similar important in the TR or GR binding.

The Met400′ in TR appears, as a surface contact in the PLIF analysis for Nq-h, Qq-d, and Fq-b and it is absent as contact in GR. For this reason, we could propose this contact as important for the TR specificity (TRS). This residue deserved special consideration in the interaction analysis of quinones by de Molfetta, de Freitas, da Silva, and Montanari (2009).

Referring the contact with Ser464′ in TR (Ser470′ in GR), it appears more frequently in TR than in GR as an important H-bonding interaction in the PLIF analysis. In GR (Figure 4(b)), there is a differential contact found in the Glu473′ (not present in TR) giving to this environment the electrostatic differential characteristic pointing to a selective binding in favor of TR.

3.4.1.2. PLIF-ContactMap and PLIF-HeatMap. The PLIF-ContactMap and the PLIF-HeatMap were created from the PLIF analysis as an ad hoc designed tool to observe all docked conformations by means of a colorful image of binding in TR and GR that confirmed and completed the previous observations. They are presented
in the Table 5 and in the Supplementary Material Table B.

In the case of PLIF-ContactMap, to each given contact, it was assigned a number representing the percentage of conformers of a quinone making a contact with a given amino acid in TR or GR. For data visualization, the numbers of contacts were highlighted by means of a color gradient from dark red (100%) to light blue (4%). The result is shown in the Table 5 (PLIF-ContactMap). Alternatively, the percentage of contacts was associated with the intensity of the color from dark to light red (PLIF-HeatMap).

Lys62 in TR makes two kinds of H-bond acceptor and surface contacts with high percentage of conformations making contacts (e.g. 64% for Nq-h). Met400 has unique and colorful annotations in TR representing the contacts made by a high percentage of quinones conformations (e.g. 55% for Nq-h). Finally, higher percentage of conformations make four kind of contacts (two H-bond side-chain acceptor and two backbone H-bond acceptor) with Ser 464 in TR (e.g. 69% for Nq-d). The PLIF-HeatMap (Supplementary Material Table B) presents a similar picture.

3.4.2. Analysis of docking in the alternatives binding sites

When the interface site was selected in the TR (Supplementary Material Table A), all quinones finally were posed near the catalytic site, indicating a tendency to bind in this subsite (Supplementary Material Figure IV). However, when the same strategy was used for GR, it is noticeable that all quinones were placed in the middle of the interface site (Supplementary Material Figure IV). Earlier studies in GR (Karplus & Schulz, 1989) shown that a putative site with allosteric properties placed in the dimer interface should be considered as a putative-binding site. Tridimensional and molecular dynamics analysis (Hikichi et al., 1995) show that the GR dimer interface is quit adequate to lodge planar molecules as they are too the aryloxy-quinones here studied. However, if we take into account the scoring obtained for all of assayed sites, the dimer interface seems not to be the best for binding.

When the NADPH was used to center a putative binding site (Supplementary Material Table A), in the case of TR the scores of all quinones ranked in lower values than in the aryloxy-quinones site near the catalytic one described in the 3.4.1 Section. In principle, then, the NADPH site could be not considered as a secondary one for binding and for inhibition, remaining as more relevant the binding near the catalytic site. On the contrary, in the GR enzyme, it seems to be a site with similar “appealing” for quinones than the active site, with similar scores for both sites. Any case, the NADPH site in GR seems to be a good alternative for binding. This result is in agreement with previous observations (Karplus et al., 1989). Then, for a molecule with a high binding energy either in the catalytic or the NADPH site in GR, it will be predictable a non-selective in TR inhibition.

3.4.3. 3D Graphical analysis and modeling

3.4.3.1. Putative modeling of selective TR inhibitors.

Taking the observations related to the catalytic and the NADPH sites together, it could be suggested that if a quinone must be designed as a noncompetitive TR inhibitor, it must be bound near the catalytic site for TR but allowing the nonproducing posing of TS2. In addition, this compound must have low binding energies in the catalytic either the NADPH GR sites.

In the Figure 5, an hypothetical complex of a TR with Nq-h posed near the catalytic site (as described in the 3.4.1 Section) with the cocryrstallized TS2 in the crystallographic pose is shown. The TR-TS2-Nq-h energy minimized model was used as putative virtual complex to show that there is enough room inside the catalytic site to lodge the TS2 and the inhibitors. Furthermore, this model could agree with a noncompetitive inhibition.

Based again in our results, to add selectivity against GR, it will be necessary that the quinone, even if it will be posed in the NADPH site or in the catalytic site, the bound will be so weak to no interfere with the GR activity. This is being sustained, for example in the case of Nq-h, by a low GR score, concomitantly with a very low GR inhibition capacity.

Moreover, if the aim of an anti-Chagasic drug should be to design optimal and selective TR inhibitors with maximal trypanocidal activity, structures like that of Nq-h could be used as leads to the improvement of the design.

3.4.3.2. Graphical analysis of best and selective TR inhibitors and comparison with the best trypanocidal assayed quinonic compounds.

In the Table 3, we add three more columns containing the score in TR and GR, and finally, the ratio between both measurements (TR/GR) as a theoretical determination of the TRS.

If we take as good specificity a ratio over 1.1, the prediction of the TRS is that Nq-h and Nq-g are selective, and all other naphthoquinones are nonselective. We were successful into predict two of three TR selective inhibitors, and all (7) nonselective TR inhibitors. It was just only one failure into predict the Nq-d as nonselective (being selective). In view of this result, we could be confident that the in silico methods have a very good capacity to predict if a molecule will be a selective TR inhibitor and to give an atomic description of the interaction.
Table 5. PLIF-ContactMap: Contact percentage detected by PLIF between a given aminoacid and all conformers of each quinone in TR and GR. Y-Axis: TR (grey) and GR (yellow) contact codes are detailed: Side chain H-acceptor (ChAcc), Backbone H-acceptor (BkAcc), and Surface contact (Surf). Blocks are colored by means of a color gradient from blue (low percentage of contact) to red (high percentage of contact). Red blocks indicate a highly frequent contacts. Black frames were used to group similar residues in TR/GR. 

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Nq-g, Nq-d, and Nq-h were further analyzed and shown in the Figures 6 and 7 (TR and GR, respectively). To do that, further energy minimization was done for the six complexes of those molecules docked in the TR and GR. The fact that—as it is clear in the Figure 6—the place occupied by quinones in the docking is in the binding site of one of the carboxylate moieties of trypanothione, could have the meaning that the anchorage of the TS2 in the presence of the quinones is prevented, generating a bad and nonproductive linkage.

The Figure 6(d) shows clearly that the Lys62, Met400′, and Ser464′ are the most frequent and intense contacts for a TR-specific binding. This conclusion reinforce the massive observations (PLIF analysis) made in the Section 3.4.1.

To compare with another interesting molecule with low score free energy to TR, we analyzed the Nq-h, the best trypanocidal molecule with low toxicity in the J-774 cells. In it best pose in TR, the nitro phenyl group is placed in the same region that the quinone ring in the Nq-h being the Nq-b quinone ring redirected toward the interface. The low score obtained for this Nq-b pose indicated that the nitro group, with its charge localized in the nitrogen and oxygen atoms, is not adequate to bound this region in which the Lys62 and the Ser464′ are sustaining the quinone rings of three best and specific TR ligand quinones above-mentioned (Nq-h, Nq-g, and Nq-d). The difference in the charge distribution (very concentrate in the nitro group and polarized in the oxygens of quinone rings) must be the key to understand the difference in their TR activity. Then, the same key moiety, which gave best trypanocidal activity to the quinones (a naphthoquinone with a nitrophenyl group), seems to be the responsible for a weak union to TR, making this design not corresponding to a selective TR inhibitor. In summary, we are showing that the action mechanism of naphthoquinones with a nitro group in it structure, making these molecules the best design for a nontoxic and trypanocidal compounds is not associated with the TR-selective inhibition. Moreover, the lack of TRS could be explained too taking into account the global electrostatic charge of both TR and GR catalytic sites. The GR-catalytic site has a global negative charge, and it will be very appealing to a negative-charged nitro compound. However, the very good specific trypanocidal properties of the Nq-b compound is related to it good reactivity in the ROS mechanisms and some capacity to lower the toxicity with respect to nifurtimox.
To associate the previous consideration with the role of different residues in the TR-selective inhibition, for the case of \textbf{Nq-h}, \textbf{Nq-g}, and \textbf{Nq-d}, we could consider for one hand (Figure 6), the presence of a network of contacts with the quinonic ring sustained by the \textbf{Lys62}, \textbf{Ser464}', and \textbf{Leu399}' residues. The alpha naphthyl moieties (\textbf{Nq-h} and \textbf{Nq-g}) contribute to the posing and are oriented to the interface (\textbf{Nq-h}) or to the catalytic site (\textbf{Nq-g}). In the case of \textbf{Nq-d}, its phenyl moiety accomplishes with the same role posing the quinonic ring near to the \textbf{Lys62}, \textbf{Ser464}', and \textbf{Leu399}'.

On the other hand, the \textbf{Met400}, in the neighborhood of the bromine atoms (\textbf{Nq-h} and \textbf{Nq-g}) makes important surface contacts (Figure 6) giving better proper specificity to the TR anchorage. In the case of the nonbrominated \textbf{Nq-d}, with its quinonic ring pointing to the polar region of \textbf{Met400}, a backbone donor H-bonding is giving the differential contact needed to confer TRS.

Once again, it was demonstrated for one hand that a molecule containing a naphthoquinone ring together to a brominated alpha naphthoxy or phenyloxy moiety seems to be a very good model to lead the design of a trypanocidal and nontoxic antitrypanosomatid (e.g. anti-Chagas) drug. On the other hand, this pharmacological property was associated with a selective TR inhibition capacity mediated by a great number of contacts with the triad \textbf{Lys62}, \textbf{Ser464}', and \textbf{Met400}, with the \textbf{Leu399}' accomplishing an important and subtle role in the spatial orientation inside the site.

Figure 6. The best scored poses in trypanothione reductase of (a) \textbf{Nq-h} (cyan rods), (b) \textbf{Nq-g} (magenta rods) and (c) \textbf{Nq-d} (green rods) (d) Ligand interaction fingerprint report: \textbf{Lys62}: ChDon and Surf; \textbf{Thr66}: ChDon; \textbf{Asn433}': ChDon and Surf; \textbf{Leu399}': BkDon; \textbf{Met400}': BkDon and Surf; \textbf{His401}': ChDon; \textbf{Lys402}': ChDon; \textbf{Asp432}': Surf; \textbf{His461}': ChDon; \textbf{Thr463}': BkDon; \textbf{Ser464}': BkDon and ChDon.

Notes: ChDon = sidechain atoms act as H-bond acceptors or donors. BkDon = backbone atoms act as H-bond acceptors or donors. Surf = surface contact interactions.
4. Conclusions

The aryloxy-quinones presented a tendency to have good docking scores in a new subsite inside the catalytic one, configuring a good model for the noncompetitive type of inhibition obtained experimentally. In this putative binding of quinones, there is enough room for the substrate trypanothione that would be anchored but in a way that would interfere with the overall catalysis.

In the TR aryloxy-quinones-binding site, the main contacts were detected with Lys62, Met400', and Ser464'. In GR, a similar sub site was occupied by quinones, but there is a differential contact found in the Glu473' giving to this environment the electrostatic differential characteristic pointing to a selective binding in favor of TR.

For most of the compounds studied, a relationship between trypanocidal activity and TR inhibition could not be observed. However, three of the naphthoquinones, namely Nq-h, Nq-g, and Nq-d, proved the hypothesis of a relationship between a good and nontoxic activity in T. cruzi, accompanied with low toxicity in mammalian cells associated with a specific TR inhibition.

Overall, our studies provided a more thorough understanding of the experimental trypanocidal results and revealed for selected compounds TR/GR as key enzymes in the action mechanisms of some aryloxy-quinones.

Interaction with other cellular targets cannot be discarded. Evidence for this is the very good and non-toxic activity of Nq-b, a nitro compound. A promising
approach to optimize these compounds are *in silico* techniques such as for example the reverse docking that allows to find other targets that could contribute to the aryloxy-quinones trypanocidal action. A strategy involving the cycles of biochemical, parasitological and *in silico* assays, appears to be the best strategy for compound optimization and required to reach the final goal of a new anti-Chagas nontoxic drugs.

### Abbreviations

| Abbreviation | Definition                                      |
|--------------|-------------------------------------------------|
| TS₂          | bis(gamma-glutamyl-cysteiny1-glycinyl) spermidine|
| RGS          | 4-N-malonyl cysteiny1-2,4-diaminobutyrate disulfide|
| DMSO         | Dimethyl sulfoxide                              |
| ROS          | Reactive oxygen species                         |
| TR           | Trypanothione reductase                         |
| GR           | Glutathione reductase                           |
| MMFF94x      | Merck Molecular Force Field                     |
| PLIF         | Protein–ligand interaction fingerprints         |
| PDB          | Protein data bank                               |
| MOE          | Molecular-operating environment                 |
| ps           | Picosecond                                      |
| FAD          | Flavin adenine dinucleotide                     |
| NADPH        | Nicotinamide adenine dinucleotide phosphate     |
| RMS          | Root mean square                                |

### Supplementary material

The supplementary material for this paper is available online at [http://dx.doi.org/10.1080/07391102.2016.1195283](http://dx.doi.org/10.1080/07391102.2016.1195283).

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