Journal of Molecular Graphics and Modelling 28 (2009) 371–381

Contents lists available at ScienceDirect

Journal of Molecular Graphics and Modelling

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

# Assaying phenothiazine derivatives as trypanothione reductase and glutathione reductase inhibitors by theoretical docking and Molecular Dynamics studies

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ARTICLE INFO

*Article history:*
Received 8 April 2009
Received in revised form 31 July 2009
Accepted 15 September 2009
Available online 23 September 2009

*Keywords:*
Chagas' disease
Phenothiazines
Trypanothione reductase
Binding affinity
Theoretical docking
Molecular Dynamics

ABSTRACT

A theoretical docking study, conducted on a sample of previously reported phenothiazine derivatives, at the binding sites of *Trypanosoma cruzi* trypanothione reductase (TR) and human erythrocyte glutathione reductase (GR), examines interaction energies (affinities) towards the parasite enzyme to check for selectivity with respect to the human counterpart. Phenothiazine compounds were previously shown to be TR inhibitors. The analysis of data collected from the docking procedure was undertaken both from the numeric and graphical standpoints, including the comparison of force field, energies, molecular contacts and spatial location of the different orientations that ligands acquired at the binding sites. Molecular Dynamics simulations were also carried out for derivatives with known quantitative inhibition kinetics ($K_i$). The results indicate that (positively) charged phenothiazines attain larger interaction energies at TR active site, in line with previous experimental information. Suitable molecular size and shape is also needed to complement the electrostatic effect, as clearly evidenced by graphical analysis of output docked conformations. Docking energies values are reasonably well correlated with those obtained by Molecular Dynamics as well as with the experimental $K_i$ values, confirming once again the validity of this type of scoring methods to rapidly assess ligand–receptor affinities. Alongside newly discovered classes of TR inhibitors, the promazine (*N*-alkylaminopropylphenothiazine) nucleus should still be considered when good candidates are sought as leaders for selective TR inhibition.



## 1. Introduction

Chagas' disease, caused by the protozoan flagellate *Trypanosoma cruzi* (*T. cruzi*) is one of the most widespread parasitic diseases in the world. Despite disease control and elimination campaigns that have been in effect for the last few years, the disease is endemic in a great portion of Latin America (8 million people are infected and 25% of the total population is at risk, with nearly 60,000 new cases and 12,500 deaths every year), giving rise to both health and economic important problems [1–3]. Notwithstanding the continued scientific research efforts, the current therapies to fight the parasitic infection are far from optimum. In effect, on the one hand, trypanosomatids have developed resistance mechanisms to a number of drugs and, on the other hand, drugs used in clinical treatments have one important drawback: high toxicity [4,5]. It follows that new and improved drugs are urgently needed.

Trypanothione reductase (TR) has long constituted an attractive target for chemotherapeutic research in relation to Chagas' and other trypanosomatid-caused diseases since there are numerous evidences indicating that the enzyme is essential for parasite survival as well as specific, i.e., it has not been found in mammals [6–10]. Instead of TR, almost all other organisms possess glutathione reductase (GR), whose catalytic function consists in the reduction of glutathione disulfide (L-γ-glutamyl-L-cysteinyl-glycine, GSSG) to yield the thiol form (GSH). The latter participates in several important metabolic functions inside the cell such as peroxide detoxication, defense against oxidative stress and synthesis of deoxyribonucleotides [11,12]. In trypanosomatids, GR is replaced by TR, which is responsible for maintaining the intracellular reduced environment via the enzymatic reduction of trypanothione disulfide ($N^1$,$N^8$-bis(glutathionyl)spermidine, $T(S)_2$), to the di-thiol species, $T(SH)_2$. It has been found also that TR, coupled with some other enzyme systems such as thioredoxin and thioredoxin peroxidase, is the principal defense in the parasite against oxidative stress, with $T(SH)_2$ effecting the protective function [13–16]. The relevant matter here is that GR and TR display mutually exclusive substrate specificity, i.e., the crossed complexes, TR-GSSG and GR-$T(S)_2$, are non-catalytic. Thus, it

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doi:10.1016/j.jmgm.2009.09.003

should be possible, in theory, to selectively inhibit the parasite function, hopefully compromising its viability, while leaving host's enzyme machinery intact.

GR and TR belong to the family of flavin-containing disulfide oxido-reductases. They are active as homo-dimmers of about 52 kDa per subunit mass and display an overall 30% of sequence identity [17–22]. In particular, 14 out of 19 residues in the substrate binding site are conserved. The catalytic site is rather complex, comprising two separate regions, i.e., the NADP site (N-site) and the active site (G-site), connected to each other by the flavin ring of FAD and a redox active disulfide bridge. The structural catalytic elements in GR and TR are conserved, namely, the redox active isoalloxazine ring of FAD, the disulfide bridge, and two couples of proton relay residues at the G-site and N-site. The proton relay function acts during the electron transfer step [23,24]. The electrons from the nicotinamide are thought to flow successively along the isoalloxazine and the disulfide bridge, finally reducing the substrate disulfide [23,24]. In addition to the active site, the dimmer interface region defines a putative binding site known as the interface or misleadingly, allosteric site. There is evidence to believe that suitable, i.e., planar ligands such as menadione (2-methyl-1,4-naphtoquinone) bound at this site, could elicit non-competitive enzyme inhibition, both in TR and GR [25]. Contrary to the active site, the interface cavity does not confer selectivity in regards ligand binding.

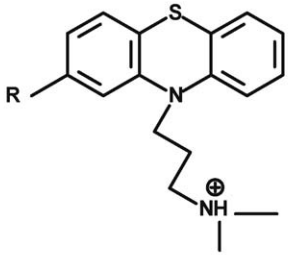
Numerous compounds of varied chemical nature have been tested as potential trypanocidal agents [5,26–28]; among them, phenothiazines are particularly interesting. They constitute a group of tricyclic neuroleptic compounds traditionally employed as antidepressant, anxiolitic and antipsychotic in clinical cases of psicosis, schitzophrenia and related disorders. A couple of decades ago it was realized that these and other tricyclic compounds, such as clomipramine, were active as anti-trypanosomal agents. Soon afterwards, a number of studies on their inhibition effect were reported [29–38]. In particular, molecular modeling techniques showed that these compounds contained some of the best-fitting probes at the active site of TR and could indeed inhibit the parasite flavoenzyme [39].

In a seminal work, Fairlamb and co-workers [40] showed that an extensive series of synthetic phenothiazines acted as reversible competitive inhibitors of TR; among them, there were commercial drugs such as chlorpromazine, trifluopromazine, thioridazine, promethazine, as well as some N-substituted and polysubstituted derivatives. In a related research, phenothiazine cationic radicals were generated by means of $H_2O_2$/peroxidase reactions [41,42]. The radical derivatives, which included promazine, chlorpromazine and trifluopromazine among others, were potent irreversible inhibitors of TR, with some of them achieving 100% of enzyme inhibition at concentrations where reversible inhibition by non-radical species would not take place [40].

It is widely accepted that productive binding, usually involving a molding work on the substrate, is a key step in enzyme catalyzed reactions [43]. This process has to be fulfilled before the chemical interconversion at the active site takes place. In this sense, ligands (e.g. inhibitors), which are able to readily accommodate at the active site at low molding intramolecular energy expenses, would form the most stable complexes with the catalytic receptor and eventually would undergo the chemical reaction [43]. As a result, it is expected that the best inhibitors will have the largest binding energies at the site of reference. Thus, an accurate energy measure of the binding step is desirable if one is to assess the relative inhibitory capacity of a given set of molecules towards particular enzyme or receptor sites.

Theoretical docking procedures [44–49] have proven suitable to adjust ligands at target sites and to yield good estimates of interaction energy (affinity) of the binding step by means of rigid or (partial) flexible treatment of ligand and protein to minimize the molecular interactions involved. In spite of the usual limitations inherent to this type of methods, such as the exclusion of solvent molecules and the neglect of entropic contributions to the binding energy, docking tools become very useful in cases where other more sophisticated calculations are not feasible due to the size of the ligand (inhibitor) sample to be studied. Alternatively, docking tools may be used in earlier steps of a strategy applied to acquire a deeper insight on ligand–receptor interaction. This is best understood in the context of modern virtual (in silico) screenings where one is interested in a rapid evaluation of very large compound samples in order to filter out uninteresting input and keep the best probes for HTS assays so as to hopefully identify good candidates as inhibition leaders. Thereafter, accurate measures of binding affinities may be obtained by means of more elaborated techniques (FEP, LRA, LIE or related calculations).

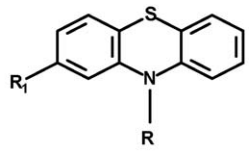
In this work, it is reported the use of theoretical docking studies in order to examine the binding at TR and GR of a sample of previously synthesized phenothiazines which were shown to inhibit both TR and *T. cruzi* in vitro [40]. Molecular structures for all 52 derivatives can be observed in Schemes 1–3. Three dimensional geometries for the whole phenothiazine sample were modeled and optimized to use as input of the docking procedure at the crystallographic enzyme structures. To check up the robustness of the docking results, Molecular Dynamics (MD) calculations in aqueous phase were carried out on selected compounds with known quantitative inhibition kinetics ($K_i$). Output orientations obtained by the docking procedure were used to generate the enzyme–ligand complexes subjected to MD calculations. The existence of correlation between experimental and theoretical results was analyzed. Comparisons were made in regard similar




| Compound | R |
|---|---|
| 1 | H |
| 2 | $CF_3$ |
| 3 | Cl |
| 4 | $COCH_3$ |
| 5 | $CCH_3{=}NOCH_3$ |
| 6 | $COCH_2CH_3$ |
| 7 | $CO(CH_2)_2CH_3$ |
| 8 | $CCH_3{=}NOBz$ |
| 9 | $CO(CH_2)_4CH_3$ |
| 10 | $CH_2OC_6H_4$-p-$C(CH_3)_3$ |
| 11 | COOH |
| 12 | $CH_2OH$ |
| 13 | $CONH_2$ |
| 14 | $COOCH_3$ |
| 15 | $CH_2NH_2$ |
| 16 | CH=NOH |

Bz: benzoyl

**Scheme 1.** Series 1 phenothiazine derivatives (promazines). (Top) Parent structure; (bottom) side chains.

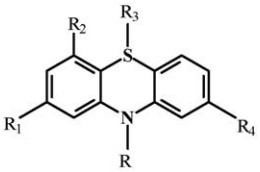



| Compound | R | $R_1$ |
|---|---|---|
| 17 | H | H |
| 18 | $(CH_2)_3NH_2$ | H |
| 19 | $CH_2C(CH_3)_2N(CH_3)_2$ | H |
| 20 | $(CH_2)_3NHCH_3$ | H |
| 21 | $(CH_2)_2C(NH_2)=NH$ | H |
| 22 | $CH_2CHCH_3N(CH_3)_2$ | H |
| 23 | $CH_2CHCH_3CH_2N(CH_3)_2$ | H |
| 24 | $(CH_2)_3Pip(CH_2)_2OH$ | $CF_3$ |
| 25 | $(CH_2)_3PipCH_3$ | $CF_3$ |
| 26 | $CO(CH_2)_2COOH$ | Cl |
| 27 | $COCH_2NEt_2$ | Cl |
| 28 | $COCH_2NHPh$ | Cl |
| 29 | $CO(CH_2)_3NEt_2$ | Cl |
| 30 | $COCH_2Cl$ | Cl |
| 31 | $CO(CH_2)_5NEt_2$ | Cl |
| 32 | $CO(CH_2)_2NEt_2$ | Cl |
| 33 | $COCH_2Br$ | Cl |
| 34 | $CO(CH_2)_2CONHNHPh$ | Cl |
| 35 | $CO(CH_2)_2CONHCH_2Ph$ | Cl |
| 36 | $CO(CH_2)_2CH_3$ | Cl |
| 37 | $(CH_2)_2NMe_2$ | Cl |
| 38 | $(CH_2)_2C(NH)_2=NH$ | Cl |
| 39 | $(CH_2)_3NH_2$ | Cl |
| 40 | $(CH_2)_3NHCH_3$ | Cl |
| 41 | $(CH_2)_3N=N(CH_2)_2CH_3$ | Cl |
| 42 | $(CH_2)_3N$ | Cl |
| 43 | $(CH_2)_2NEt_2$ | Cl |

Compounds 18-25, 27, 29, 31-32, 37-40 and 42-43 where modeled with positive (+1) net charge.
Pip: piperidine; Ph: phenyl; Et: ethyl; Me: methyl

**Scheme 2.** Series 2 phenothiazine derivatives. (Top) Parent structure; (bottom) side chains.

studies performed previously on some other TR inhibitors; in particular the very well known nitrofuran compounds [50–51]. Based on interaction energy results and graphical analysis, hypotheses are advanced to hopefully gain deeper insight on the determinants of inhibitor specificity between TR and GR.




| Compound | R | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| 44 | $(CH_2)_3NMe_2$ | H | Cl | | H |
| 45 | $(CH_2)_3NMe_2$ | $COCH_3$ | H | | $COCH_3$ |
| 46 | $(CH_2)_3NMe_2$ | Cl | H | | $COCH_3$ |
| 47 | $(CH_2)_3NMe_2$ | Cl | H | | $CCH_3$=NOBz |
| 48 | $(CH_2)_3NMe_2$ | Cl | H | O | H |
| 49 | $COCH_3$ | $COCH_2Cl$ | H | | H |
| 50 | $COCH_3$ | $COCH_3$ | H | | H |
| 51 | $COCH_3$ | Cl | H | | $COCH_3$ |
| 52 | $COCH_3$ | $COCH_3$ | H | | $COCH_3$ |

Compounds 44-48 were modeled with positive (+1) net charge.
Bz: benzoyl; Me: methyl

**Scheme 3.** Series 3 phenothiazine (polysubstituted) derivatives. (Top) parent structure; (bottom) side chains.

## 2. Methods

Geometry optimized molecular structures for all phenothiazine compounds in schemes 1–3 were obtained by means of PM3 semi-empirical quantum chemical calculations in vacuo [52]. Most of the ligands contain side chains carrying alkyl amine groups able to acquire positive partial charge at physiological pH hence models for both neutral and charged species were constructed. The enzyme structures for human GR and *T. cruzi* TR, in complex with their cognate substrates, corresponded to the available crystallographic coordinates [20,22]; PDB codes 1GRA and 1BZL, respectively.

For one selected phenothiazine derivative (promazine, compound **1** in Scheme 1), the optimum geometry conformation of the radical species was also obtained. This was intended to compare the binding of the free radical with that of the closed shell species. In order to adequately describe geometry minima, the treatment of the radical species (in this case a cation-radical which could possibly bear two positive charges) demanded the application of more sophisticated theoretical calculations than those provided by semi-empirical theory. Thus, GAMESS program [53] was employed to perform DFT calculations using the B3LYP [54–56] hamiltonian. In addition, for some derivatives (compounds **1–3** in Scheme 1 and **22** and **23** in Scheme 2) the binding at the interface site was also studied.

Molecular docking calculations for all phenothiazine derivatives at TR and GR active sites were undertaken using program DOCK3.5 [44,45]. Prepared ligand molecules were docked using the contact and force field scoring scheme, that is, by means of contact and energy grids. Subsequently, energy minimization was performed on each orientation docked at the binding site, and a final energy score calculated. Reported orientations were filtered taken into account a threshold value of binding energy (force field score).

For each ligand, the analysis of the energy results was made considering both the docked orientation with the lowest binding energy and the energy average over all orientations reported. Besides, based on the RMSD (Root Mean Square Deviations) values yielded by the program, clusters of orientations were obtained for each ligand, so as to identify different binding modes. Orientations were assigned to a different cluster when the RMSD value was greater than 3.0 Å. Molecular contacts between ligand orientations and residues at the active sites were also measured, with a cut off radius of 4.0 Å. The results were also graphically analyzed using molecular graphics software such as VMD1.8 [57] and O6.1 [58].

GROMOS96 [59] was employed for the Molecular Dynamics (MD) simulations. Both enzyme–ligand and free (non-bound) ligand molecular systems were studied for the selected phenothiazine derivatives. Output (minimum energy) ligand orientations obtained by the docking procedure were used to seed the simulations. In all cases, periodic boundary conditions were applied to octahedral boxes encompassing the entire enzyme–ligand complexes. The same box dimensions were used for the free ligands. Water was added to fill the spheres using the SPC potential. Non-bonded interactions were treated with the twin range method; van der Waals and electrostatic interactions were evaluated every time step within a 10 Å shell while electrostatic interactions alone were updated every 50 time steps for those atoms in the spherical shell found between 10 and 20 Å. Prior to the molecular dynamics simulation itself, each system was energy minimized using the steepest descent and conjugate gradient algorithms. Thereafter, systems were coupled to a heat bath with $\tau = 0.01$ where the simulation temperature was held at 300 K except during the initial heating (solvent relaxation) phase when the temperature was slowly raised in steps along a 20 picoseconds (ps) scheme. During this phase, solute atoms were restrained to their original positions by strong harmonic restraints, which were

successively weakened and finally removed at the end of the scheme. Trajectories were continued for additional 500 ps. Non-bonded energy data were collected throughout the last 100 ps, after thermal equilibration was attained. SHAKE algorithm was enabled to speed up simulations.

Ad hoc scripts written in PERL, AWK and BASH aided the numeric analysis of the docking and MD calculations.

As a complementary analysis, a QSAR-3D study was undertaken using CoMFA methodology [60] by means of Sybyl 6.8 software [61]. To seed the algorithm, a common substructure, the phenothiazine ring, was selected as pharmacophore and used as template to align all inhibitor conformations as obtained from the docking calculations. The structure–activity relationships were derived using PLS and the resulting model was validated by means of both internal ($q^2$ coefficient derived from PLS on 70% of data) and external ($r^2$ coefficient from 30% of remaining data as reported in [62]) cross-validation procedures.

## 3. Results and discussion

In Table 1 results are shown for non-bonded force field energies (total and electrostatic and van der Waals contributions) and overall number of molecular contacts of phenothiazine derivatives at TR active site. Data correspond to the lowest energy (best score) orientation of each ligand. In Table 2 the same information is presented for the docking in GR. Table 3 includes the energy differences of ligands in both enzymatic active sites. For those ligands whose side chains are able to protonate at physiological pH, only the results for the charged species are included. For the sake of brevity, results corresponding to averages over all docked orientations of each ligand are not shown here (either in the tables or the text), although it has to be said that nearly identical trends are verified. Table 4 provides the experimental inhibition data for the phenothiazine sample in TR. Finally, Table 5 includes the energy results of the MD simulations.

As stated before, TR inhibition kinetics of phenothiazines is known. These compounds display competitive inhibition, that is, they compete with the natural substrate for binding at the active site, more specifically at the so-called *Z*-site, a region where aromatic and non-polar residues abound [40]. This is the reason why the present work was focused primarily on TR and GR active sites. Note that we are assuming that the kinetics of inhibition of GR is very similar to TR, an idea that makes sense given the apparent structural and functional similarity between both flavoenzymes. However, for consistency sake, the binding of a few derivatives was also tested at the interface site. The results (not included here) show that phenothiazine derivatives are not able to penetrate the interface cavity. In effect, the fact that promazine (compound **1** in Scheme 1), the smallest structure of the entire sample, was one of the tested compounds, clearly indicates that bulkier derivatives will not bind there either. Although this result is somewhat expected, it is very important from the methodology standpoint. Had the docking calculations been able to dock the compounds at the interface site with interaction energies similar to those at the active site then the results presented here would have become meaningless.

Insofar the energy results are concerned, the whole phenothiazine sample was able to dock at both enzyme active sites. In the case of TR, this is the expected outcome according to experimental data accepting that the compounds have to bind first in order to inhibit the enzyme. For the lowest energy binding modes, non-bonded total energies range approximately from −21 to −40 kcal/mol, with most of the compounds having energies ranging between −27 and −33 kcal/mol as depicted in Fig. 1. In GR, energies range between similar values: −23 and −38 kcal/mol, with the majority of the derivatives between −28 and −33 kcal/mol (see again Fig. 1).

**Table 1**
Non-bonded electrostatic ($E_{el}$), van der Waals ($E_{vdw}$) and total ($E_{tot} = E_{el} + E_{vdw}$) energies for the lowest energy orientation of phenothiazines docked at the active site of trypanothione reductase. Energy values in kcal/mol.

| Compound | $E_{tot}$ | $E_{el}$ | $E_{vdw}$ |
|---|---|---|---|
| 1 | −27.52 | −3.44 | −24.08 |
| 2 | −29.44 | −5.74 | −23.69 |
| 3 | −29.98 | −8.82 | −21.16 |
| 4 | −31.1 | −6.53 | −24.57 |
| 5 | −32.96 | −7.52 | −25.44 |
| 6 | −26.76 | −7.89 | −18.87 |
| 7 | −29.45 | −6.69 | −22.77 |
| 8 | −33.04 | −5.87 | −27.16 |
| 9 | −34 | −6.9 | −27.1 |
| 10 | −39.96 | −10.74 | −29.22 |
| 11 | −28.1 | −1.73 | −26.37 |
| 12 | −28.75 | −5.82 | −22.93 |
| 13 | −29.29 | −4.42 | −23.87 |
| 14 | −33.29 | −8.74 | −24.55 |
| 15 | −31 | −11.84 | −19.16 |
| 16 | −32.2 | −7.63 | −24.57 |
| 17 | −20.93 | −0.17 | −20.77 |
| 18 | −27.74 | −12.6 | −15.14 |
| 19 | −27.45 | −8.97 | −18.48 |
| 20 | −25.45 | −6.55 | −18.9 |
| 21 | −22.79 | −8.14 | −14.65 |
| 22 | −24.98 | −7.51 | −17.47 |
| 23 | −25.71 | −3.13 | −22.57 |
| 24 | −31.77 | −7.91 | −23.86 |
| 25 | −32.32 | −6.67 | −25.65 |
| 26 | −21.95 | −0.86 | −21.09 |
| 27 | −28.72 | −4.99 | −23.72 |
| 28 | −26.78 | 0.23 | −27.01 |
| 29 | −34.52 | −9.03 | −25.5 |
| 30 | −22.98 | −0.89 | −22.08 |
| 31 | −28.98 | −7.02 | −21.97 |
| 32 | −30.25 | −5.97 | −24.28 |
| 33 | −21.49 | 0.23 | −21.72 |
| 34 | −31.94 | −1.89 | −30.04 |
| 35 | −29.35 | −1.37 | −27.99 |
| 36 | −22.18 | −0.27 | −21.45 |
| 37 | −27.18 | −3.31 | −23.88 |
| 38 | −27.51 | −6.14 | −21.37 |
| 39 | −25.35 | −3.93 | −21.42 |
| 40 | −31.28 | −5.6 | −25.68 |
| 41 | −22.59 | −0.3 | −22.29 |
| 42 | −29.27 | −5.26 | −24.01 |
| 43 | −29.72 | −4.21 | −25.51 |
| 44 | −23.97 | −2.72 | −21.25 |
| 45 | −33.09 | −11.05 | −22.04 |
| 46 | −32.73 | −5.7 | −27.03 |
| 47 | −32.56 | −5.95 | −26.61 |
| 48 | −29.01 | −6.79 | −22.22 |
| 49 | −23.93 | −0.66 | −23.28 |
| 50 | −21.61 | −1.68 | −19.93 |
| 51 | −24.14 | −1.51 | −22.63 |
| 52 | −24.74 | −1.13 | −23.61 |

Overall energy results would indicate a marginally better binding of phenothiazines at GR (compare energy differences in Table 3), suggesting a higher affinity for GR. There is a narrow average energy gap of less than 2 kcal/mol in favor of the host enzyme. This can be readily appreciated in Fig. 2 where a dispersion graph of total non-bonded energies is presented for the whole sample of compounds in TR and GR. Note that dots are fairly distributed up and below the $y = x$ curve, with a bias towards the superior triangle indicating slightly lower binding energies (higher in absolute value) in GR.

Approximately 40% of the whole phenothiazine sample display interaction energies actually lower (high in absolute value) in TR than in GR indicating a better binding in the former. This proportion is increased (nearly 50% of whole sample), when only charged derivatives are considered and reaches a maximum (75%)

**Table 2**
Non-bonded electrostatic ($E_{el}$), van der Waals ($E_{vdw}$) and total ($E_{tot} = E_{el} + E_{vdw}$) energies for the lowest energy orientation of phenothiazines docked at the active site of glutathione reductase. Energy values in kcal/mol.

| Compound | $E_{tot}$ | $E_{el}$ | $E_{vdw}$ |
|---|---|---|---|
| 1 | −23.39 | −5.42 | −17.97 |
| 2 | −28.08 | −3.71 | −23.37 |
| 3 | −26.47 | −13.66 | −12.81 |
| 4 | −30.48 | −2.88 | −27.6 |
| 5 | −32.93 | −5.87 | −27.06 |
| 6 | −27.36 | −0.11 | −27.09 |
| 7 | −30.34 | −4.37 | −25.98 |
| 8 | −27.96 | −4.32 | −23.64 |
| 9 | −30.72 | −1.59 | −29.13 |
| 10 | −31.84 | −7.14 | −24.7 |
| 11 | −30.06 | −2.64 | −27.41 |
| 12 | −27.44 | −1.459 | −25.982 |
| 13 | −27.56 | −0.83 | −26.72 |
| 14 | −32.33 | −5.66 | −26.6 |
| 15 | −26.52 | −10.22 | −16.3 |
| 16 | −33.11 | −6.42 | −26.69 |
| 17 | −25.48 | −2.24 | −23.25 |
| 18 | −37.86 | −22.27 | −15.59 |
| 19 | −26 | −7.95 | −18.05 |
| 20 | −27.19 | −10.98 | −16.21 |
| 21 | −28.81 | −9.39 | −19.42 |
| 22 | −26.48 | −2.46 | −24.03 |
| 23 | −25.95 | −6.38 | −19.57 |
| 24 | −34.71 | −4.44 | −30.27 |
| 25 | −33.12 | −5.82 | −27.31 |
| 26 | −35.56 | −11.27 | −24.29 |
| 27 | −30.08 | −4.13 | −25.95 |
| 28 | −28.5 | −0.51 | −28 |
| 29 | −32.36 | −5.46 | −26.89 |
| 30 | −27.98 | −2.14 | −25.84 |
| 31 | −30.86 | −5.34 | −25.52 |
| 32 | −28.34 | −5.68 | −22.66 |
| 33 | −27.9 | −1.55 | −26.35 |
| 34 | −29.16 | −0.92 | −28.24 |
| 35 | −28.48 | −1.2 | −27.27 |
| 36 | −29.15 | −0.34 | −28.81 |
| 37 | −28.49 | −3.67 | −24.82 |
| 38 | −28.78 | −4.64 | −24.13 |
| 39 | −34.48 | −12.4 | −22.08 |
| 40 | −30.76 | −5.42 | −5.34 |
| 41 | −29.98 | −0.78 | −29.2 |
| 42 | −33.56 | −10.15 | −23.42 |
| 43 | −31.25 | −2.91 | −28.34 |
| 44 | −32.13 | −4.19 | −27.94 |
| 45 | −32.6 | −3.37 | −29.24 |
| 46 | −33.76 | −4.19 | −29.57 |
| 47 | −38.43 | −4.23 | −34.2 |
| 48 | −34.34 | −5.28 | −29.05 |
| 49 | −31.1 | −2.46 | −28.64 |
| 50 | −30.59 | −2.61 | −27.98 |
| 51 | −31.02 | −1.18 | −29.85 |
| 52 | −30.57 | −1.01 | −29.55 |

among the promazine (*N*,*N*-dimethylaminopropyl) derivatives with compounds **1**, **3**, **8**, **10** and **15** (see Scheme 1) presenting the larger energy gaps between TR and GR. In contrast, for those derivatives which are not able to protonate at physiological pH (compounds **26**, **28**, **30**, **33**–**36**, **41** and **49**–**52**) it is observed that, barring compounds **34** and **35**, all of them display larger interaction energies in GR. The highest gap (14 kcal/mol) is verified for compound **26** which in fact carries a net negative charge.

This is quite different a scenario than the one that was previously obtained when a similar work scheme was applied to a series of nitrofuran compounds [50]. Nearly all nitrofurans studied were found to bind better at GR active site with an energy gap that averaged 5 kcal/mol in favor of the host enzyme. A fundamental difference between the phenothiazines assayed here and the nitrofuran derivatives studied elsewhere is that the majority of phenothiazines are able to acquire positive charge at physiological

**Table 3**
Non-bonded electrostatic (el), van der Waals (vdw) and total (el + vdw) energy difference for the lowest energy orientations of phenothiazines docked at trypanothione reductase (TR) and glutathione reductase (GR) active sites. Differences measured as TR–GR. Values in kcal/mol.

| Compound | $\Delta E$ | | |
|---|---|---|---|
| | $E_{tot}$ | $E_{el}$ | $E_{vdw}$ |
| 1 | −4.13 | 1.98 | −6.11 |
| 2 | −1.36 | −2.03 | −0.32 |
| 3 | −3.51 | 4.84 | −8.35 |
| 4 | −0.62 | −3.65 | 3.03 |
| 5 | −0.03 | −1.65 | 1.62 |
| 6 | 0.6 | −7.78 | 8.22 |
| 7 | 0.89 | −2.32 | 3.21 |
| 8 | −5.08 | −1.55 | −3.52 |
| 9 | −3.28 | −5.31 | 2.03 |
| 10 | −8.12 | −3.6 | −4.52 |
| 11 | 1.96 | 0.91 | 1.04 |
| 12 | −1.31 | −4.361 | 3.052 |
| 13 | −1.73 | −3.59 | 2.85 |
| 14 | −0.96 | −3.08 | 2.05 |
| 15 | −4.48 | −1.62 | −2.86 |
| 16 | 0.91 | −1.21 | 2.12 |
| 17 | 4.55 | 2.07 | 2.48 |
| 18 | 10.12 | 9.67 | 0.45 |
| 19 | −1.45 | −1.02 | −0.43 |
| 20 | 1.74 | 4.43 | −2.69 |
| 21 | 6.02 | 1.25 | 4.77 |
| 22 | 0.77 | −0.67 | 1.46 |
| 23 | 0.97 | −1.13 | 2.1 |
| 24 | 5.07 | 1.43 | 3.63 |
| 25 | 0.8 | −0.85 | 1.66 |
| 26 | 13.61 | 10.41 | 3.2 |
| 27 | 1.36 | −0.86 | 2.23 |
| 28 | 1.72 | 0.74 | 0.99 |
| 29 | −2.16 | −3.57 | 1.39 |
| 30 | 5 | 1.25 | 3.76 |
| 31 | 1.88 | −1.68 | 3.55 |
| 32 | −1.91 | −0.29 | −1.62 |
| 33 | 6.41 | 1.78 | 4.63 |
| 34 | −2.78 | −0.97 | −1.8 |
| 35 | −0.87 | −0.17 | −0.72 |
| 36 | 6.97 | 0.07 | 7.36 |
| 37 | 1.31 | 0.36 | 0.94 |
| 38 | 1.27 | −1.5 | 2.76 |
| 39 | 9.13 | 8.47 | 0.66 |
| 40 | −0.52 | −0.18 | −20.34 |
| 41 | 7.39 | 1.08 | 6.91 |
| 42 | 4.29 | 4.89 | −0.59 |
| 43 | 1.53 | −1.3 | 2.83 |
| 44 | 8.16 | 1.47 | 6.69 |
| 45 | −0.49 | −7.68 | 7.2 |
| 46 | 1.03 | −1.51 | 2.54 |
| 47 | 5.87 | −1.72 | 7.59 |
| 48 | 5.33 | −1.51 | 6.83 |
| 49 | 7.17 | 1.8 | 5.36 |
| 50 | 8.98 | 0.93 | 8.05 |
| 51 | 6.88 | −0.33 | 7.22 |
| 52 | 5.83 | −0.12 | 5.94 |

pH, the charge sitting at the side chain amino group (yielding the quaternary ammonium); see Schemes 1–3. The comparison between the neutral and charged species of each ligand in TR active site makes evident that the latter form more stable complexes (data not shown). The energy gap in favor of TR is about 5 kcal/mol. This is in excellent agreement with previous experimental results where the contribution of the quaternary ammonium to the binding in TR was estimated to be 5.5 kcal/mol by comparing the binding of the charged species and the respective ether analogues [63].

Charge effect in GR does not present such a uniform trend as in TR. For most phenothiazine derivatives, a charged amino group effectively decreases affinity (about 5 kcal on average) as compared to the neutral species, while for some others the effect

**Table 4**
*T. cruzi* TR inhibition available data for phenothiazine derivatives. Values in μM.

| Compound | $I_{50}$[a] | $K_i$[a] |
|---|---|---|
| 1 | 108 | 59.1 |
| 2 | 110 | 30.2 |
| 3 | 35.4 | 10.8 |
| 4 | 502 | |
| 5 | 351 | |
| 6 | 357 | |
| 7 | 301 | |
| 8 | 204 | |
| 9 | 171 | |
| 10 | 161 | |
| 11 | 3911 | |
| 12 | 1187 | |
| 13 | 458 | |
| 14 | 348 | |
| 15 | 219 | |
| 16 | 145 | |
| 17 | N.I.[b] | |
| 18 | 1412 | |
| 19 | 1176 | |
| 20 | 1028 | |
| 21 | 992 | |
| 22 | 271 | 216 |
| 23 | 249 | 127 |
| 24 | 73.7 | 21.2 |
| 25 | 72 | 23.0 |
| 26 | 1484 | |
| 27 | 829 | |
| 28 | 256 | |
| 29 | 320 | |
| 30 | 862 | |
| 31 | 457 | |
| 32 | 802 | |
| 33 | 251 | |
| 34 | 151 | |
| 35 | 188 | |
| 36 | 95 | |
| 37 | 150 | |
| 38 | 307 | |
| 39 | 139 | |
| 40 | 126 | |
| 41 | 95.5 | 18.7 |
| 42 | 80.8 | |
| 43 | 81.3 | |
| 44 | 765 | |
| 45 | 472 | |
| 46 | 175 | |
| 47 | 199 | |
| 48 | 76.6 | |
| 49 | 436 | |
| 50 | 2811 | |
| 51 | 402 | |
| 52 | 49.1 | |

[a] According to Ref. [40].
[b] No inhibition.

**Table 5**
Non-bonded interaction energies obtained by docking and Molecular Dynamics (MD) for the selected phenothiazine derivatives in complex with trypanothione reductase (TR) and glutathione reductase (GR). Inhibition constants ($K_i$) are also included when available. Docking values correspond to the lowest energy orientations of compounds. MD values are averages over the last 100 ps of simulation, include solvent contribution and represent differences with respect to the isolated ligands in solution. Energy values in kcal/mol, $K_i$ in μM.

| Compound | GR | | | TR | | |
|---|---|---|---|---|---|---|
| | Energy | | $K_i$ | Energy | | $K_i$ |
| | Docking | MD | | Docking | MD | |
| 1 | −23.39 | −22.42 | | −27.52 | −27.06 | 59.10 |
| 2 | −28.08 | −27.68 | | −29.44 | −28.77 | 30.20 |
| 3 | −26.47 | −27.90 | | −29.98 | −34.31 | 10.80 |
| 22 | −26.48 | −20.98 | | −24.98 | −18.40 | 216.00 |
| 23 | −25.95 | −18.92 | | −25.71 | −25.75 | 127.00 |

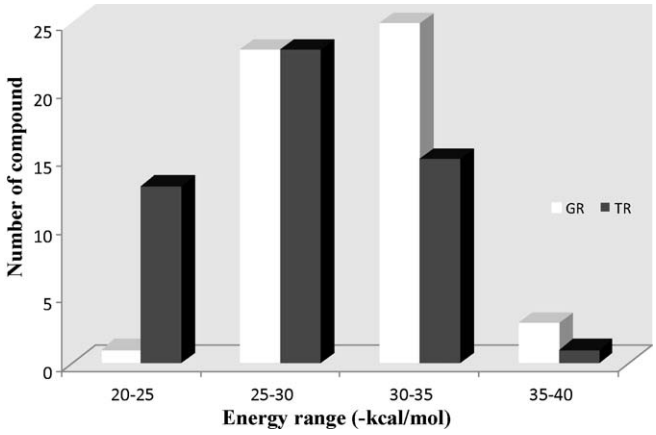



**Fig. 1.** Bars graph depicting number of phenothiazine derivatives docked at trypanothione reductase (TR) and glutathione reductase (GR) active sites versus total non-bonded interaction energy ranges measured for the lowest energy binding modes.

on affinity is nearly negligible or even the opposite, i.e., binding at GR active site is favored (data not shown). The overall result, however, is that the relative affinity for TR is increased when the derivatives bear a positive charge and for those derivatives where the charged species still display higher interaction energies in GR, the energy gap with respect to TR becomes significantly narrower (less than 2 kcal/mol).

An interesting case is compound **15** (see Scheme 1) which eventually could bear and additional positive charge at physiological pH [40]. However, contrary to what one could advance the interaction energy for this compound in TR is about the same as the rest of the promazine series indicating that an augmented ligand electrostatic field does not necessarily enhance affinity towards TR. In the same vein, the cation-radical species of compound **1** (modeled either with a net charge of +1 or +2 at physiological pH) does not attain a better binding in TR as compared the closed shell species (net charge of +1 at physiological pH). This result (not shown) suggests that the superior inhibitory capacity of the cation-radical species [41] should not be produced by an enhanced affinity for TR active site. Rather, as it was previously hypothesized, the cation radical may be able to elicit irreversible changes inside the active site so as to compromise the correct enzyme function. The fact that a supplementary positive charge does not improve affinity towards TR may be explained in the light of the rather hydrophobic nature of TR active site which lacks suitable (i.e., ionic or polar) residues for establishing additional contacts to stabilize the second charge. Remember that the crystallographic structure of the natural substrate $T(S)_2$, in complex with *T. cruzi* TR, does not

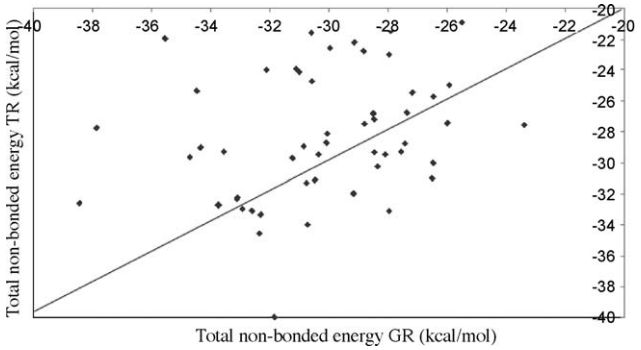



**Fig. 2.** Dispersion graph of the lowest energy binding mode for each phenothiazine derivative docked at trypanothione reductase (TR) and glutathione reductase (GR) active sites. The line $y = x$ is drawn to ease visualization.

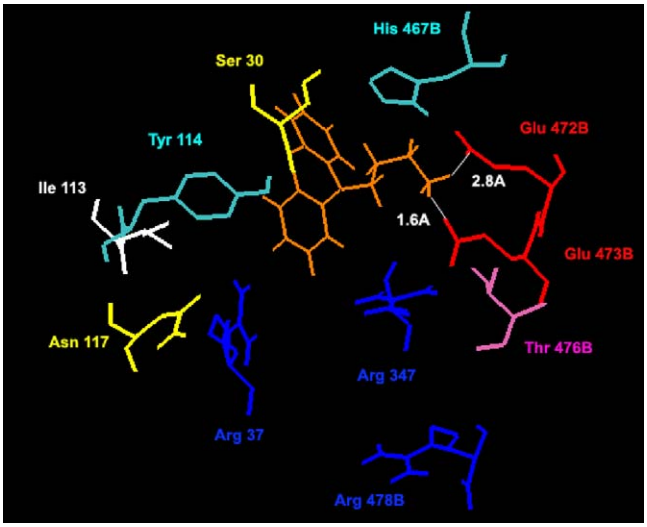



**Fig. 3.** Lowest energy orientation of phenothiazine derivative **18** docked at the active site of glutathione reductase. Distances between the charged side chain amino group of ligand and carboxylate groups of protein residues Glu 472B and Glu 473B are indicated.

show a particular molecular interaction between the spermidine positive charge and any active site residue such as the non-conserved glutamate (Glu 18).

All the aforementioned results are in accord with previous experimental work [63–67], which suggested the key role of the electrostatic nature of inhibitors in terms of affinity to TR. Specifically, the charged dimethylamino group found in compounds of series 1 (see Scheme 1), was recently shown to be essential to afford TR inhibition [68]. However, electrostatics alone is not always the determining factor in terms of relative affinity towards GR and TR. The picture is not that simple and the interplay with the steric factor should be taken into account as pointed out by the graphical analysis of the docking binding modes.

Phenothiazine charged derivatives with rather small side chains are able to locate in GR the positive charge in order to avoid the electrostatic repulsion with basic residues in the active site, namely, Arg 37, Arg38, Arg 347, Arg 478B (numbers as in the crystallographic data of the human enzyme). As a result, the energy binding gap between TR and GR is reduced and even reverted making inhibitor binding more favorable for GR. This effect is apparent in the case of compound **18** whose interaction energy difference (10 kcal/mol) in favor of GR is nearly as steep as that of compound **26** discussed above. Fig. 3 shows the docking result for compound **18** in GR. It is readily appreciated that strong interactions may be established between the protonated side chain amino group at 10-position of the inhibitor tricycle and the conserved acid residues Glu 472B y Glu 473B (numbers as in the crystallographic structure of the human enzyme).

On the contrary, derivatives with bulkier side chains, typically aromatic or heterocyclic in nature, are prevented from acquiring similar binding modes as compound **18**. As a consequence, the ligand positive charge cannot be stabilized and interaction energies are accordingly lower. This situation is best exemplified by compound **10** which comprises a very bulky substituent at 10-position of the phenotiazine tricycle. The graphical analysis for this derivative makes apparent that the lowest energy binding mode is not able to avoid electrostatic repulsion with the arginine residues in GR. Thus it came to no surprise that for this compound the energy gap between TR and GR is the largest (over 8 kcal/mol) among all inhibitors assayed. It is no coincidence either that the only two neutral phenothiazines displaying better interaction energies in TR (compounds **34** and **35**) are those having aromatic side chains which, still fitting in TR, make them too big to adopt binding modes with adequate interaction energies in GR. Remember that GR active site is narrower and more polar in nature than TR active site.

The corollary of this reasoning is that a fair amount of phenothiazine inhibitors are unable to establish enough molecular contacts at GR actives site so as to make binding favorable. This is an important difference with the binding of nitrofurans where the nitro group acts as a strong anchor to GR active site preventing a selective binding towards TR [50]. In the same vein, the effect of the steric factor is much stronger for phenothiazines as compared to nitrofurans for which a clear relationship between size and affinity towards TR was not found. The difference here is the combined bulkiness of the phenothiazine nucleus and the side chain groups which results in an overall large molecular size which makes difficult for ligands to orient adequately in GR active site. In particular, some phenothiazine derivatives (such as compounds **8** and **10** mentioned above) possess a rather globular and compact topology, much more in line with TR active site, a trait not to be found among nitrofurans where elongated geometries prevail, allowing ligands to enter (at least partially) GR active site and establish stable contacts therein [50]. The size difference between the two ligand populations is also the main reason why phenothiazines establish more electrostatic and van der Waals interactions in TR active site. The magnitude of the total electrostatic interaction of phenothiazines is about 5 kcal/mol in both enzymes while it is just 2 kcal/mol lower in TR than GR when it comes to total van der Waals interaction. Both results are much better than the ones previously obtained for nitrofurans [50].

Statistical tests yielded good (positive) correlations ($R^2 = 0.91$) between total number of molecular contacts and total interaction energies of assayed compounds at both active sites. Good correlations are also obtained now discriminating molecular contacts established with particular residues at the active site such as Arg 37, Arg 347, Ser 30, Tyr 114, His 467B in GR, and Glu 18, Trp 21, Ser 14, Tyr 110, His 460B in TR**.**

As far as phenothiazine binding modes in TR is concerned, graphical inspection suggests that ligands adopt two principal orientations. The first, maps in the vicinity of the natural substrate ($T(S)_2$) binding region near the enzyme disulfide bridge. There, derivatives are in contact with the hydrophobic pocket surrounded by residues Glu 18, Trp 21, Leu 17, Tyr 110, Met 113 and Phe 114

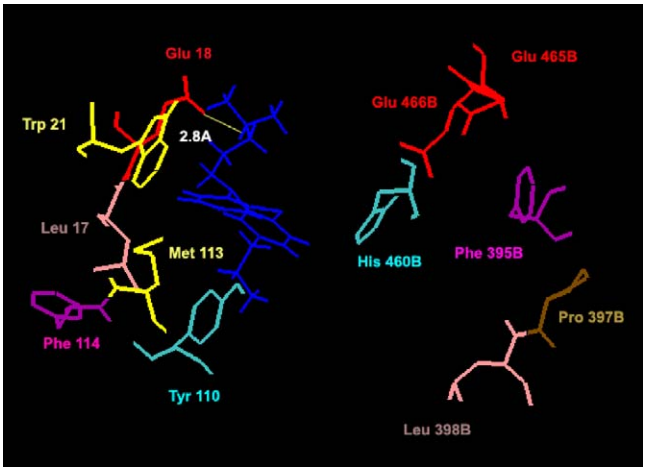



**Fig. 4.** Lowest energy orientation of phenothiazine derivative **15** docked at the active site of trypanothione reductase. The observed orientation is representative of one of the two general binding modes for the whole phenothiazine series, i.e. in the binding region of the natural substrate ($T(S)_2$). The distance between the charged side chain amino group of ligand and the carboxylate group of protein residue Glu 18 is indicated.

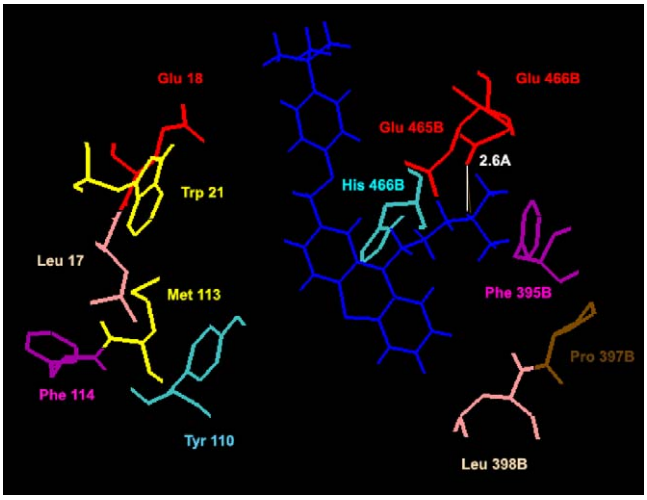



**Fig. 5.** Lowest energy orientation of phenothiazine derivative **10** docked at the active site of trypanothione reductase. The observed orientation is representative of one of the two general binding modes for the whole phenothiazine series, i.e. near the *Z*-site. The distance between the charged side chain amino group of ligand and the carboxylate group of protein residue Glu 465B is indicated.

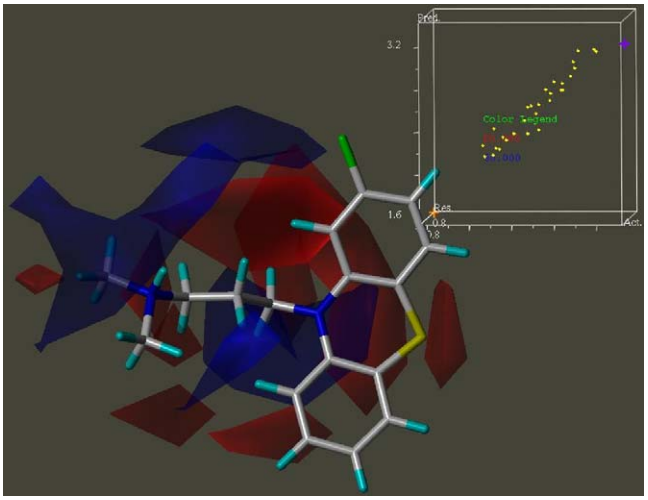


**Fig. 6.** CoMFA electrostatic field contour plots of phenothiazine derivative **3**. Blue contours indicate ligand regions where positive electrostatic groups increase activity whereas red contours indicate likewise for negative electrostatic groups: the scatter plot shows the predicted versus actual inhibition activity ($I_{50}$) of the compound sample in trypanothione reductase. Residuals are plotted in the third dimension.

(numbering as in the *T. cruzi* crystallographic structure). As shown in Fig. 4, this binding mode allows for the establishment of a strong interaction between the charged amino group in the inhibitor and the carboxylate group of residue Glu 18. Here, the distance of inhibitors to the active site disulfide bridge is irrelevant given that phenothiazines (in contrast to other inhibitors like nitrofurans) do not undergo any chemical interconversion at the active site and inhibition is based solely on competition with the natural substrate. The second binding mode maps to the *Z*-site, referred in previous sections. At this location, hydrophobic interactions may be established between the tricylic phenothiazine ring and residues Phe 395B, Pro 397B y Leu 398B – numbering as in the *T. cruzi* crystallographic structure – as well as of ionic type between the positive side chain amino group at 10-position of the tricycle and the carboxylic groups of Glu 465B and Glu 466B (numbering as in the *T. cruzi* crystallographic structure). This is depicted in Fig. 5. In a very recent work [69], the very same acid residues were found to stabilize the binding of a set of diaryl sulfide based inhibitors of TR from *T. brucei* by simultaneous interactions with the *N*-alkyl side chain of the inhibitors anilino moiety.

Yet, there are quite a few ligands which are not able to acquire binding modes in which the positive charge is prone to establish strong interactions with TR active site, case in point compound **39**, one of the smallest in the whole sample. Among the charged derivatives, this compound displays the lowest selectivity towards TR (and one of the lowest considering the whole set), with a 9 kcal/mol energy difference in favor of GR. Such a difference is explained by graphical inspection again: in TR, the compound does not form any hydrogen bond or salt bridge with active site residues while in GR, it is docked in such a way that the binding is stabilized by means of molecular interactions with residues Glu 472B and Glu 473B – numbering as in the human crystallographic structure – homologues to the above mentioned Glu 465B and Glu 466B. As discussed above, the fact that a positive charge does not always attain strong molecular interactions in TR is not surprising given that a similar effect is found for $T(S)_2$, the natural substrate. At variance with $T(S)_2$, which can benefit from its bulky structure and fill a good deal of the active site cavity, it is likely that smaller ligands, such as some of the ones assayed here, need a stronger electrostatic anchor in TR, provided by only one or by a stretch of acid residues.

Phenothiazine ligands present a broad range of possibilities in regards side chain substitution (see Schemes 1–3). For this reason, the graphical analysis further explored the influence of substituent nature and position in regards binding (interaction) energies. At 10-position of the tricyclic ring, those derivatives with tertiary amines, in particular the *N*,*N*-dimethylaminopropyl group found in the promazine series, are the ones with the highest binding energies in TR. The presence of aromatic bulky groups at the same position significantly increases relative affinity towards TR as well. Such is the case of compounds **34** and **35**, discussed above, which despite lacking a positive net charge attain larger interaction energies in TR. As far as position 2 in the phenothiazine nucleus is concerned, aromatic and bulky groups would also be favored if one is interested in higher affinities for the parasite enzyme as seen with compounds **8** and **10**, already mentioned before. In the same way, electronegative substituents such as Cl and F (compounds **2** and **3** in Scheme 1) are preferred in this position. Similar laboratory

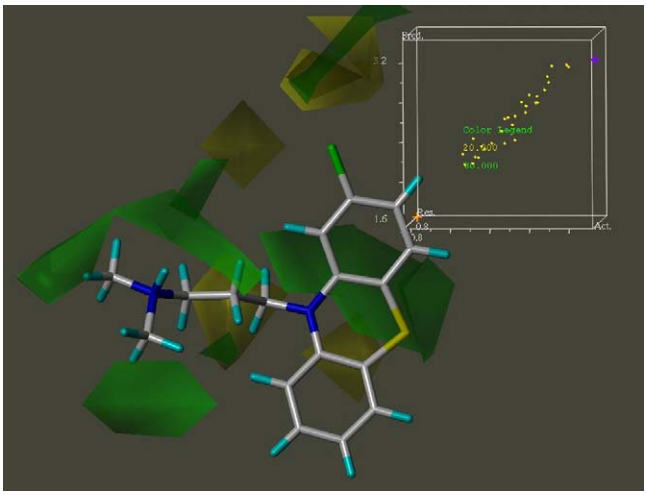


**Fig. 7.** CoMFA steric field contour plots of phenothiazine derivative **3**. Green contours indicate ligand regions where bulky groups increase activity whereas yellow contours indicate likewise for small groups. The scatter plot shows the predicted versus actual inhibition activity ($I_{50}$) of the compound sample in trypanothione reductase. Residuals are plotted in the third dimension.

results were previously reported [70], once again evidencing the agreement between experimental and theoretical (docking) results.

CoMFA electrostatic and steric contour plots are presented in Figs. 6 and 7, respectively, with one of the most active derivatives, compound **3** as reference. Note the good quality of the model as evidenced by the predicted and actual inhibitor activity values (scatter plots). Values of $q^2$ and $r^2$ coefficients were 0.54 and 0.92, respectively. The most conspicuous result in Fig. 6 is the electrostatic field favoring a positive electron density in the whereabouts of side chains at 10-position of the tryclic ring. This effect is, for the most part, caused by the *N*,*N*-alkylaminopropyl moiety present in all series 1 (promazine) compounds which, allowing for a couple of exceptions, showed high levels of TR inhibition (see Table 4). Note that the blue surface is widely spread due to the heterogeneous docking location of substituents at said position of the aromatic nucleus. The electrostatic negative contour corresponds mainly to the tricyclic ring location, in particular the 2-position of the ring where several active derivatives, case in point compound **3**, were substituted by chlorine or fluorine (see Schemes 1–3). The steric field contours depicted in Fig. 7 evidence a similar result where green areas are fairly in accord with the distribution of substituents at 10-position and, to a lesser extent, at 8-position of the aromatic nucleus. Small side chains appear to be preferred on the other side of the aromatic tricyclic, i.e. the 2-position, even though a couple of compounds with bulky groups at this ring location (see above) attained high docking interaction energies in TR.

Substitution at other positions of the tricyclic ring, especially with bulky side chains, is not helpful to increase affinity towards TR as indicated by the very poor results obtained for compounds **44**–**52**, that is, the polysubstituted phenothiazine series (compare energy differences in Table 3). For nearly all compounds in this series, and regardless of net charge, interaction energies are larger in GR than TR. However, positive charged polysubstituted compounds follow the general trend described at the beginning of the section and display larger interaction energies in TR as compared the neutral derivatives. More specifically, those derivatives with the *N*,*N*-dimethylamino propyl group at 10-position (compounds **45** and **46**) are the ones with the largest energies at TR active site.

The graphical analysis explains once again the energy results found for the polysubstitued derivatives. In effect, for those compounds with net charge, the positive center binds far away (at the opposite region of the active site cavity) the basic residues in GR namely Arg 37, Arg 38 and Arg 347 (numbers as in the crystallographic human structure). Strong interactions with acid active site residues such as Glu 472B and Glu 473B (which are located opposite to the basic residues) are not established either. In regard TR binding, none of the charged polysubstituted derivatives is able to benefit from the electrostatic factor, barring compound **45** whose amino group shows a strong interaction with residue Glu 466B. Accordingly, this compound is, among the polysubstituted derivatives, the one with higher selective binding towards TR.

Contrary to other TR inhibitors, a good deal of experimental inhibition data is available for phenothiazines. Kinetics ($K_i$) data, in particular, are known for some of the compounds studied here namely compounds **1**–**3** and **22**–**23** (see Schemes 1 and 2). This allows for a comparison with the theoretical results herein reported. A good correlation ($R^2 = 0.95$) is found between inhibition constants ($K_i$) and docking (entalphy) total energies (seen in Table 1). As seen in Fig. 8, the best fit is obtained with a regression curve of exponential type, a somewhat expected result considering the theoretical relationship between kinetic constants and free binding energies (approximated here by enthalpy energies). To further attest to the validity of the docking results, it would be

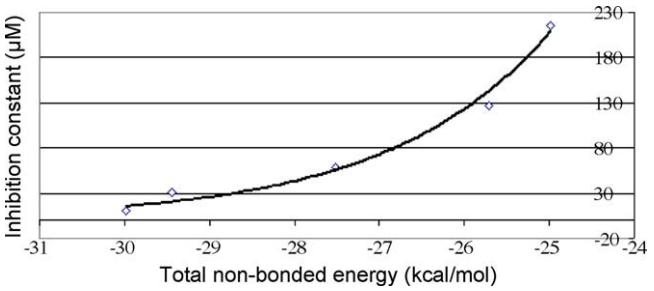



**Fig. 8.** Inhibition kinetic constants ($K_i$) plotted against docking interaction energies for selected phenothiazine derivatives in trypanothione reductase. Best correlation trendline is drawn.

interesting to extend the comparison to a larger group of phenothiazines and, if possible, to GR. Unfortunately, additional inhibition kinetics, in particular for GR, has not become available for phenothiazines so far. An alternative way to test the docking energy results is what we did here, i.e., to perform more accurate and realistic theoretical calculations such as Molecular Dynamics and analyze the degree of agreement with the docking calculations. The detailed MD analysis will be published elsewhere hence only the relevant point will be discussed here. From Table 5, one can observe that the total interaction energies calculated by Molecular Dynamics (as the difference between the bound and free ligand results) and docking are consistent. A linear correlation test yields an $R^2$ coefficient of 0.81 which is a bit far from perfect but evidences a reasonable proportionality nonetheless. Additionally, when one analyzes the relationship between interaction energy data from MD and the experimental $K_i$ values, an exponential regression curve is again verified with an $R^2$ coefficient of 0.95. Thus, the MD results are also in line with the experimental information. Allowing for the fact that the phenothiazine sample used for the comparison is rather small, these results are promising and give support to the docking based methodology and work scheme pursued here.

## 4. Conclusions

The binding affinity of a sample of phenothiazine derivatives towards TR and GR was assessed by standard molecular docking and MD calculations. Output analysis was conducted both from the energy and structural standpoints. Overall, the phenothiazine ligands displayed nearly an equal capacity (as measured by interaction energies) to bind at both enzymes, at variance with previous results found for other inhibitors, i.e., nitrofurans, which presented a marked preference for the host enzyme [50]. In the same way, phenothiazines proved not able to bind at the interface (non-selective) binding site either of TR or GR. The importance of this result should not be overlooked in the context of selective inhibition. Binding at the (non-specific) enzyme interface would act as a diversion from the expected binding pathway precluding achievement of selectivity. Such a dual binding mode was previously postulated for naphthoquinones and nitrofurans [25,50].

The (five) phenothiazines herein studied by MD (see above) do not inhibit GR experimentally [40]. In a previous work [71], we compared the interaction energies of the physiological substrates, namely GSSG and $T(S)_2$, both in TR and GR and found that the energy gap between the catalytic complexes (GR-GSSG and TR-$T(S)_2$) and the non-catalytic ones (GR-$T(S)_2$ and TR-GSSG) is roughly four times larger than the difference verified for the aforementioned phenothiazines when interaction energies in TR and GR are compared. Even for the phenothiazine subset with the largest interaction energies in TR (i.e., the promazine series), the

energy breach with respect to GR, measured both by docking and MD, is smaller than those of the natural substrates. Thus, the theoretical results would not rule out the possibility that some inhibition effect would take place at the human receptor.

One of the most relevant results of this work relates to the concordance of the simple docking calculations with both, experimental and MD data. Although more elaborated docking methods and implementations are available, the good correlations found here allow us to conclude that even the simplest scoring methods would suffice if one is interested in a rapid evaluation of binding affinities of ligands to receptor (e.g. enzyme) sites. This first step is to be ensued by more real theoretical calculations should one be interested in determining accurately ligand–receptor energy interactions and use the data (and a specific methodology) to predict, for example, binding free energies of new compounds. In point of fact, we have followed this strategy for some of the phenothiazine compounds presented here and will report the results in a future communication.

Results for the charged phenothiazine subset, in particular, the promazine series (Scheme 1) confirm the importance of charge as a discriminating factor in terms of ligand specificity towards TR as hinted previously [50,63–68]. However, the docking results also make evident that charge alone does not suffice to drastically alter binding selectivity but a complementation with molecular size and shape is required. In this sense, the role of volume and steric factors is straightforward, suggesting that it is required a fine-tuning of ligand topology in order to achieve rewarding results. Specifically, one should stick to large and rather globular side chains. Otherwise, chances are that a positive charged ligand would orient itself in GR active site in such a way to be able to avoid electrostatic repulsion (from the conspicuous stretch of arginine residues) and, at the same time, find strong binding anchors, i.e., the conserved glutamic residues.

New classes of compounds with potent TR inhibition activity have been identified in the last few years. As opposed phenothiazines, for which some studies are available [72–75], these compounds have not been tested yet as in vivo inhibitors of *T. cruzi*. Yet, the majority of them display lower $I_{50}$ and/or $K_i$ than the phenothiazine set assayed here. Such is the case of aminodiphenylsulfides, quaternary arylalkylammonium phenothiazines and 2-chlorophenyl phenyl sulfides, polyaminoguanidines and biguanidines, 2-iminobenzimidazoles, and diaryl sulfides. Most of these inhibitors share one thing in common: the physiological active species is likely to bear a protonated amino group. This, again, is proof that charge is the one trait that needs to be taken into account first, if one is to find truly selective (competitive) inhibitors of *T. cruzi* TR.

## Acknowledgments

This work was partially supported by the ChagaSpace Project (NASA-USA, donation number: NAG5-11146) and PEDECIBA-Química (University of the Republic, Montevideo-Uruguay). F.I. is indebted to Dr. P. Denis from DETEMA (Faculty of Chemistry, Montevideo, Uruguay) for his help with DFT calculations and to Dr. H. Cerecetto for his advise on ligand protonated states. M.P. thanks Prof. E. Barreiro from LASSBio (Faculty of Pharmacia, RJ-Brasil) for kindly allowing the use of Sybyl 6.8 software. O.T. is grateful to NFR (Swedish Research Council) for sustained financial support.

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