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Conformational analyses and docking studies of a series of 5-nitrofuran- and 5-nitrothiophen-semicarbazone derivatives in three possible binding sites of trypanothione and glutathione reductases

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Abstract

To explore three possible binding sites of trypanothione and glutathione reductase, namely, the active, the dimer interface and the coenzyme NADPH binding site, a series of eight compounds, nitrofurans and nitrothiophenes derivatives, were docked, using their crystallographic and modeled conformations. Docking results showed that, for both families and both enzymes, compounds are more likely to bind in the interface site, even though there is some probability of binding in the active site. These studies are in agreement with experimental data, which suggest that these class of compounds can act either as uncompetitive or mixed type inhibitors, and also with the finding that there is an α-helix which connects the active with the interface site, thus allowing charge transference between them.

Keywords: Turncoat inhibitors; Chagas' disease; Sleeping sickness; Docking; Nagana

1. Introduction

The flagellate protozoa trypanosomes are the causative parasites of several tropical diseases, as African sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodiense*) and Chagas' disease (*Trypanosoma cruzi*) in humans, and nagana (*Trypanosoma congolense* and *Trypanosoma brucei*) in cattle. According to the World Health Organization around 16–18 million people are infected with *T. cruzi*, 2–3 million individuals have the clinical symptoms of the chronic stage of Chagas' disease and, every year, are reported 21,000 deaths and 300,000 new cases [1]. Moreover, Chagas' disease is transmitted not only by Triatomine insects but also by blood transfusion, this latter mechanism is responsible for the occurrence of Chagas' disease in regions where it is not endemic [2].

Two drugs, nifurtimox® (4-[(5-nitrofurfurylidene)-amino]-3-methylthiomorpholine 1,1-dioxide) and benznidazole® (*N*-benzyl-2-nitro-1-imidazoleacetamide) (Scheme 1) have been shown to cure at least 50% of recent infections [3].

However, these drugs have several important drawbacks namely, they produce serious side effects [4,5], they have to be given for long periods, and their usefulness in the indeterminate or chronic stage of the infections has also been questioned [6]. As for blood treatment before transfusion the only drug available as a chemoprophylactic agent is Gentian Violet which is carcinogenic in animals and there is some concern in its use [7]. African sleeping sickness shares a similar state with respect of available drug treatment, this includes suramin, pentamidine and difluoromethylornithine, which are ineffective against more virulent strains and are toxic [8]. Clearly exists a need for safer and more efficacious chemotherapeutic agents against trypanosomatids.

The *Trypanosomas* are one of the very few genera that do not use the glutathione/glutathione reductase (GR) system for protection against oxidative stress [9], instead, they rely on trypanothione and trypanothione reductase (TR) to protect

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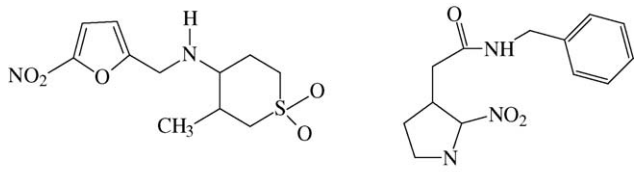
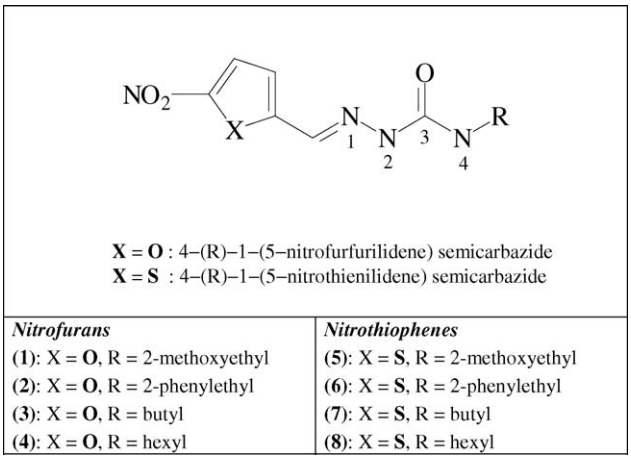


Scheme 1.

them [10]. GR catalyzes the regeneration of two molecules of GSH from GSSG (glutathione disulfide), and TR catalyzes the reduction of trypanothione disulfide (TS_2) to trypanothione, a covalent conjugate of glutathione and spermidine [$T(SH)_2$, N^1,N^8-bis(glutathionyl)spermidine]. Both, GR and TR are homodimeric FAD-dependent NADPH oxide-reductases that, in spite of sharing close structural and mechanistic similarities, are mutually exclusive respect to their disulfide substrates, this mutual substrate exclusivity indicates that selective ligand design should be possible, making TR a potential target for drug design [11]. Moreover, the design of potential TR inhibitors must take into account the effects on GR, and as one of the goals of the present work was to develop a strategy for the theoretical evaluation of the drug binding and its agreement with experimental data, thus gaining more insight of the inhibition mechanism, a docking study was undertaken, with a series of eight compounds, four 5-nitrofurans and four 5-nitrothiophens which were modeled, starting from the crystal structures of three of them. Previous docking studies were done only in the active site [12–15], in this work and in order to search for competitive inhibitors, three different binding sites were explored, namely, the active (AS), the NADPH (NS) and the interface site (IS), of the target proteins, TR and GR.

The molecules studied, shown in Fig. 1, were designed based on the knowledge of the binding patterns of the complexes TS_2–TR, GSSG–GR, GSP–TR, TS_2–GR and GSSG–TR [16], where TS_2 and GSSG are the natural substrates of TR and GR, respectively and GSP is glutathionylspermidine. The nitrofuran and thiophene groups were used to mimic the redox properties of Nifurtimox® [11,17], the remaining of the molecule was designed in order to bind in an hydrophobic



Nitrofurans	*Nitrothiophenes*
(1): X = **O**, R = 2-methoxyethyl	**(5)**: X = **S**, R = 2-methoxyethyl
(2): X = **O**, R = 2-phenylethyl	**(6)**: X = **S**, R = 2-phenylethyl
(3): X = **O**, R = butyl	**(7)**: X = **S**, R = butyl
(4): X = **O**, R = hexyl	**(8)**: X = **S**, R = hexyl

Fig. 1. The 5-nitrofuran and 5-thiophen semicarbazones studied.

pocket which is only present in TR. Their synthesis was described elsewhere [16] and showed some inhibitory activity against TR, GR and *T. cruzi* growth in vitro [14,16].

2. Experimental

2.1. X-ray measurements

Of all compounds shown in Fig. 1, only (**1**), (**2**) and (**6**) could be crystallized, their 3D structure determined and the crystal data deposited with the Cambridge Crystallographic Data Centre, CCDC 253154, 253155, 253156. Fig. 2 shows the ORTEP representations of the compounds. It should be pointed out that in (**6**) there were two crystallization water molecules.

In all cases the molecules are joined through hydrogen bonds, giving rise to different supramolecular arrangements. In (**1**) and (**2**) the molecules are arranged in dimers (centrosymmetric in (**1**)), whereas in (**6**) the supramolecular arrangement is in a chain fashion that includes the two water molecules.

2.2. Calculations

The crystallographic results were used as templates to model the compounds that did not crystallize and also as starting models for quantum chemical calculations and the resulting conformations used for performing the docking studies.

The fact that compound (**2**) (see Section 3) presents two different conformations in the asymmetric unit, and that its thiophen analog, (**6**), shows only one, which is different from those of (**2**), prompted us to make a detailed analysis in order to explore all the possible conformations to be used in the docking studies. Initially the geometry of the two independent molecules of (**2**) were optimised using molecular mechanics with the MM+ force field [18]. Then, conformational analysis of the MM+ optimised structures was carried out using molecular dynamic simulation (MD) in gas phase at 1000 K [19,20]. The structures were then submitted to a double geometry optimization, first using MM+ force field and then using the semiempirical AM1 method with MOPAC 7.01 [21,22]. The obtained conformations were organized in increasing order of heat of formation values to select the ones that were going to be used in the docking studies.

The conformation modeling for the remaining molecules of the series was performed using geometry optimization by AM1 method, taking into account the crystallographic results and the structural knowledge acquired in the analysis of (**2**).

2.3. Docking

Docking, performed using the DOCK 4.0 program [23], was carried out using three protein crystal structures obtained from the Protein Data Bank under 1GRA entry, which is human GR with substrate and NADPH, for the active and the NADPH sites; 1XAN, which is GR complexed with a xanthene inhibitor, for the interface site, and 1NDA, which is TR from *T. cruzi* without ligands, was used for all the three sites of TR. The docking calculations were validated by reproducing several

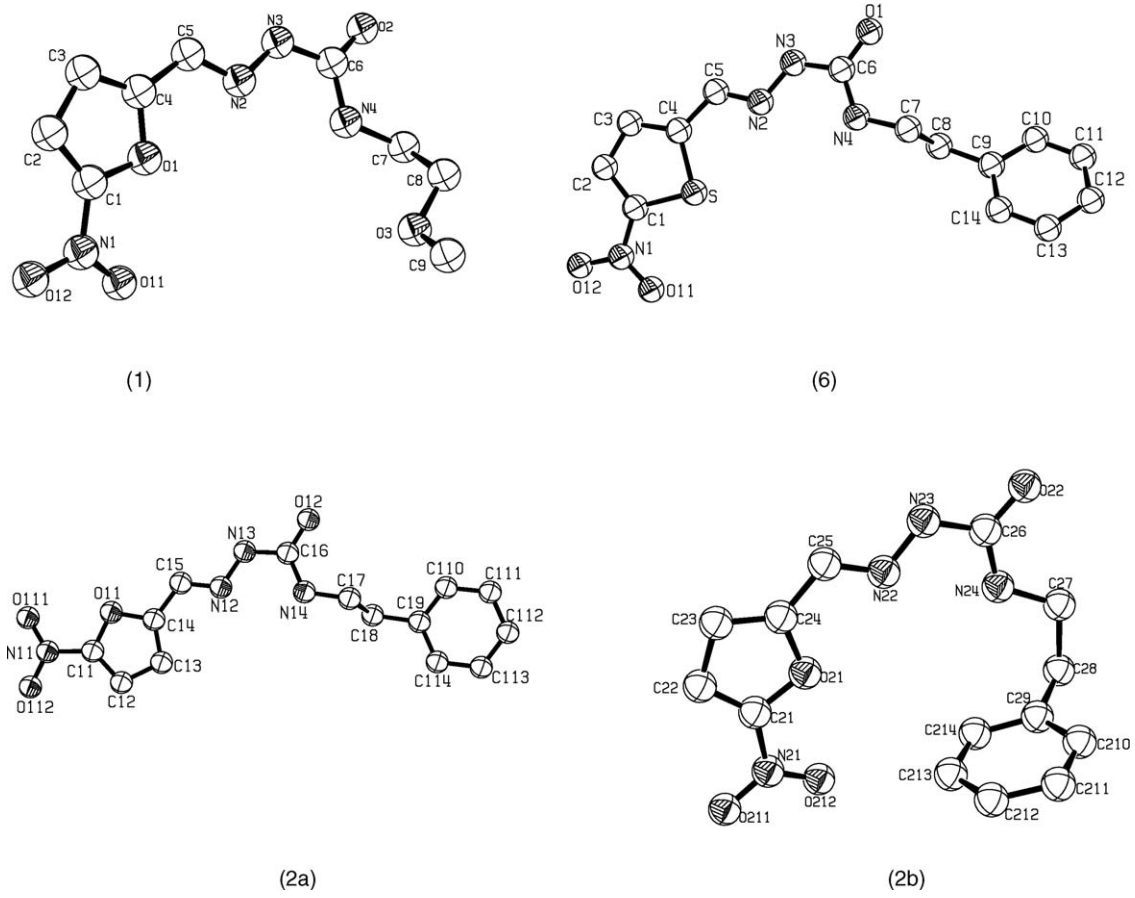


Fig. 2. X-ray crystal structures of (**1**), two independent molecules of (**2**) and (**6**).

crystal structure complexes: 1GRA for the AS and NS sites of GR and 1XAN for the IS site, whereas, for TR, AS was validated using the TS_2 substrate of 1BZL, NS using the NADPH from 1TYP (TR form *Crithidia fasciculata*) and the IS site was constructed by analogy with that of GR.

DOCK defines the ligand-binding site using a set of spheres and creating a negative image of the site. Different sizes of cut-off radii were used to describe the shape characteristics of the sites, for AS a 13 Å radius sphere, centered around the disulfide bridge, was chosen, this corresponds to Cys52-Cys57 for TR and Cys58-Cys63 for GR, in this way all main residues were included. It should be noted that, this site has a different polar character being (−) in TR and (+) in GR, and that it is bigger in TR than in GR. In NS a 12 Å cut-off radius, centered in Phe198, was chosen for TR, whereas, in GR two superposed spheres of 8 Å, centered in Arg218 and Arg224, forming approximately an ellipsoid, were used. In both enzymes, the IS site has two-fold symmetry, the main difference being that in GR there is a disulfide bridge which covalently connects both monomers, while in TR the monomers are joined by non-covalent interactions. As the 1XAN crystal structure shows the xanthene inhibitor in the interface site, this information was used to chose a 10 Å cut-off radius centered on His75-His82 for GR, and a 12 Å cut-off radius centered on His72 for TR.

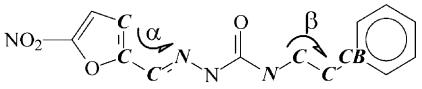
In order to obtain a meaningful statistical result, we set a energy cut-off in order to select a set of 15–20 complexes with the most favorable scores, in each calculation. This score is an estimation of the ligand–protein interaction energy, which is calculated using the ligand atoms and the potential energy grid, generated by the chemgrid subroutine of the program (see DOCK 4.0 Reference Manual). The steps followed for analysis and selection of the complexes constructed were: first a graphical analysis, using the O program [24], mostly in order to prevent the selection of complexes presenting steric clashes, then, complexes with almost the same ligand–site relative orientation were clustered together and within each cluster the complex with the best fitting was preserved for further analyses. Following, mean interaction energies (total, Coulomb and van der Waals) were calculated for all the selected complexes. Finally, to establish the binding patterns, defined as the binding modes with enough statistical weight, a series of parameters were considered: (i) favorable energy score; (ii) complementary atomic contacts; (iii) high percentage of repetition in a docking calculation; (iv) being a binding mode representative of the complete series of compounds studied. MOPAC charges were used for the ligands in the docking calculations.

3. Results and discussion

3.1. Ligands conformations and selection

The most striking result, of the crystallographic studies, was the fact that, as shown in Fig. 2, the two independent molecules in the asymmetric unit of compound (**2**) have rather different

Table 1
Torsion angles, α and β (°) that characterize the models



Conformation	α (°)	β (°)
A-I	−0.14	−179.29
B-I	−179.00	−176.26
A-II	0.87	−60.85
B-II	168.45	−60.67

conformations, one in a extended fashion, (**2a**), and the other in a folded fashion, (**2b**). Moreover, the crystallographic conformation found for (**6**) is a combination of these two, that is, the phenyl ring has similar orientation as that in (**2a**) and the nitrothiophen ring that of (**2b**). Finally, (**1**) presents a crystallographic conformation that resembles that of (**2b**).

As mentioned earlier, the conformational search and selection was started by studying compound (**2**), for which two torsion angles, α and β (Table 1), were selected to characterize the conformations. These conformations, named A-I, B-I, A-II and B-II (Table 1), where A, B stands for an α torsion angle of c.a. 0° or 180°, respectively, and the roman number I and II indicates the extended and folded conformation respectively, represent the whole conformational space. Moreover, for a sake of completeness the "mirror conformers" of the four models were taken into account and added to the set, thus, eight conformations of (**2**) and (**6**) were used in the docking studies.

The crystallographic conformation of (**1**) is close to that of the B model and the overall structural conformations obtained for it can be described with the A and B models, as for the remaining five compounds of the series. So that, for all of them, the A and B models were constructed and their geometry optimized, which together with their mirror images were used in the docking studies.

3.2. Docking studies

After the graphical analysis of the enzyme–compound complexes, the same ligand conformation and relative orientation, for the complete series, were selected for AS and IS of TR and GR. In the case of NS, for GR and TR, it was not possible to recognize a common complexation pattern for the complete series. Moreover, due to the fact that the extended conformations (A-I and B-I) of (**2**) and (**6**) presented bumping problems in the sites, they were not considered for further studies (see Fig. 3).

For AS of TR and GR the B conformation was the one selected (Fig. 4a and b), as the docking calculations showed a percentage of repetition, in general, higher than 50%. It should be pointed out that in AS–GR 100% of repetition was reached in most cases. This relative orientation, which has the nitrofuran ring pointing to the catalytic disulfide (c.a. 4.5–5.0 Å) is the same that was previously suggested, for TR [14], as a possibility.

For IS two different situations were found, for IS–GR the A conformation was the one selected for the series (Fig. 4c),

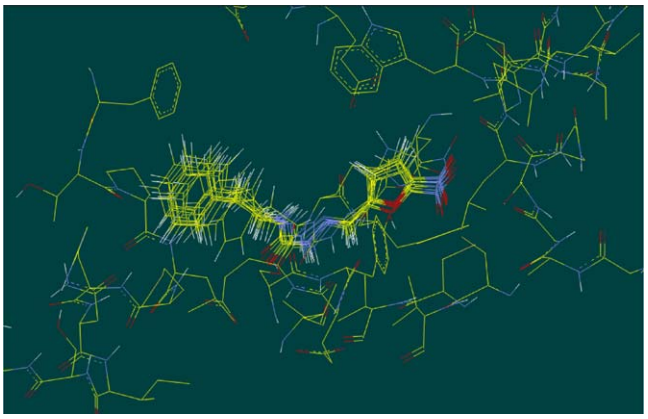


Fig. 3. Superposition of the complexes, generated in the docking calculation, of the A-I conformation of (**2**) in AS–GR, illustrating the bumping problems (left side) detected in the studies of the extended conformations of (**2**) and (**6**).

whereas for IS–TR the B conformation was selected for (**1**), (**3**), (**4**), (**5**), (**7**) and (**8**) (Fig. 4d) and the A for (**2**) and (**6**) (Fig. 4e). This could be ascribed to their different sizes, nevertheless, in all cases the nitrofuran ring is contacting the multipolar ring shown in Fig. 5. The percentage of repetition of the relative orientation selected for each compound was higher than 67%, in both enzymes.

Regarding the docking results shown in Fig. 4c and d it should be noted that there seems to be two "different" orientations, actually, due to the two-fold symmetry of the IS they are equivalent.

In Table 2(a) the mean total energies of the docking calculations, 15–20 complexes for each compound of the series, show that the scores in the different sites present a similar profile for each compound, suggesting a family behavior. Analysis of Table 2(b), shows, on one hand that, regarding the total energy and the van der Waals interaction, the most favorable site is IS, followed by NS and AS ($|IS| > |NS| > |AS|$) for TR and

Table 2
Mean values of interaction energies (kcal/mol): (a) mean total energy for each compound of the series and (b) mean energies for the series, discriminated by type, in the studied sites

Compounds	TR			GR		
	AS	NS	IS	AS	NS	IS
(a) Mean total energies						
1	−18.85	−22.82	−28.64	−25.65	−24.16	−38.41
2	−21.83	−28.94	−31.79	−28.37	−28.20	−44.38
3	−18.53	−22.37	−28.83	−26.03	−25.40	−36.55
4	−18.52	−21.98	−30.79	−24.72	−21.96	−37.07
5	−17.68	−23.67	−28.63	−24.94	−22.40	−33.45
6	−20.20	−24.10	−32.40	−29.96	−24.72	−41.33
7	−17.77	−24.16	−29.48	−25.17	−24.52	−33.40
8	−17.70	−23.73	−31.47	−25.48	−21.14	−34.69

Interaction types	TR			GR		
	AS	NS	IS	AS	NS	IS
(b) Mean energies for the series						
Total	−18.88	−23.97	−30.25	−26.29	−24.06	−37.41
Electrostatic	−3.14	−2.27	−4.48	−2.72	−2.03	−6.26
van der Waals	−15.75	−21.70	−25.78	−23.57	−22.03	−31.15

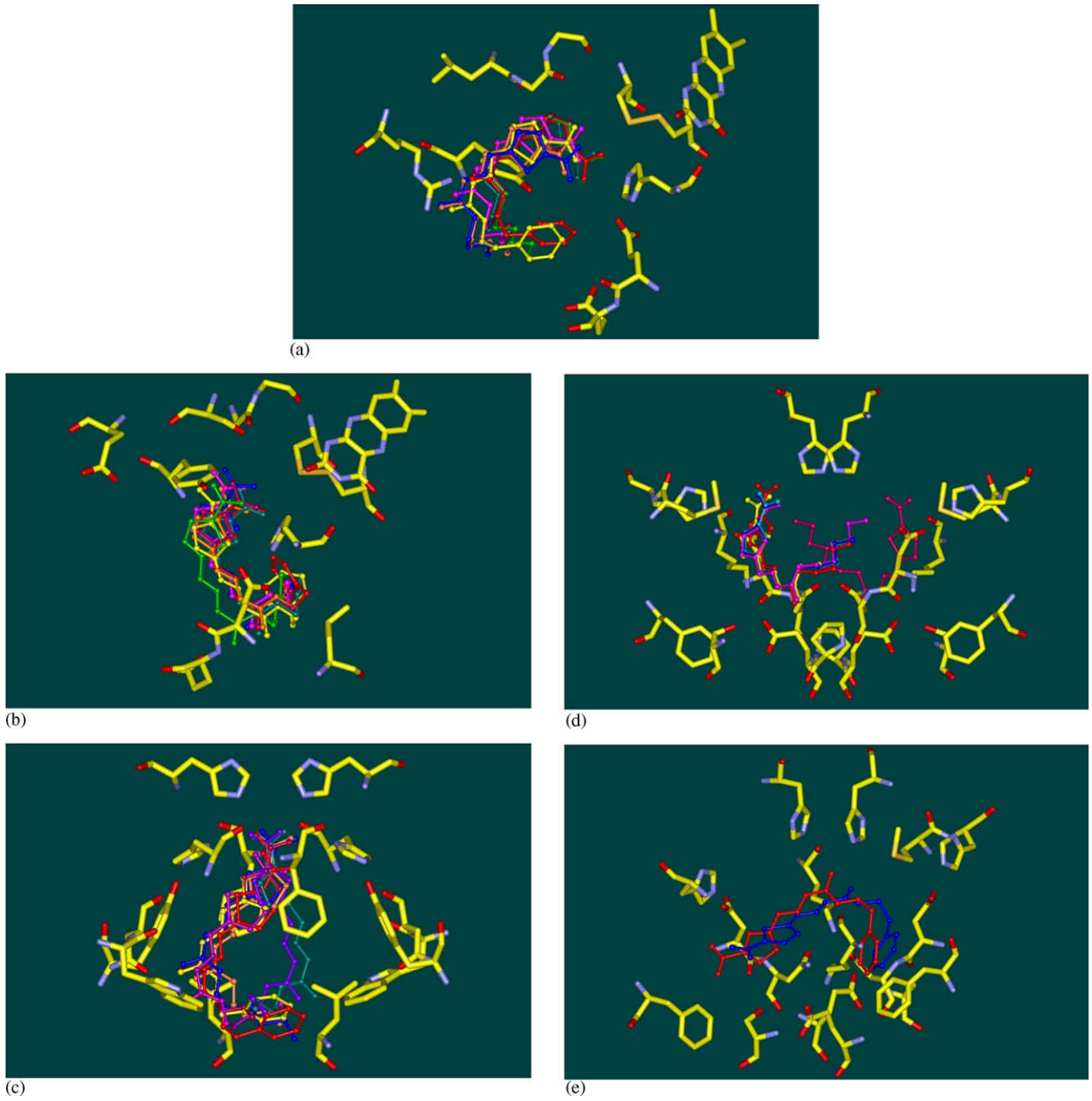


Fig. 4. Superposition of the selected complexes for the complete series, in each site. (a) In AS–GR, (b) in AS–TR, (c) in IS–GR, (d) (**1**), (**3**), (**4**), (**5**), (**7**) and (**8**) in IS–TR and (e) (**2**) and (**6**) in IS–TR.

$|IS| > |AS| > |NS|$ for GR and that, on the other hand, the electrostatic interaction shows the same tendency for both enzymes, that is $|IS| \gg |AS| > |NS|$. Overall it can be seen that IS appears as the most favorable binding site.

Experimental data of inhibition for these compounds [14] showed a mean inhibition of c.a. 48.0% in TR (TR inh%) and c.a. 74.0% in GR (GR inh%). The factor of inhibition difference, F_{id} [=(TR inh%/GR inh%) × 100], being of 64.9. A similar factor, F_{be}, can be calculated using the total energies given in Table 2(b), being 71.8; 99.6 and 80.9, for AS, NS and IS, respectively. It is worth noting that there is a good correlation between the experimental factor, $F_{id,}$ and the calculated, F_{be}, in AS and IS.

Although the superposition of the main chain of both enzymes is relatively close [25] the position of the residues (c.a. 50% non-conserved) gives rise to different IS cavities where to locate the ligands. IS–TR presents two multipolar rings, one per monomer, which are formed by Gln68, His72, Glu75, His400 and Asp431 (Fig. 5), the two His72 are at a distance of 4.0–4.5 Å between them and close to the two-fold symmetry axis, these five residues having alternated H-bond donor/acceptor properties. Three other residues can be associated to the multipolar ring, Lys408 (interacting with Glu75), Glu71 (interacting with Lys408) and Ser403 (interacting with His400). It is found that these eight residues are highly conserved in the different TR enzymes from *T. cruzi*, *T. brucei*,

Table 3
Main residues in contact with the studied series in IS of TR and GR

Moiety	IS–TR residue: range of distances (Å)		IS–GR residue: range of distances (Å)	
Nitrofuran/thiophene ring	Gln68: 6.5–7.0 His72: 4.5–5.0 His72′: 5.0–5.5	Glu75: 3.5–4.0 His400: 4.0–4.5 Asp431: 4.5–5.0	His75: 2.5–3.0 His75′: 2.5–3.0 Phe78: 3.5–4.0	Phe78′: 3.5–4.0 His82: 2.5–3.0 His82′: 2.5–3.0
Semicarbazide fragment	Asn432: 3.0–3.5 Lys61′: 4.0–4.5	Glu435′: 2.0–2.5 Pro434′: 3.5–4.0	Tyr407: 3.5–4.0 Phe78: 5.0–5.5	Asn71′: 3.5–4.0
Terminal fragment	Pro370′: 3.0–3.5 Asn432′: 4.5–5.0	Asp431′: 3.0–3.5	Trp70′: 4.5–5.0 Asp441′: 4.0–4.5	

The distances are given in ranges and are relative to non-H atoms.

T. congolense, *Crithidia fasciculata* and *Leishmania donovani* [25]. Only His72 is substituted by non-conservative mutations in the last three parasites, moreover, *T. cruzi* and *T. brucei*, in which His72 is present, are specially sensitive to nitrofuran compounds, which can act as turncoat inhibitors of TR [26,27].

The IS–GR presents two identical sequences of polar residues, with alternated H-bond donor/acceptor profile, forming two multipolar semicircles that surround the symmetry axis of the cavity (Fig. 6): Tyr407, Asp81, Arg413, Glu77, His374 and His75′ (which belongs to the other monomer). In the crystal structures of 1GRA and 1XAN, in which the IS is, respectively, unoccupied and occupied by a molecule of xanthene, several water molecules are found between Arg413 and Glu77, filling the gap shown in Fig. 6, thus making the multipolar semicircle to be continuous.

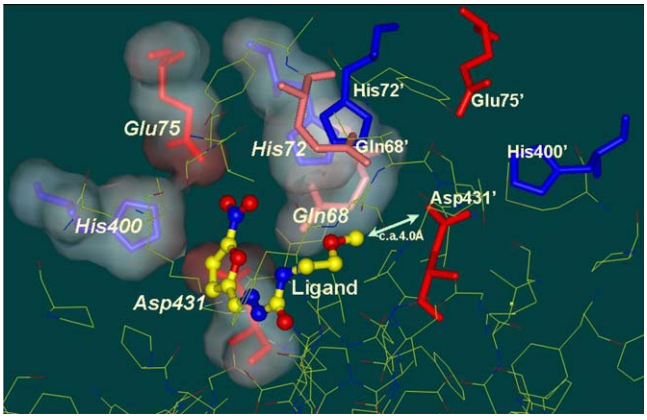


Fig. 5. (**1**) In the interface site of TR. The nitrofuran ring is located in the center of one multipolar ring, shown with van der Waals surfaces. The symmetry related multipolar ring is shown as rods.

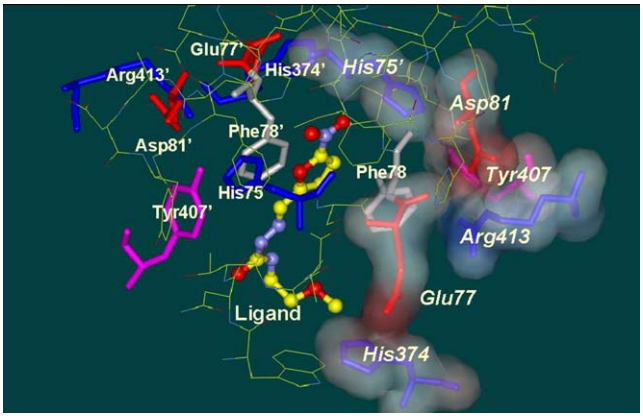


Fig. 6. Interface site of GR, the nitrofuran ring of (**1**) is located in the center of the cavity formed by the monomers. The residues that form one of the multipolar semicircles are shown with van der Waals surfaces and the other in rods.

In Table 3 the main residues, of IS–TR and IS–GR, that are in contact with the three moieties that form the compounds, are shown. Two fragments are specially interesting, the nitro-ring, in which resides the redox activity of the ligand, and the terminal fragment, on which the molecular design has been focused. In TR the nitro-ring is centered in one of the multipolar rings, close to both His72 residues, whether in GR it is centered in the cavity, thus, interacting with both His75 residues. The semicarbazide fragment interacts, in TR and GR, with residues that are on the side of each cavity. Finally, the terminal fragment, in TR is located near the center of the ellipsoidal cavity, interacting with part of the other multipolar ring, meanwhile in GR it is interacting with residues located in the opposite side to that of the nitro-ring.

Moreover, the analyses of the crystal structures of TR and GR show that AS and IS are connected by an α-helix which leaves an internal tunnel that could allow charge transference between both sites [28]. In TR the α-helix goes from Cys57 (which is part of the catalytic disulfide bridge) in AS to Phe79 in IS, passing through His72, the residue contacted by the nitro group of the docked ligands. In GR the α-helix goes from Cys63 in AS to Tyr85 in IS, passing through His75, again, the residue contacted by the nitro group of the docked ligands.

4. Conclusions

The docking results suggest a general tendency of the studied compounds to form complexes in IS of both TR and GR indicating, also, a prevailing role of the nitro-furan or thiophene moiety over the rest of the molecule, in the selection of a non-competitive binding way. It seems that the most relevant interaction is the contact between the nitro-furan and thiophen moiety with the histidine residues present in IS of both enzymes, His75 in GR, and His72 in TR. It should be pointed out that, as for nitrofurans there are experimental data showing that they can bind in IS of both enzymes [29–31], whether there is nothing regarding nitrothiophenes.

At the light of these results, and in order to reach a better binding in IS–TR together with a selective inhibition of TR over

GR, the design of new compounds of this type should considerate the possibility of locating as terminal fragment an H-bond donor in a slightly longer terminal group in order to reach a distance of c.a. 2 Å from Asp431′ of TR, instead of the c.a. 4 Å (see Fig. 5), and so interacting with it and causing a much better selectivity for TR.

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