Computer assisted design of potentially active anti-trypanosomal compounds ☆

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Abstract

A computer assisted molecular modeling was used to design molecules having a shape complementary to the active site of glutathione reductase (GR) and trypanothione reductase (TR). The designed 5-nitro compound derivatives, were obtained from structural knowledge gleaned on glutathione (GSSG), trypanothione ($T[S]_2$) and GSP disulfide (glutathionylspermidine disulfide). These molecules form complexes with the enzymes GR and TR. The theoretical lead compound was: N1-[1-(5-nitro-2-furyl)methylidene]-N4-{4-[3-(2,2,2-trifluoroacetyl)hexahydro-1-1-pyrimidynil]butyl} semicarbazide (NPIPCO). A multidisciplinary team developed around the efforts to synthesize this lead. In this work we report on eight compounds that were synthesized in the pathway to NPIPCO: 4-(2-methoxyethyl)-1-, 4-butyl-1-, 4-hexyl-1- and 2-methoxphenyl-1-(5-nitrofurfurilidene) semicarbazides and the corresponding 5-nitrothiophenes. These substances are expected to act as pro-transition state analogues. Enzymologic studies proved that many of these compounds are inhibitors of TR. Furthermore, they showed inhibitory activity on *Tripanosoma cruzi* growth in vitro.

Keywords: Trypanosomiasis; Drug design; Molecular modeling; Anti-trypanosomal compounds; Inhibition assays

1. Introduction

Computer assisted molecular design is having an important impact in the quest for new drugs. This is particularly true when specific enzymes are known to be playing key roles in metabolic pathways controlling in part the malfunction. The situation improves if

☆ Presented at the XXVIth International Congress of Theoretical Chemists of Latin Expression, held in Caxambú, MG, Brazil, 3–9 September, 2000.

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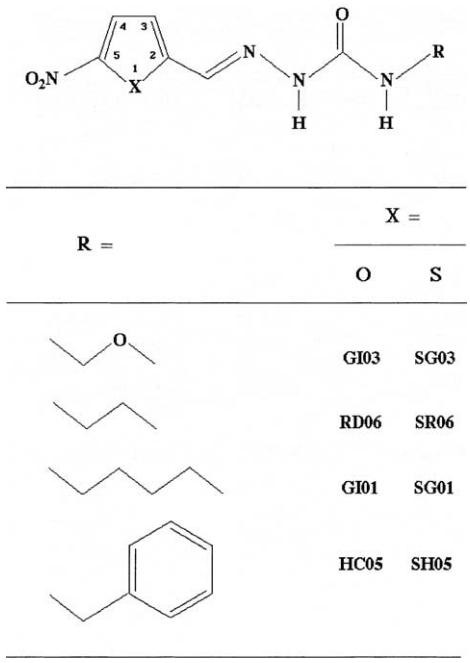

Fig. 1. Synthesized 5-nitrofuranic and 5-nitrotiophenic semicarbazide derivatives.

a three-dimensional structure of the protein is known. This case allows for an active-site-specific molecular design. This work contains the results accomplished from a multi-disciplinary approach to obtain drugs having anti trypanosomatic activity. The difficulties encountered in actual synthetic pathways have prevented full completion of the aims this research program had. However, work carried out so far contains unique experiences derived from the global approach, and the intermediate products have interesting properties. We report here the approach and results obtained so far.

Trypanosomiasis and leishmaniasis are infectious diseases still affecting large human and cattle populations in extended regions of the world. Although there is extensive experimental work on drug design, there is still a need to develop novel compounds [1–4].

Glutathione reductase (GR) in mammalian cells and trypanothione reductase (TR) in trypanosomatid parasites, participate in the defense against free radical attack. GR helps reduce glutathione disulfide (GSSG) while TR catalyzes the reduction of trypanothione disulfide ($T[S]_2$). Both use NADPH (nicotinamide adenine dinucleotide phosphate) as a coenzyme. These enzymes have a striking specificity for their cognate substrates [1–4], which is a key feature for designing selective inhibitors that may lead to new drugs [5].

One of the most important chemotherapeutic agents used against American trypanosomiasis (Chagas' disease) is Nifurtimox®. This nitrofuran derivative acts by mechanisms involving reduced oxygen metabolites, namely, superoxide, hydrogen peroxide and hydroxyl radicals [3,6]. Unfortunately, Nifurtimox® is not effective against chronic Chagas' disease and, furthermore, it causes unpleasant toxic effects. One way to circumvent these hindrances involves the development of a much more specifically targeted antiparasitic therapy. In fact, the work of several authors [3,4,7–9] provides a basis for designing selective inhibitors, which would bind at the trypanothione reductase active site, e.g. inhibitors which bear a positive charge and/or a flexible side chain. As we presently know, hydrazonoamine-substituted nitrofurans and quinones inhibit TR more strongly than GR [8]. In the same vein, phenothiazine inhibitors of TR were tested for in vitro activity against *Tripanosoma cruzi* and other trypanosomatids [9]. The search for selective inhibitors of trypanothione reductase, especially redox-active compounds, remains a desirable and, as it is shown here, feasible target.

The computer assisted design of a 5-nitrofuran semicarbazide named as NPIPCO, N1-[1-(5-nitro-2-furyl)methylidene]-N4-{4-[3-(2,2,2-trifluoroacetyl)-hexahydro-1-1 pyrimidyn-il] butyl} semicarbazide was a follow up of a detailed study of GSSG-GR, $T[S]_2$-TR, cross-binding complexes GSSG-TR and $T[S]_2$-GR, and a model of glutathionyl-spermidine disulfide (GSP disulfide). This approach is based on the transition state analog strategy [10–12]. Here, we report on intermediate compounds obtained in order to get a synthesis of NPIPCO as a real compound.

Full synthesis of NPIPCO has turned out to be difficult. Among the intermediates, we have synthesized four nitrofuranic compounds: 4-(2-methoxyethyl)-1-, 4-hexyl-1-, 4-butyl-1-, and (2-phenylethyl)-1-, -(5-nitrofurfurilidene) semicarbazides, henceforth denominated GI03, RD06, GI01 and HC05, respectively. A complementary series of four 5-nitrothiophenic semicarbazides, namely SG03, SR06, SG01 and SH05 was also synthesized. Both series are shown in

Fig. 1. These molecules share structural properties with previously proposed (and tested) compounds by Fairlamb and coworkers [1,2,7,13–15].

The enzymologic work reported here indicates that, to different degrees, the new nitrofuran derivatives are TR inhibitors. Furthermore when tested on *T. cruzi* cultures, the 5-nitrofuranic compounds were found to be active in vitro [16]. The experimental observations (voltametric and lipophilicity measurements) and the theoretical calculations (partially reported here) strongly suggest that the new nitrofurans form anion radicals. The chemical syntheses of RD06, SR06, GI01, SG01, GI03, SH03, HC05 and SG05 are briefly described.

2. Chemistry

2.1. Computer assisted design

The docking of GSSG, $T[S]_2$, GSP disulphide and the putative substrate NPIPCO and complexes with GR and TR were performed using the molecular graphics facility of TOM/mdFRODO package [17,18]. The DOCK3.5 program [19] was used to study the interactions of the modeled structures in the putative TR active site. The combined use of both programs is ideal to design molecules when significant knowledge about the active site is available, as is the case of GR and TR. Interestingly, the docked coordinates were compared with recent work by Krauth-Siegel and coworkers [5] with excellent scores.

The work was initiated and pursued with a model protein structure [20] constructed in our group at an early stage of this project. The model TR coordinates were then checked with more recent X-ray data on *Crithidia fasciculata* (E.C. 1.6.4.8). The active site coordinates of the model *Tripanosoma congolense* [21] overlap nicely with the structure of *C. fasciculata* TR, hence, all docking work can be transferred to the crystal structure.

The approach used to construct the molecules does not require extensive screening of compound databases, as the chemical structures are derived in part from knowledge extracted from special redox properties and surface complementarity criteria [20–22]. The salient points are [12,20–22]: the model structure of trypanothione is docked at the active site of TR; this model is used to design a new molecule. Instead of the substrate disulfide group, the putative molecule was given a nitrofuran head. We assume that the $-NO_2$ group will act as an electron attractor and thereby may bind at the active disulfide S-R-center of TR. This might happen once the reduced form nicotinamide adenine dinucleotide phosphate is oxidized by the cofactor (FAD). The negative charge derived from the redox step should flow toward the active disulfide-bridge. NPIPCO has at the opposite end a positive group that is assumed to interact with the TR negative binding site but not with GR. The molecular groups serving as connectors to the nitro group are hydrophobic. This would increase the global binding power of this model system. It is important to note that the actual modeling of trypanothione structure required the knowledge of GSSG, the substrate of GR.

Binding of the substrate GSSG to GR was also examined in order to incorporate to some extent specificity [20]. As outlined by Karplus and Schulz [23], more water-mediated than direct hydrogen bonds are involved in the interaction between GSSG and GR. The knowledge of the polypeptide structure alone does not suffice to predict GSSG binding accurately. This renders all docking calculations rather difficult without actual X-ray coordinates for the protein. In this work, no solvent molecules were included. The modeled complex was refined by graphically docking the four terminal moieties of GSSG into the binding site. The final spatial position of the substrate model was assigned in agreement with a number of experimentally determined distances verified between GSSG and several protein residues [11,23]. Residues in GSSG named GluII, CysII and GlyII are located near the enzyme disulfide-bridge (Cys58-Cys63); CysII contain the sulfur atom to be covalently linked to Cys58 during catalysis. GluI, CysI and GlyI represent the remaining half of the molecule (not shown). The docking pinpoints the shortest possible distance between those two sulfur atoms (not shown). The docked species is then thought to represent a productive complex.

Taking as a starting point the docked GSSG (or the presently available crystal coordinates that differ little from ours), a possible structure of $T[S]_2$ was constructed and placed in the model of TR from *T. congolense.* As the long and flexible spermidine

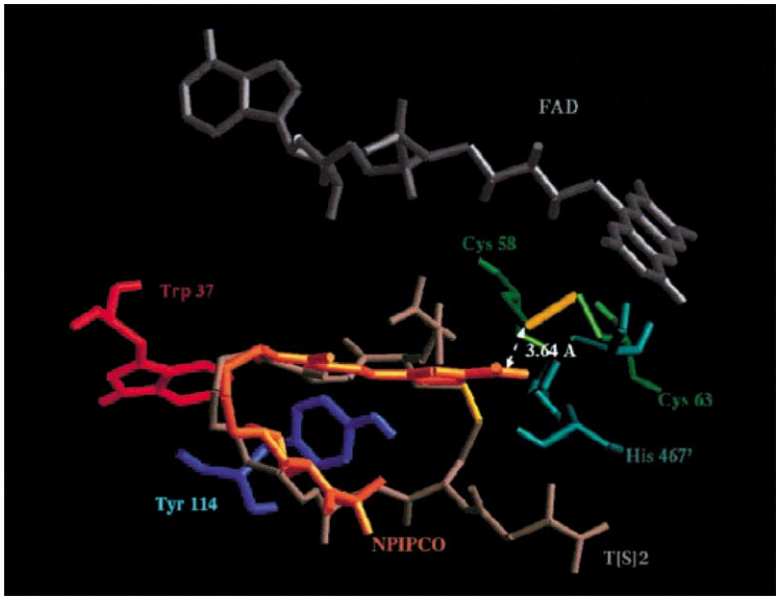

Fig. 2. NPIPCO (N1-[1-(5-nitro-2-furyl) methylidene]N4-{4-[3-(2, 2,2,-trifluoroacetyl) hexahydro-1-pyrimidinyl] butyl} semicarbazide) model folded into a conformation following the $T[S]_2$ leader at trypanothione reductase (*T. congolense*) active site. The pyrimidine ring is making contacts with Trp 37 and Met 117. The arrow denotes the distance between the nitro functional group and the catalytic sulfur.

moiety links the glycine residues of this disulfide ligand, a molecular mechanics refinement was necessary to have an unstrained structure. The MM2G force field [22] was used to obtain a relaxed conformation of the spermidine moiety while the other atoms of $T[S]_2$ were kept fixed. Then, the resulting geometry was molded in accordance with the binding site of TR and docked at the active site. Thereafter, this new

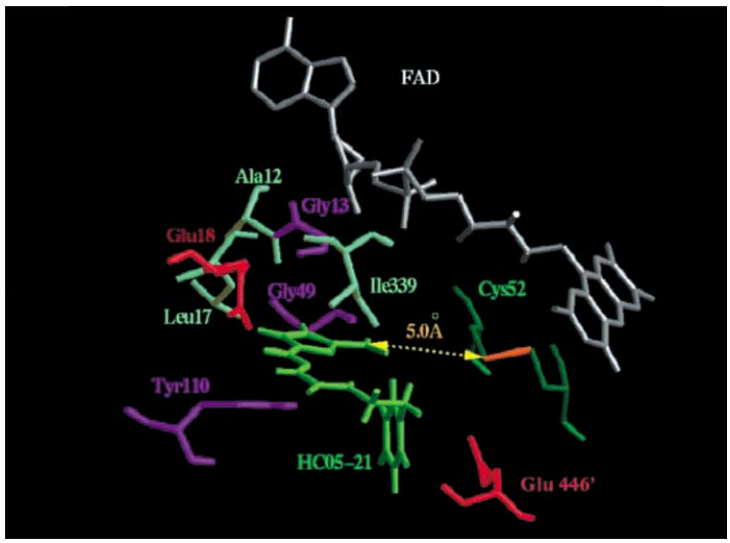

Fig. 3. HC05-21 (crystallographic folded conformation) docked into the putative active site of trypanothione reductase (*T. cruzi*). Residues making contacts are shown. As in NPIPCO (the pro-transition state analogue guiding compound), the nitro functional group is located in the neighborhood of a redox complex. The distance between the nitro functional group and the catalytic sulfur is displayed over the yellow arrow.

conformation was removed from the enzymatic surroundings and underwent a MM2 force field optimization involving the entire ligand geometry, in vacuo. In the next step, the geometry of this optimized species was docked back into the active site of TR and the new spatial positions were compared with the starting configuration. Subsequently, quantum chemical semiempirical AMPAC/AM1 [24] calculations were carried out to check for energy differences. The geometry-relaxed structure actually differs significantly from the active site-adapted conformation.

A model of GSP disulfide was constructed from the $T[S]_2$ complex and this configuration was adjusted to fit into the active site of TR from *T. congolense*. Later checks with the crystallographic coordinates for the complex TR-GSP disulfide corresponding to *C. fasciculata* [25] showed good structural agreement.

As for the crystal structure of GSP disulfide, in a similar fashion to $T[S]_2$, we ran a molecular mechanics minimization on the ligand structure in vacuo. The resulting geometry was placed back at the active site of TR (from *C. fasciculata*). Next, the optimized structure was energetically compared with those of the crystal- and model-bound conformations using again the AM1-hamiltonian. From this analysis, we conclude that the geometry of the docked complex may be considered more like a transition structure than a minimum energy structure, since the latter cannot be docked without important steric constraints.

As mentioned earlier, NPIPCO design was made by taking as a template the conformation of trypanothione adapted to fit the putative site. The building was done under the assumption that the intramolecular degrees of freedom should permit sufficient flexibility so the guiding compound NPIPCO may fit the binding site while still interacting with the disulfide active bridge (Cys58-Cys63). This flexible species will be referred to as a pro-transition state analog to distinguish it from rigid molecules having the conformation of a transition state analog that are usually used as haptens to produce catalytic antibodies. NPIPCO contains four molecular fragments:

(i) a nifurtimox related moiety (N1-[1-(5-nitro-2-furyl)] methylidene), which actually corresponds to the 5-nitrofuran derivatives of Henderson et al. [7];
(ii) a semicarbazide molded in the same conformation as the amide linkage between GlyI and spermidine in $T[S]_2$ docked into TR;
(iii) this moiety was elongated by a chain of four carbon atoms to mimic the arm of spermidine;
(iv) a hexahydro pyrimidine ring designed as a basic center to be protonated at physiologic pH. The template compound bears a resemblance with compound VII of Henderson et al. [7], but with important differences in the second fragment. Fig. 2 depicts this compound docked in the active site of the *T. congolense* model active site and overlaid on $T[S]_2$ to help viewing.

The nitrofuran moiety was located as near as possible the catalytically active bridge Cys58-Cys63. If NPIPCO were to bind at the real active site in such a position, one would expect formation of a charge transfer complex between the nitrofuran and the sulfur atom nearest to it. The pyrimidine moiety makes a number of contacts with Trp 37, Tyr 114 and Met 117 belonging to the specificity pocket of TR (numbering as in the model structure, CF, Fig. 2). The binding site of this new guiding compound is compared with a recent X-ray structure of a TR–mepacrine complex by Krauth-Siegel and coworkers [5]. Residues Trp 37(21), Tyr 114(110) and Met 117(113) appear to be playing a role similar as in the case of the complex with the real substrate (numbers in parentheses correspond to those of the *T. cruzi* TR–mepacrine complex). Mepacrine binds to trypanothione reductase with the acridine ring close to the hydrophobic region formed by Trp 37(21) and Met 117(113). Contacts at less than 4.0 Å were analyzed for our complex of NPIPCO with TR and compared with those reported by Jacoby et al. [5]; we have 14 out of 30 contacts found by Jacoby et al., they involve Met, Ser, Trp and Tyr residues (not shown). There are three contacts with Cys58(52) that are not described for the mepacrine complex (not shown).

Knowledge of the electronic mechanism has also been used for the design. As shown by Mannervik and coworkers [26–28] the kinetics of GR is branched, switching between ping-pong and sequential-ordered mechanisms. Depending upon many factors, such as the substrate concentrations, direction of catalysis, and side chain composition, one of the two branches may dominate the other or the ensemble may show

Table 1
Biological (in vitro growth inhibition of *T. cruzi*) and enzymological (TR and GR inhibition) activity for the complete set of eight synthetic 5-nitrofuranic and 5-nitrothiophenic derivatives. Percentages of TR and GR inhibition are expressed as mean ± standard deviation from triplicate assays. In vitro activity data were adapted from Ref. [16]

	X	% TR inhibition at 50 μM	% GR inhibition at 50 μM	% Growth inhibition at 10 μM[a]
GI03[b]	2-Methoxyethyl	> 30.6 ± 1.6	60.4 ± 3.9	50/47
SG03[c]		58.1 ± 4.6	69.8 ± 4.8[d]	0/1
RD06	Butyl	44.8 ± 7.7	75.3 ± 5.8	95/89
SR06		64.6 ± 4.8	80.8 ± 5.5	3/13
GI01	Hexyl	50.7 ± 3.9	83.9 ± 6.8[d]	93/90
SG01		39.1 ± 8.2	61.1 ± 0.5	4/00
HC05	2-Phenylethyl	57.2 ± 1.9	82.0 ± 0.9[d]	16/15
SH05		37.9 ± 5.5	78.0 ± 0.7	3/9

[a] Day 5/Day 7.
[b] First row: 4-(X)-1-(5-nitrofurfurilidene) semicarbazide.
[c] Second row: 4-(X)-1-(5-nitrothienylidene) semicarbazide.
[d] $n = 2$.

combinations with varied weights. For instance, the mutant Tyr177Gly in GR (*E. coli*) produces a change of mechanism from the ping-pong scheme to a sequentially ordered one [29]. Changes in the coenzyme concentration also produces changes in the kinetics, as shown by Rakauskiené [30]. Due to the evident structural and functional properties shared with TR, it is likely that these mechanistic considerations may apply to the parasite enzyme. In particular, under physiologic conditions, Borges et al. [31] showed TR kinetics is of a ping-pong type. Elements for a theoretical explanation to such behavior were recently proposed [32]. The same transposed hydride transfer step is in action for both kinetic paths [32,33] in order to accomplish the redox reaction. The oxidized $NADP^+$ unbinding rate would control the global kinetic type: fast unbinding compared with substrate binding would lead to the ping-pong, while slow unbinding would create conditions to form a binary complex between protein and coenzyme and thereafter a tertiary complex with the substrate [33].

The hypothesis behind the present molecular design was to simulate the space distribution of $T[S]_2$ (as modeled here) with a nitro-derivative interacting with the active disulfide bridge, an intermediate molecular compound mimicking the flexibility of the spermidine moiety (this idea has been exploited by other workers too) and a molecular group able to interact with the residues making up the specificity pocket (Tyr, Met and Trp). Thus, besides the surface complementarity principle used in the construction of the template (an idea implicit in the refine tool found in TOM [17]), the pro-transition state analog was designed to subvert the electronic mechanism of TR, namely, to use the disulfide bridge as an electron-donor site.

A set of nitro-derivatives of thiophene was designed to check for differential effects.

3. Experimental protocols

3.1. Synthesis of 5-nitrocompounds

GI03, SG03, RD06, SR06, GI01, SG01, HC05 and SH05 were prepared in three steps according to a classic synthetic procedure indicated in Ref. [16]. In the first step, the carbamates were obtained by reaction of phenyl chloroformate with the corresponding amines in methylene dichloride and triethylamine. In the second step, the semicarbazides were synthesized by condensation of the starting carbamates with an excess of hydrazine monohydrate at 80–90 °C. In the third step, the reaction of semicarbazides with 5-nitro-2-furaldehyde diacetate, in 50% sulfuric acid, led to the nitro compounds GI03, RD06, GI01 and HC05. The thio derivatives SG03, SR06, SG01, and SH05 were prepared in an alternative third step by reaction of semicarbazides, from the second step of the procedure, with 5-nitrothiophene-2-carboxaldehyde in toluene, catalyzed with *p*-toluene sulfonic

Table 2
Total energies and Coulomb and Lennard-Jones partial energies for the selected docked compounds GI03 and HC05-21. The internal structures for GI03 and HC05-21 correspond to the crystallographic coordinates presented in this same work. All energies are given in kcal/mol

	X	Number of contacts	Total energy	Coulomb	Lennard-Jones	
					Repulsive	Attractive
GI03[a]	2-Methoxyethyl	103	− 18.991	0.343	21.769	− 43.170
HC05[a,b]	2-Phenylethyl	134	− 25.264	− 2.581	23.759	− 46.442

[a] 4-(X)-1-(5-nitrofurfurilidene) semicarbazide.
[b] Crystallography, folded conformation.

acid. In all the cases, overall good yields were obtained. Each compound was identified by ^{1}H-NMR, IR and MS and its purity determined by TLC, HPLC and microanalysis. The complete set of spectral and analytical analysis is available on request from Cerecetto's group.

3.2. Electrochemical studies

Voltametric responses for semicarbazides derivatives were measured by cyclic voltametry. Experiments were carried out using a Weenking POS 88 instrument with a Kipp Zenen BD93 recorder, in *N*,*N*-dimethylformamide (Aldrich, spectroscopy grade).

Tetrabutylammonium perchlorate (Fluka) (ca. 0.1 mol/ml) was the supporting electrolyte and purged with nitrogen at room temperature. A three-electrode cell configuration was used, with mercury dropping working electrode, a platinum wire auxiliary electrode and a saturated calomel reference electrode. Voltage scanning rates ranged from 0.1 to 0.5 V/s.

3.3. EPR measurements

Radicals were generated by electrolytic reduction in situ, at room temperature by means of an EPR 106 spectrometer, using a rectangular cavity with a 50 kHz field modulation, in dimethylsulfoxide (Aldrich, spectroscopy grade). The hyperfine splitting constants were estimated to be accurate within an error of 0.05 G. Spectral plots are available on request from Olea's group.

3.4. Lipophilicity studies

Reversed-phase TLC experiments were performed on pre-coated TLC plates SIL RP-18W/UV_{254} (MachereyNagel) and eluted with methanol:water (50:50, v/v). Plates were developed in a closed chromatographic tank, dried and the spots were located under UV light. The R_f values were averaged out of two to three determinations, and converted into R_M via the relationship

$$R_M = \log[(1/R_f) - 1]$$

3.5. TR and GR inhibition assays

Activity was determined by monitoring the decrease in absorbance at 340 nm using either a Thermomax microtitre plate reader (Molecular Devices) or a Beckman DU640 spectrophotometer. Recombinant *T. cruzi* TR [31] (active site concentration 4 nM) was equilibrated in 40 mM (Na^+) Hepes, pH 7.8, 1 mM EDTA and 150 μM NADPH at 27 °C for 5 min prior to addition of inhibitors (50 μM final concentration from 2.5 mM stock solutions in DMSO). After further incubation for 5 min reactions were initiated with 50 μM $T[S]_2$ (Bachem). Human recombinant GR activity was measured in a similar fashion by adding the compounds to reaction mixtures containing 100 mM (Na^+) Hepes, pH 7.8, 0.5 mM EDTA, 8 nM GR and 150 μM NADPH that were initiated with 100 μM glutathione disulfide (Sigma). Reaction rates were followed in triplicate by measuring the linear decrease in $OD_{340\ nm}$. Percent inhibition was calculated relative to control assays, which contained an equivalent volume of DMSO.

Table 3
Electrochemical (redox potential), lipophilic (R_M) and electrostatic (AM1 LUMO energies and nitro group charges) results obtained for the complete set of eight synthetic 5-nitrofuranic and 5-nitrothiophenic derivatives

	X	First redox potential E_{pc} 1/V SCE[a]	Lipophilicity (R_M)	E_{LUMO}/Q_{NO2}
GI03[b]	2-Methoxyethyl	−0.86	0.07	−1.35/−0.066
SG03[c]		−0.78	0.35	−1.61/−0.103
RD06	Butyl	−0.86	0.43	−1.42/−0.067
SR06		−0.79	0.75	−1.66/−0.102
GI01	Hexyl	−0.83	1.06	−1.42/−0.068
SG01		−0.78	1.38	−1.66/−0.102
HC05	2-Phenylethyl	−0.87	0.68	−1.39/-0.067
SH05		−0.79	1.06	−1.64/−0.105

[a] First row: 4-(X)-1-(5-nitrofurfurilidene) semicarbazide.
[b] Second row: 4-(X)-1-(5-nitrothienylidene) semicarbazide.
[c] Peak potentials (±0.01 V) measured at a scanning rate of 0.2 V/s.

3.6. Pharmacology

The capacity of the synthesized compounds to act as enzyme inhibitors either of TR or GR was evaluated using recombinant *T. cruzi* TR and human GR. The results are expressed as percentages of inhibition and summarized in Table 1.

4. Results and discussion

For GR-GSSG, TR-T[S]$_2$ and TR-GSP disulfide, a validation procedure [12] was performed based on the available X-ray information on the structures of: GR, GR-GSSG, TR and TR-GSP disulfide [5,8,15,23,25,34–41]. We determined both quantitatively and qualitatively the contacts existing between each particular substrate and their respective enzyme active sites [12].

Additionally, by using graphical resources, we exchanged the substrates T[S]$_2$ and GSSG between TR and GR, obtaining crossed-complexes, namely, GR-T[S]$_2$ and TR-GSSG. According to kinetic studies [13], these complexes were not catalytic. This part of work was intended to seek for structural parameters defining the substrate specificity displayed by the proteins being investigated.

The molecular modeling was the starting point for the experimental work reported here. Now, with the models reasonably validated, it remains to be seen whether the intermediate compounds share the presumptive properties. Namely, whether they have some inhibitory properties.

The results shown in Table 1 indicate that all compounds are active as TR inhibitors, with 5-nitrofurans having a TR inhibitory capacity greater than 38% at 50 μM.

In the light of these results, a relationship with structural properties was sought by performing additional crystallography for the free compounds GI03 and HC05 followed by docking studies. In Table 2, are shown the total and partial energies of HC05 and GI03 docked at *T. cruzi* TR putative active site (crystal structure).

Free GI03 crystallizes as one independent molecule in the asymmetric unit. Using now this geometry, GI03 can fit into TR's putative active site in only one arrangement, with a total binding energy of −19.0 kcal/mol and making 103 contacts (see Table 2). The main energetic component is the attractive Lennard-Jones energy with a value of −43.2 kcal/mol. Free HC05 crystallizes as two independent molecules in the asymmetric unit, one extended and one folded conformation. Each conformer could be docked in two ways, resulting in four possible complexes. The docked structures derived from the folded HC05 are energetically more stable and the number of contacts is larger than the corresponding structures for the extended conformations. The more stable putative complex (dubbed HC05-21) was

selected. The complex with the enzyme can be seen in Fig. 3. In this conformation, HC05-21 makes 134 contacts with a total binding energy of −25.3 kcal/mol (see Table 2). The main energetic component is the attractive Lennard-Jones energy with a value of −46.4 kcal/mol.

The distances between the nitro moiety and the disulfide catalytic bridge (Cys52 and Cys58) are 4.5 and 5.0 Å for both GI03 and HC05-21 putative complexes, respectively, suggesting the possibility of a 'subversive' reduction of 5-nitrofuran derivatives by TR which could reinforce their oxidative stress mechanism.

As stated by Benson et al. [42], the hydrophobic wall formed by Leu 17, Trp 21, Tyr 110, Met 113 and Phe 114 of TR (numbering as in the crystal structure of *T. cruzi*) is of particular interest. The contact region with the enzyme, for both GI03 and HC05-21, is mainly this hydrophobic region, Tyr 110, Leu 17, Ile 339 being some of the closest residues in the putative docked complexes. HC05 shows 30% more contacts with TR than GI03 and a corresponding increase in the total binding energy of 24%. Additionally, HC05 shows a global lipophilicity (expressed in Table 3 as the R_M coefficient defined in Section 3) greater than GI03. These facts may explain the superior TR inhibitory activity of the 4-(2-phenylethyl)-nitro-derivative, HC05, with respect to GI03 or 4-(2-methoxyethyl)-nitro-derivative.

All synthetic compounds tested were marginally better inhibitors of GR than TR. This result is not bad news in itself, since the lead compound (NPIPCO) should have a volume large enough so as to reduce its accessibility to the active site of GR, thereby increasing selectivity towards TR. This factor has not yet been fully exploited in the compounds discussed here. According to the original design strategy, the presence of a bulky functional group could be necessary to confer more binding energy to the putative active site of TR. Recently, Marsh and Bradley [43] concluded that the access to the active site of TR by large aromatic groups seems to be readily tolerated. This fact strongly suggests that the modulation of TR inhibition must be made taking into account steric bulk properties. In addition, the electrostatic component to the binding specificity should not be forgotten. Again, the lead compound was designed in order to acquire a positive charge at physiologic conditions, which would enhance the binding specificity to TR. The (neutral) synthetic intermediates cannot benefit from this trait.

As far as in vitro *T. cruzi* growth inhibition is concerned, the 5-nitrofurans appear to be better growth inhibitors than the corresponding 5-nitrophenes [16].

The experimental EPR and voltametric results along with the molecular orbital calculations (LUMO energies and charges of the nitro group) (cf. Table 3), support the hypothesis of a free radical forming nitrofuran fragment. This, in turn, would mean that the main mechanism of action of the synthesized compounds could be exerted by free radical formation. In previous reports, we pointed out the free radical production of another set of 5-nitrofurans derivatives, analogues to Nifurtimox® [44,45], and stressed the relationship between the electronic properties and the inhibition capability of in vitro *T. cruzi* growth. Nevertheless, the electronic redox properties of HC05 are similar to those of RD06, GI03 and GI01. According to the lipophilicity index (R_M) reported in Table 3, the 5-nitrofuran derivatives could be ordered as: GI03 < RD06 < HC05 < GI01. These results suggest a selective uptake by the parasite.

5. Conclusions

First, inhibitors of TR were obtained by means of knowledge assisted design. The entire modeling task was developed, under the control of TOM program [17], by using exclusively geometric constraints. The ligand structures docked into these enzymes' active sites do not correspond to energy minima of the free molecules. For the design of subversive substrates, one follows the approach conceived by Henderson et al. [7]. The design of transition state analogues [10] leads to molecules having a rather rigid geometry which may follow from the corresponding saddle point calculated with quantum chemical techniques [11,46–48]. One can also design pro-transition state analogs as particular (flexible or semirigid) molecules that can be molded into the active site of a given enzyme at low intramolecular energetic expenses. If the enzyme contains a ligand with properly oriented nitrogen, one obtains a pseudo-substrate, otherwise one obtains a competitive

inhibitor. The use of the DOCK program in this context is complementary to our approach. Both, TOM and DOCK emphasize the shape factor. The most probable location and orientation at the active site are assigned by the knowledge-based procedure. Otherwise, a blind search with DOCK may not be satisfactory.

Second, some of the new synthesized compounds proved to be potentially good antichagasic drugs, displaying high levels of in vitro *T. cruzi* growth inhibition. Their apparent redox activity is clearly due to the 5-nitrofuran substructure included in the design. Depending on the accessibility to the target, inhibition of TR could reinforce the oxidative stress elicited by free radicals, with a corresponding decrease in the $T(SH)_2$ level inside the parasites.

In view of the experimental and theoretical results gathered here, the compounds seem to have a propensity to form free radicals. A conjecture can be advanced as to the effect of such radicals: they may act as enzyme inhibitors. If this is the case, the present experiments do not rule out such a hypothesis.

The results reported here, strongly suggest that a balance between the inhibition of TR and the oxidative stress activity, together with a differential uptake by the parasite is desirable to have an adequate candidate for a drug.

In conclusion, the potentiality of these compounds to inhibit TR and promote free radical formation could be combined with an adequate accessibility to the parasite to have an adequate antichagasic drug. The partially successful results herein reported are encouraging. A full synthesis of the lead compound, and the related family proposed elsewhere, may lead to actual selective trypanocidal drugs. This stands up as a prediction that can be experimentally tested.

Acknowledgements

AHF is supported by the Wellcome Trust. OT thanks NFR (Swedish Research Council) for financial support. Most of the work carried out in South America was funded by SAREC/SIDA.

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