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Charge is the Major Discriminating Factor for Glutathione Reductase Versus Trypanothione Reductase Inhibitors

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Abstract—Benson et al. (*Biochem. J.* **1992**, *286*, 9) reported three novel competitive inhibitors of trypanothione reductase (TR), which were selected to complement a hydrophobic region identified on the TR structure which was not present on human glutathione reductase (hGR). Benson et al. also noted that chlorpromazine, a tricyclic antidepressant known to have trypanocidal activity, was an inhibitor of TR. Here we show that chlorpromazine is a competitive inhibitor of TRs from *Crithidia fasciculata* ($K_i = 14$ μM) and *Trypanosoma cruzi* ($K_i = 10$ μM), but the drug binds >50-fold more weakly ($K_i = 762$ μM) to hGR. Analogues of chlorpromazine differing in the length of the side chain carrying the positively charged R-group are also selective TR inhibitors whereas, a tricyclic structure carrying a negatively charged side chain is a competitive inhibitor with selectivity for hGR ($K_i^{hGR} = 165$ μM vs. $K_i^{TR} = 1400$ μM). This finding suggests that simple charge characteristics, rather than differences in hydrophobicity, may account for a significant portion of the selectivity of this series of inhibitors for these two enzymes. Electrostatic analysis of the structures of TR and hGR thus provides a rationale for these results, and offers a new principle for inhibitor design. The principle gains further support from the observation that all known tricyclic competitive inhibitors of TR are positively charged. In order to investigate the in vivo relevance of our findings we have examined the effect of chlorpromazine and its negatively charged analogue on the growth of *C. fasciculata* parasites. Consistent with our kinetics, chlorpromazine (50 μM) inhibited the growth of parasites by 50%, while no measurable decrease in parasite growth rate was noted in the presence of the negatively charged inhibitor (400 μM). Furthermore, the highly similar inhibitory profiles of *C. fasciculata* TR and *T. cruzi* TR suggest that drug-design studies using the structurally better-studied *C. fasciculata* TR are also relevant to the human pathogen *T. cruzi*.

Introduction

The enzyme trypanothione reductase (TR) from trypanosomatid parasites catalyses the NADPH-dependent reduction of trypanothione disulfide [$T(S)_2$; Fig. 1], a metabolite which in these parasites replaces the otherwise prevalent glutathione disulfide (GSSG). Because of the known sensitivity of such parasites to oxidative stress, and because of the distinct specificities of trypanothione reductase and the human counterpart glutathione reductase (hGR), TR is an attractive target for rational drug design to combat Chagas' disease and other diseases caused by trypanosomes.[2,3] In order to provide structural information to guide the inhibitor design process, multiple structural studies have been carried out on TR from both the insect pathogen *Crithidia fasciculata*,[4–7] [Strickland, C. L.; Karplus, P. A., unpublished work] and the human pathogen *T. cruzi*.[8] These structures, together with the known structure of hGR,[9–11] may be used to guide selective inhibitor design.

A variety of promising lead compounds have recently been reported.[1,12–19] Here we follow up the molecular modeling and kinetics study that used structural information to identify three selective TR inhibitors, with the most active, clomipramine, having $K_i = 6$ μM.[1] All of the TR inhibitors reported by Benson et al.[1] contained a significant hydrophobic region and a net positive charge. The authors hypothesized that the large hydrophobic portion of these compounds would bind to the spermidine subsite of TR, which is much more hydrophobic than the equivalent region of hGR. Moreover, the positively charged R-group would extend toward the site normally occupied by the amino group of one of the γ-glutamyl moieties of $T(S)_2$ (Fig. 1). In the model of Benson et al.,[1] selectivity between TR and hGR is only due to the hydrophobic portion of the inhibitor.

Mepacrine, the first detected tricyclic competitive inhibitor of TR,[13] carries a positively charged R-group. In accordance with the results on mepacrine, we will provide evidence that the positively charged R-group

†The first two authors contributed equally to this work.
Abbreviations: hGR, human glutathione reductase; TR, trypanothione reductase; $T(SH)_2$, reduced trypanothione; $T(S)_2$, trypanothione disulfide; GS-I, glutathione-I in GSSG; GS-II, glutathione-II in GSSG.

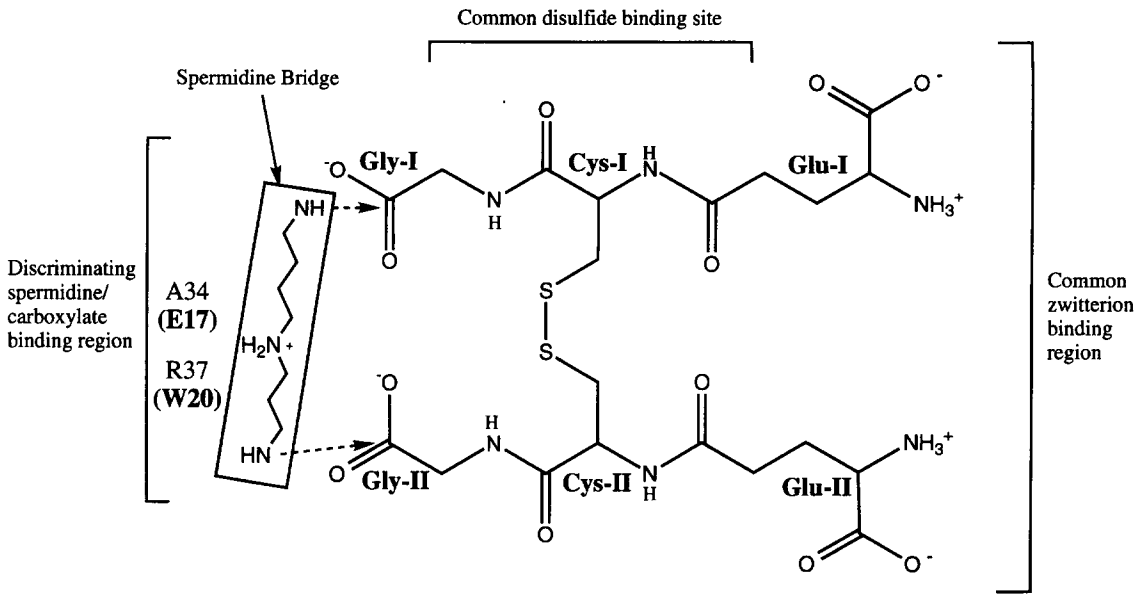
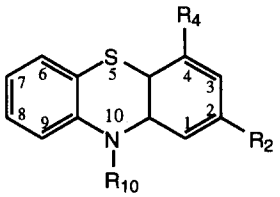


Figure 1. A comparison between GSSG and $T(S)_2$. $T(S)_2$ is GSSG plus a spermidine bridge that joins the Gly-I and the Gly-II ends of GSSG. The active sites of TR and hGR are almost identical in the regions that bind the disulfide (Cys-I and Cys-II) and zwitterionic (Glu-I and Glu-II) portions of the two substrates. The residues that differ between hGR and TR are shown close to the spermidine moiety, with the ones in bold characters corresponding to TR.

of the inhibitor plays a major role for selective binding to TR (Fig. 1). To test this hypothesis, we have studied the inhibition patterns of four further tricyclic compounds. The series is based on the lead molecule chlorpromazine (**1**) (Fig. 2), a known antidepressant, which has also been shown to inhibit the proliferation of *T. cruzi* and which Benson et al.[1] noted to be an inhibitor of TR. The inhibition patterns we see support our hypothesis that the charged R-group is crucial for the discrimination between TR and hGR.

Results and Discussion

The four compounds used in this study are all close analogues of chlorpromazine (**1**) each varying from another in the series by a single change of ring substituent, side-chain length, or side-chain charge (Fig. 2). The K_i-values measured for these compounds against *Cf*TR, *Tc*TR, and hGR (Fig. 2) show that all of the compounds act as competitive inhibitors of both TRs and hGR, with **1** being the best TR inhibitor with



Compound	Name	R_{10}	R_2	R_4	K_ihGR (μM)	K_i*Cf*TR (μM)	K_i*Tc*TR (μM)
1	Chlorpromazine	$\overset{+}{N}H(CH_3)_2$	Cl	--	762	14	10
2	2,4-Dichloropromazine	$\overset{+}{N}H(CH_3)_2$	Cl	Cl	900	68	65
3	2-Chloro-10-(2-dimethylaminoethyl) phenothiazine	$\overset{+}{N}H(CH_3)_2$	Cl	--	440	24	17
4	2-Chloro (10-(2-2-carboxyethyl)) 10-H-phenothiazine	COO^-	Cl	--	165	1400	not determined

Figure 2. Structures and K_i values for the inhibitors used in this study.

$K_i^{TR} \approx 10$ μM. The three positively charged compounds (**1–3**) are all reasonable inhibitors of TR with K_i values <70 μM, and the K_i values for *Cf*TR and *Tc*TR exhibit quite similar magnitudes. The similarity of the *Cf*TR and *Tc*TR K_i values agrees with the observation that nearly all active site residues are conserved between these species, given that there is near 70% overall identity. It also supports the idea that *Cf*TR is a reasonable model system for designing potential anti-trypanosomal drugs.

Compounds **1–3** are rather selective inhibitors of TR with the K_i^{hGR} values 14- to 76-fold higher than the K_i^{TR} values. These properties are very similar to those measured by Benson et al.[1] for the positively charged tricyclics clomipramine, amitriptyline, and trifluoroperazine which have even larger differences in ring- and side-chain structure, and suggests that the selective inhibitory properties are not very sensitive to the detailed spatial relationships of this class of inhibitors. This agrees with the recent results of Evans and Croft.[19]

Compound **4**, on the other hand, shows dramatically different properties, having a K_i for hGR of 165 μM and showing a nine-fold selectivity for hGR over TR. Although it does not bind very tightly, **4** provides a rare non-peptidic inhibitor of hGR that is competitive with GSSG. Comparison of the properties of **4** to those of the most similar positively charged compound, **3**, shows that the charge reversal has altered the specificity by a factor of ca. 130. If, as proposed by Benson et al.,[1] the charged side chain were directed to the conserved Glu-I or Glu-II binding pockets (Fig. 1) one would have expected compound **4** to show poorer inhibition of both TR and hGR. We conclude that despite the design principle that led to their discovery,[1,20] the charge is much more important than the hydrophobic moiety for discriminating selectively between TR and hGR. The size and nature of the hydrophobic moiety are, however, quite important for binding affinity.

Our in vivo studies with *C. fasciculata*, in which the inhibitory effect of compounds **1** and **4** on parasitic cell growth was investigated, have shown that 25, 50, and 75 μM of compound **1** inhibited cell growth by 30, 50, and 90%, respectively, whereas neither 200 μM nor 400 μM of compound **4** caused any notable decrease in cell growth. The toxic levels of compound **1** are comparable with its observed K_i, and the non-toxicity of compound **4** matches well with its much weaker inhibition of TR. Taken together, the results are consistent with the toxicity of compound **1** being due to its inhibition of TR. However, it is also possible that the lack of toxicity observed for compound **4** is due to poorer transport across the cell membrane or other issues related to bioavailability or pharmacokinetics.

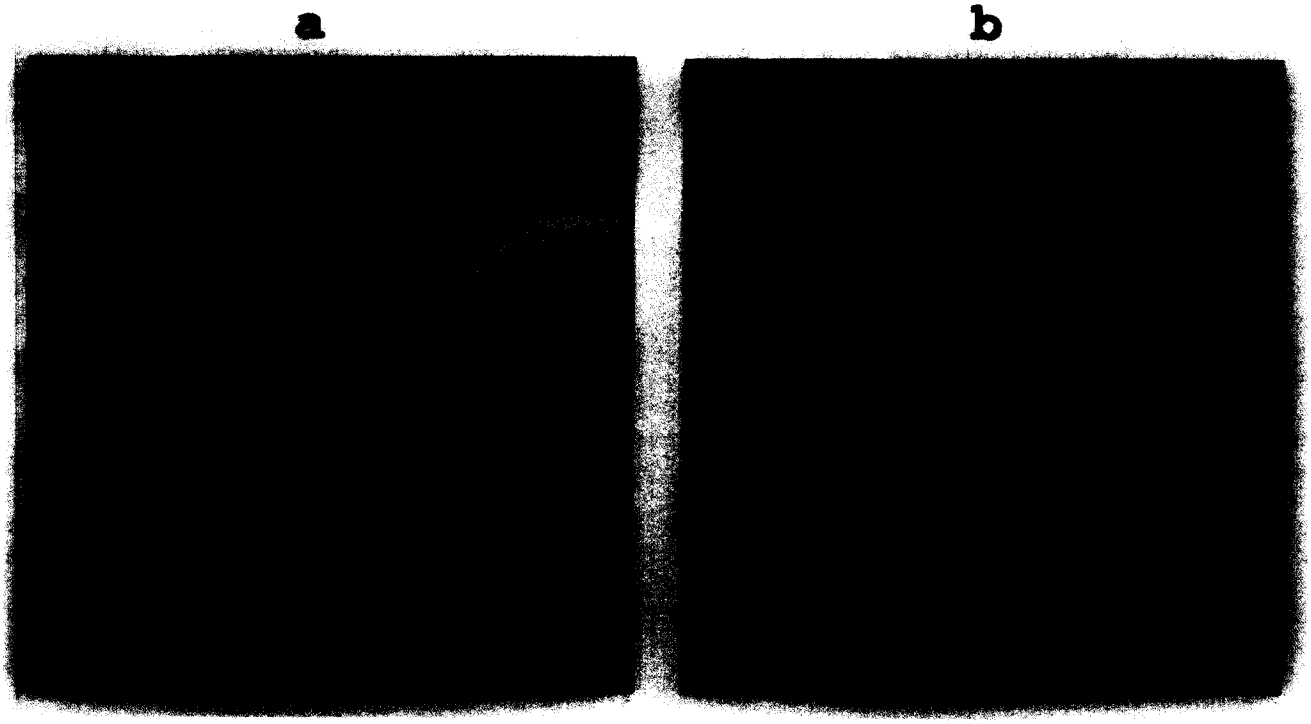


Figure 3. Electrostatic properties of *Cf*TR and hGR near their disulfide binding sites. (a) *Cf*TR. A ribbon diagram of *Cf*TR is shown viewed directly down the ≈2-fold axis of the dimer. The white arrows point to the corresponding active disulfide bridges located at the catalytic centers. The electrostatic potential is shown for a plane perpendicular to the dimer axis and intersecting the disulfide substrate binding site. The coloring of the electrostatic potential (in units of kT/e) is as follows: >5=blue; 5 to 0.5=light blue; 0.5 to −0.5=black; −0.5 to −1=green; −1 to −5=yellow; <−5=red. (b) hGR displayed as in (a). The electrostatic features at the substrate binding sites show that *Cf*TR is highly complementary to positively charged ligands whereas that of hGR is complemetary to negatively charged ligands. Similar calculations with *E. coli* glutathione reductase, which accepts both $T(S)_2$ and GSSG as a substrate, show that it has intermediate electrostatic properties, in agreement with a recent study by Smith et al.[43]

Because no crystal structures have been reported for inhibitors of this class bound to TR, the mode of binding remains unknown. Thus far, soaks and co-crystallization experiments of *Cf*TR with **1** and hGR with **4** (see methods) have been unsuccessful. Our kinetic results indicate that the positively charged side-chain is much more important for specificity than the tricyclic moiety, and that the charged group probably interacts with the distinctive region of the active site which in TR is centered on Trp-20 that interacts with the spermidine bridge of the substrate (see Fig. 1). In this regard it should be noted that the secondary or tertiary amines found in these inhibitors differ from primary amines in that the positive charge is diffusely distributed over the many methylene protons surrounding the formally charged nitrogen. Based on other characterized enzymes which bind substituted amines, like acetylcholinesterase[21] and trimethylamine-dehydrogenase,[22] such amines interact with Trp residues and not the formally negatively charged Asp or Glu side chains. This would seem to relate to the diffuse nature of the positive charge, which can interact well with the electron-rich platform provided by the face of aromatic groups.[23] Automated docking studies with compound **1** do not favor this orientation, although they show it is plausible. Using the 'contact only', 'force field only', or 'contact plus force field' options, a highest-ranked binding mode placing the amine R-group near Trp-20 was only the 69th, 45th, or 56th best mode, respectively. When electrostatic properties were also used ('contact plus Delphi') a solution showing this mode was among the top 10 docking results. Clearly the docking results are quite sensitive to the parameters used and do not provide strong evidence for or against any particular binding mode.

A cursory inspection of the electrostatic isopotential surfaces for both *Cf*TR and hGR shows that the former enzyme has negative values for the potential at the active site whereas the latter enzyme has positive values for the potential at the active site (Fig. 3). This observation is consistent with a global electrostatic complementarity between *Cf*TR and the three positively charged compounds tested in this study, **1–3**. In a similar manner, we observe a global electrostatic complementarity between hGR and the negatively charged inhibitor **4**. The importance of this correlation is emphasized by the finding that most of the recently discovered inhibitors of TR with $K_i < 100$ μM[1,13–15,17–18,24] have a net positive charge. That finding further suggests that the success of many TR inhibitors, such as kukoamine[25] and the peptide-based inhibitor benzoyl-Leu-Arg-Arg-β-naphthylamide,[17] may be due to the simple combination of a hydrophobic moiety with a positively charged R-group.

The studies here enlarge the extensive inhibition studies already carried out on TR[1,12–19] which uniformly show that good competitive inhibitors of TR are all positively charged compounds with a large hydrophobic group. Although the current study does not involve a large number of compounds, it was designed to specifically test the relative importance of charge and hydrophobicity on the specificity of inhibition of TR vs. hGR. The strength of this analysis is that the four compounds studied provide three matched pairs differing in one parameter at a time: side-chain charge (**3** vs. **4**), side-chain length (**1** vs. **3**), and specific structure of the hydrophobic group (**1** vs. **2**). The results are quite unambiguous: although the details of side-chain length and hydrophobic group structure modulate the affinity of inhibitors of TR, it is the ligand charge which is most important for TR vs. hGR selectivity.

Experimental

Reagents

All reagents used were of the highest purity available and were purchased from Aldrich Chemical Company, Fisher Scientific, and Sigma Chemical Company. $T(S)_2$ was purchased from Bachem Bioscience Inc. Chemical structures and names of the inhibitors are presented in Figure 2. Inhibitor **1** was purchased from Aldrich Chemical Company. Inhibitors **2** and **3** were gifts from Research Biochemicals International (RBI). Inhibitor **4** was synthesized as follows: a suspension of 2-chloro-10-(2-cyanoethyl)-10-H-phenothiazine (Aldrich; 52 mg, 0.184 mmol), and 25% aq NaOH (1.25 mL) in MeOH (4.2 mL) was heated at reflux for 4 h. After concn in vacuo, the resulting residue was dissolved in H_2O (10 mL) and washed with 3×10 mL Et_2O. The aq layer was acidified to pH 2 with 2 N HCl, and extracted with 7×10 mL $CHCl_3$. The combined organic layers were dried over Na_2SO_4, filtered, and concd in vacuo. Flash chromatography of the residue (SiO_2, 3:97 MeOH:CH_2Cl_2 followed by 8:92 MeOH:CH_2Cl_2) afforded **1** (23 mg, 41%) as a tan solid: mp 153–154.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.90 (t, 2H, $J = 7.3$ Hz), 4.19 (t, 2H, $J = 7.3$ Hz), 6.85–7.26 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 32.16, 42.47, 115.49, 115.60, 122.74, 123.33, 123.92, 125.21, 127.57, 127.73, 128.11, 133.36, 143.85, 146.00, 177.07; IR ν^{CDCl_3} cm^{-1}: 3050, 2920, 1690, 1455; MS (*m/z*) 306, 308 (^{35}Cl [M]$^+$, ^{37}Cl, [M]$^+$), 305 (base), 307 (^{35}Cl [M-H], ^{37}Cl [M-H]).

Enzyme Kinetics

Crithidia fasciculata trypanothione reductase (*Cf*TR) was prepared from an *E. coli* expression system as described by Strickland et al.[26] Human glutathione reductase (hGR), overexpressed in *E. coli*,[27,28] was prepared as described by Savvides and Karplus.[29] Recombinant *Trypanosoma cruzi* TR (*Tc*TR) was purified as described.[30] Steady-state kinetic constants were $K_m = 56$ μM and $k_{cat} = 10500$ min^{-1} for *Cf*TR, $K_m = 18$ μM and $k_{cat} = 5000$ min^{-1} for *Tc*TR, and $K_m = 65$ μM and $k_{cat} = 12500$ min^{-1} for hGR.

Enzyme kinetics experiments were carried out at 25 °C. The activity of both TRs and hGR was monitored from the rate of conversion of NADPH to NADP$^+$ as measured by the decrease in absorbance at 340 nm. The reaction mixture for hGR assays was 1 mL of the

following ingredients: 0.2 M KCl, 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA, 0.1 mM NADPH, 30–1000 μM GSSG, and 35 nM bovine serum albumin (this stabilizes hGR without interfering with the assay).[31] For *Cf* TR, the assay mixture consisted of 0.1 M Hepes (pH 7.8), 0.5 mM EDTA, 0.25 mM NADPH, and 12–400 μM $T(S)_2$. The reactions were initiated by the addition of NADPH. For *Tc* TR, the kinetic measurements were carried out in 40 mM Hepes, 1 mM EDTA, (pH 7.5), 0.1 mM NADPH and 40–200 μM $T(S)_2$, and the reactions were started by the addition of $T(S)_2$. In each case, measurements were carried out for three concentrations of inhibitor. The possibility for inhibitor-induced NADPH oxidase activity was eliminated by the fact that none of the inhibitors acted as an electron acceptor in assays that were carried out in the absence of $T(S)_2$. Kinetic constants, K_m and k_{cat}, were calculated by fitting the reaction rates to the Michaelis–Menten equation by a non-linear least-squares method. For the inhibition studies of *Cf* TR and hGR, 10 mM stock solns of **1–3** were prepared in distilled water, whereas a 3 mM stock soln of **4** was made in 0.1 M Tris–HCl at pH 8. For the inhibition studies of *Tc* TR, stock solns of **1–3** were prepared by dissolving the compounds in a small volume of ethanol and then diluting the soln to 10 mM with distilled water. Inhibitors were added to the above assay mixtures at the desired concentrations, keeping all other parameters the same. The K_i values were estimated by fitting the kinetic data to the Michaelis–Menten equation for competitive inhibition.

In vivo inhibition of *C. fasciculata*

The *Crithidia fasciculata* strain used in this study was a gift from Christopher Walsh (Dana–Farber Cancer Inst). Stock cell cultures were kept at −80 °C in 15% glycerol. Cell culture media contained 5 g/L yeast extract, 4 g/L tryptone, 15 g/L sucrose, 2.5 g/L triethylanolamine (2,2′,2″-nitrilotriethanol), 4.6 mL/L Tween-20, 200 mg/L streptomycin sulfate, and 2 mg/L hemin. *C. fasciculata* parasites were introduced to 3 mL aliquots of cell culture media in 10 mL glass tubes to achieve a cell concn of $\approx 10^6$ cells/mL. Cell populations were measured using a hemacytometer. Compound **1** was added to the cell cultures from a 10 mM aqueous stock soln to achieve final concns of 25, 50, and 75 μM. To overcome solubility problems, a 4 mM stock solution of inhibitor **4** was prepared in 1 M Tris–HCl pH 8.5, and cell cultures containing 200 and 400 μM of inhibitor **4** were set up. The pH of the Tris-containing cultures was adjusted to 7.7 by addition of concentrated hydrochloric acid. In this case control experiments contained Tris at concentrations and pH equivalent to those of cell cultures containing compound **4**. All cell cultures were grown in triplicate in a water bath at 26 °C. Cell growth was assessed from absorbance (A) measurements at 595 nm using a Shimadzu UV160U spectrophotometer. A_{595} measurements were taken until readings reached a maximum suggesting cessation of cell growth due to overpopulation. In the case of the cultures containing inhibitor **4** and Tris a lag in cell growth was observed which can be attributed to the presence of Tris as revealed by our control experiments.

Crystallization trials

Crystals of hGR and *Cf* TR were grown as previously described.[26,29] Crystal soaking experiments for hGR inhibitors were done at saturating concns of inhibitor in the crystal artificial mother liquor. The concentration of inhibitor used in the *Cf* TR soaks was typically 10 mM, but concns up to 100 mM have been tried. Cocrystallization of *Cf* TR in the presence of **1** was done using a soln of 10 mg/mL *Cf* TR with 10 mM **1**, or 4 mg/mL *Cf* TR with 10 mM **1** under various conditions.

Computational analyses

Automated docking was carried out using the program DOCK[32] (version 3.0) and the crystal structures of *Cf* TR [Strickland, C. L.; Karplus, P. A. unpublished work] and **1**.[33] All four scoring options were used: 'contact only', 'contact and Delphi', 'contact and force field' and 'force field only'. The scoring option 'contact and Delphi' requires electrostatic calculations carried out with Delphi.[34]

The electrostatic calculations were done using the finite-difference algorithm Delphi of Honig et al.[35–37] after the polar hydrogens were added to the *Cf* TR [Strickland, C. L.; Karplus, P. A. unpublished work] and hGR[9] (Protein Data Bank entry 3GRS) dimers using the program Sybyl.[38] The Debye–Hückel model was used with the region within the protein approximated by a cavity of low dielectric constant $\varepsilon_{prot} = 4$, and surrounding water modeled as a high-dielectric medium, $\varepsilon_{solv} = 80.0$, containing counterions at an ionic strength of 0.1 M. Atoms were assigned point charges using the united-atom approximation of the AMBER program[39] and calculations were done on a cubic grid of $65 \times 65 \times 65$ nodes. Initially, 200 iterations using a linear approximation of the Poisson–Boltzmann equation and 50 iterations of a non-linear approximation, were carried out with the protein region occupying 30% of the grid volume. These results were subsequently used to define the boundary conditions of a second calculation in which the protein occupied 66% of the grid volume. For this purpose, the *focusing* feature available within Delphi was used. Visualization of the electrostatic properties was carried out using the IBM Visualization Data Explorer™ (DX)[40] and a set of chemistry applications modules developed at the Cornell Theory Center by Gillilan.[41] Construction of the molecular surfaces of the enzymes was accomplished using the program SURF.[42]

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design. We also thank Christopher Walsh for providing us with the strains of *Crithidia fasciculata* used in this study. Compounds 2,4-dichloropromazine and phenothiazine, 2-chloro-10-(2-dimethylaminoethyl) HCl were provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N0IMH30003. Support of the Cornell NMR Facility by the NSF (CHE 7904825; PGM 8018643) and NIH (RR02002) is gratefully acknowledged.

Note Added In Proof

A 2.9 Å resolution structure of the complex between the tricyclic inhibitor mepacrine and *T. cruzi* TR has recently been published. [Jacoby, E. M.; Schlichting, I.; Lantwin, C. B.; Kabsch, W.; Krauth-Siegel R. L. "Crystal structure of the Trypanosoma cruzi Trypanothione Reductase–Mepacrine Complex", *Proteins: Structure, Function and Genetics* **1996**, *24*, 73]. Mepacrine was modified to fit the relatively weak electron density, in an orientation which is consistent with the conclusions presented here regarding the origins of binding strength and specifity.

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