Use of an Additional Hydrophobic Binding Site, the Z Site, in the Rational Drug Design of a New Class of Stronger Trypanothione Reductase Inhibitor, Quaternary Alkylammonium Phenothiazines§

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Received April 5, 2000

Improved rationally designed lead drug structures against African trypanosomiasis, Chagas disease, and leishmaniasis were obtained against trypanothione reductase from *Trypanosoma cruzi*. Substituted-benzyl [3-(2-chloro-4a,10a-dihydrophenothiazin-10-yl)propyl]dimethylammonium salts, synthesized by Menschutkin quaternization of the tertiary alkylamine ω-nitrogen atom of chlorpromazine, were linear, competitive inhibitors of recombinant trypanothione reductase from *T. cruzi*, with either trypanothione disulfide or *N*-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide disulfide as substrate. The permanent positive charge on the distal nitrogen atom of the tricyclic side chain contribution to binding was estimated as $\geq$5.6 kcal·mol^{-1} by comparison with the analogue with the cationic nitrogen atom of the quaternary replaced by an ether oxygen atom. A further major contribution to improving K_i values and inhibition strength was the hydrophobic natures and structures of the *N*-benzyl substituents. The strongest inhibitor, the [3-(2-chloro-4a,10a-dihydrophenothiazin-10-yl)propyl]-(3,4-dichlorobenzyl)dimethylammonium derivative (K_i 0.12 μM), was ~2 orders of magnitude more inhibitory than the parent chlorpromazine. Several of these quaternary phenothiazines completely inhibited *T. brucei* parasite growth in vitro at <1 μM. Antiparasite activity was not solely determined by inhibition strength against trypanothione reductase, there being a strong contribution from hydrophobicity (for example, benzhydryl-quaternized chlorpromazime had ED_{50} < 1 μM). Although active against *Leishmania donovani*, none of the analogues showed major improvement in this activity relative to chlorpromazine or other nonquaternized phenothiazines. The *p-tert*-butylbenzyl-quaternized analogue very strongly inhibited (ED_{50} < 1 μM) growth of the amastigote stage of *T. cruzi*.

Introduction

Pathogenic parasites of *Trypanosoma spp.* and *Leishmania spp.* cause major tropical diseases such as African sleeping sickness, Chagas disease, and leishmaniasis. Infection can be severely debilitating, even fatal, and the few drugs available are ineffective and often toxic. For example, the second stage of African sleeping sickness, in which the trypanosome has invaded the central nervous system and which is refractory to drugs such as suramin or pentamidine, is treated with organoarsenicals, a regimen leading to 4−8% deaths in treated patients. Resistance and toxicity are common for the drugs available for these diseases.[1]

There is an absolute biochemical difference between host and trypanosomal/leishmanial parasites, the latter containing not glutathione reductase (GR) but rather the analogous trypanothione reductase (TR).[2] Parasite TR differs from host GR in not processing glutathione diulfide (GSSG). Conversely, host GR does not reduce trypanothione disulfide ($T[S]_2$).[3,4] Such mutually exclusive rejection of cognate substrates between host and parasite boded well for selective inhibitor design.[3,4,5] Efficient selective blockade of TR would be expected to compromise the redox defenses of the parasites. An appropriate TR inhibitor might be a drug in its own right or for co-administration with a redox-active drug, such as nifurtimox, which may even show synergy, allowing lowered doses of redox drug.[6] It has been proposed that any rationally designed inhibitor of TR must attain >85% inhibition for activity as an antileishmanial drug but that clinically usable TR inhibitors may not need to be quite as effective in vivo.[7]

The initial use of rational drug design approaches against TR[8] led to the discovery of phenothiazine, imipramine, and other tricyclic structures as specific inhibitors of TR over host GR. Other tricyclic-based inhibitors are now known,[6,9,10] and rational drug design against TR has been reviewed.[11] It has been suggested[6,8] that such tricyclics may bind to TR with the tricyclic group lodged against the hydrophobic wall formed by W21, M113, etc., and the aminopropyl side chain

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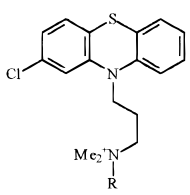
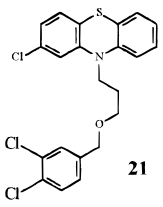
§ Abbreviations: GR, glutathione reductase; GSSG, glutathione disulfide; GSH, reduced glutathione; rms, root-mean-square; $T[S]_2$, trypanothione disulfide; $T[SH]_2$, reduced trypanothione as dithiol; TR, trypanothione reductase; $(ZCGdmapa)_2$, *N*-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide disulfide.

10.1021/jm000156+ CCC: $19.00
Published on Web 07/22/2000

Chart 1. Structures of Ligands Used in This Study





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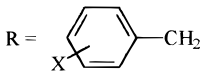
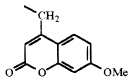
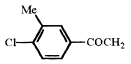
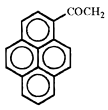


Compound	X =
1	H
2	pCH$_3$
3	4-Cl
4	3-Cl
5	4-Br
6	3,4-dichloro
7	pNO$_2$
8	3,4-dimethyl
9	3,5-dimethoxy
10	pentafluoro
11	isopropyl
12	4-benzyloxy
13	4-t-butyl
14	
15	Ph$_2$CH
16	
17	
18	
19	
20	CH$_3$ chlorpromazine methiodide
22	

extending toward E466′ and E467′. Nearby lies an additional hydrophobic cavity, not used in T[S]$_2$ binding, but which may be accessed by some hydrophobically functionalized phenothiazines, and which we have designated the Z site.[6] This site, in the region of F396′, was first postulated in the design and introduction of *N*-carbobenzoxy (Z)-substituted substrates of TR.[12] The region, since recruited in explanations for binding of other ligands,[6,13] is approximately bounded by F396′, P398′, and L399′.

From such modeling studies, it was logical to try to invoke additional binding interactions through this Z site and to introduce a permanent positive charge to interact with E466′ and/or E467′ (as the ω-*N*-amino group of chlorpromazine may well bind to TR in its protonated form). Ligand design studies indicated that it should be possible to more effectively sequester the TR active site than the first-generation[6,8] tricyclics by quaternization of the tertiary amino site of chlorpromazine with suitable hydrophobic groups to give substituted-benzyl [3-(2-chloro-4a,10a-dihydrophenothiazin-10-yl)propyl]dimethylammonium salts. We now report enzyme inhibition analyses and quantitative ligand-docking studies, as well as antiparasite data for initial compounds in this class, quaternary arylalkylammonium chlorpromazines (the structures are given in Chart 1).

Results and Discussion

Enzyme Inhibition. The values obtained for the specific activity (259 units·mg^{-1}), K_m for T[S]$_2$ (4.9×10^{-5} M) and k_{cat} (193 s^{-1}) of the isolated TR, were comparable to reported values (284 units·mg^{-1} for specific activity, $K_m = 4.5 \times 10^{-5}$ M (T[S]$_2$), and $k_{cat} =$ 237 s^{-1} for *T. cruzi* TR from cultured epimastigotes[4]), and the preparation showed no detectable substrate activity with GSSG. The product was homogeneous by the criterion of SDS−PAGE, and the molecular weight was similar to literature values. The K_m and k_{cat} values determined against (ZCGdmapa)$_2$ were also similar to literature values.[12]

Inhibition type was assessed by plotting data as: (a) Lineweaver−Burk plot, 1/*v* versus 1/[S] at various [I]; (b) Dixon plot, 1/*v* versus [I] at various [S]; (c) Cornish−Bowden plot, [S]/*v* versus [I] at various [S]. This combination of three classes of plot allows inhibition type to be deduced unambiguously[14] and indicated that the compounds studied are linear competitive inhibitors of TR against T[S]$_2$ or (ZCGdmapa)$_2$ as substrates. Figure 1 shows a typical set of diagnostic plots for **6**. Values of IC$_{50}$ and K_i determined against *T. cruzi* TR are collected in Table 1.

To date derivatives of phenothiazines and related tricyclic antidepressants,[6,8,10] plus dimeric and polyamine variants of them,[13,15,16] are the most potent TR inhibitors reported. Clomipramine is a linear, competitive inhibitor of TR ($K_i = 6.5$ μM).[8] Under comparable conditions it did not inhibit human erythrocyte or yeast GR, even at millimolar levels. Chlorpromazine and its derivatives show comparable activity without inhibition of GR.[6] The compounds of the present study provide improvements relative to the parent tricyclic phenothiazine nucleus in potency against TR.

Quaternary arylalkylammonium chlorpromazines (Table 1) are stronger TR inhibitors than the parent chlorpromazine framework. When quaternization was by means of a methyl group (**20**, $K_i = 1.1$ μM) the potency increased 10-fold over chlorpromazine. When quaternization was by means of a benzyl group (**1**, $K_i =$ 1.3 μM) the compound was about 8-fold more potent than chlorpromazine. Appropriate mono- or disubstitution of the aromatic ring with small groups gave further improvements, most of the compounds in Chart 1 tested showing enhancements in inhibition by about 1 order of magnitude, some up to 20-fold relative to chlorpromazine, e.g. the 3,4-dimethyl (**8**) and dimethoxy (**9**). The 3,4-dichloro compound (**6**) showed the greatest improvement in inhibition, about 2 orders of magnitude ($K_i =$ 0.12 μM). Use of benzhydryl (**15**) or naphthyl (**16**) or replacement of the aromatic ring with a benzyloxybenzyl (**12**) group showed an improvement of inhibition of TR by ∼20-fold compared to chlorpromazine.

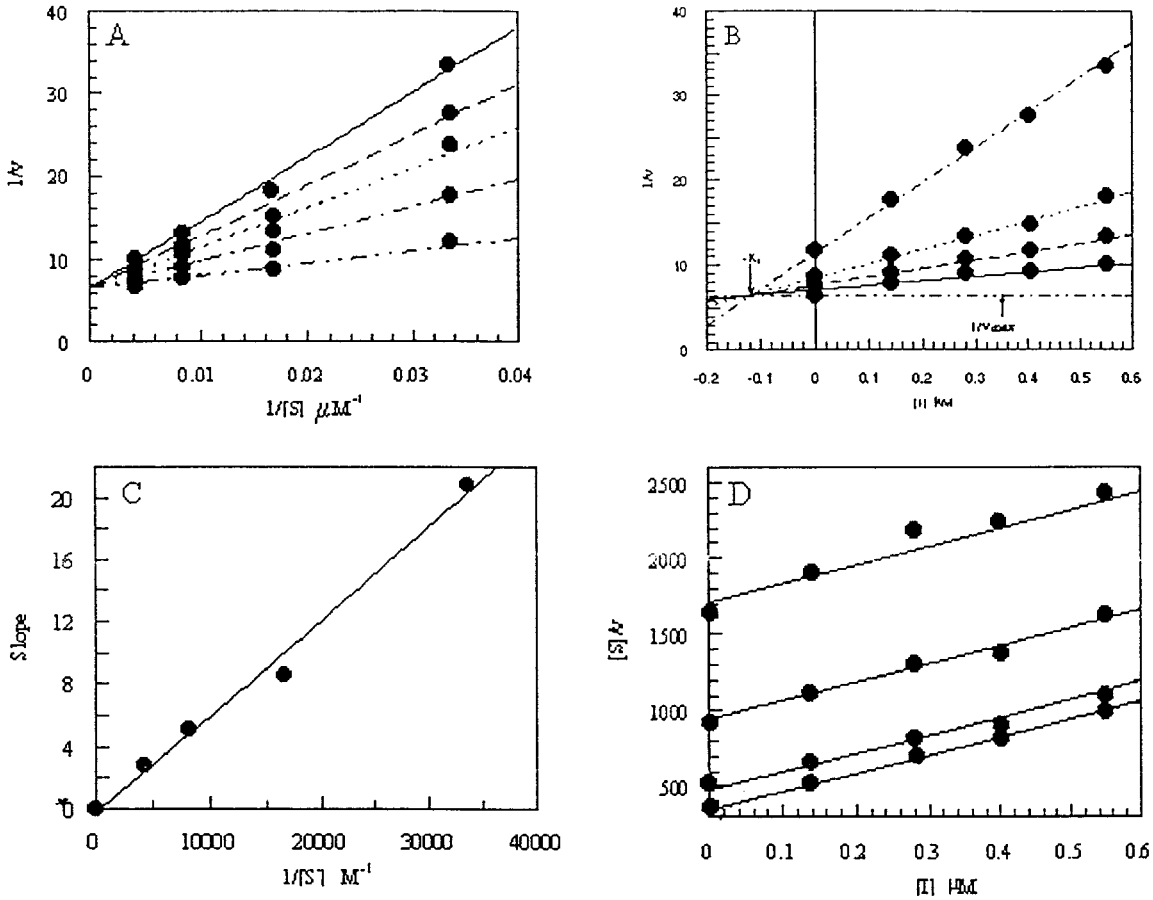


Figure 1. Diagnostic plots for determination of inhibition type for inhibitor **6.** A. Lineweaver−Burk plot of 1/*v* (initial velocity) versus 1/[S] ((ZCGdmapa)$_2$ substrate) at pH 7.25, 25 °C in 0.02 M Hepes buffer containing 0.15 M KCl, 1 mM EDTA, and 0.1 mM NADPH. Points are experimental; lines are theoretical for linear competitive inhibition by **6** with 0.12 ± 0.01 μM, V_{max} = 0.154 ΔA/min, and K_m = 21.5 ± 2.6 μM. The inhibitor concentrations were 0.56, 0.4, 0.28, 0.14, and 0 μM, respectively. B. Dixon plot of 1/*v* versus inhibitor **6** concentration, [I], at pH 7.25, 25 °C in 0.02 M Hepes buffer containing 0.15 M KCl, 1 mM EDTA, and 0.1 mM NADPH. Points are experimental; lines are theoretical for linear competitive inhibition with K_i = 0.12 ± 0.01 μM, V_{max} = 0.154 ΔA/min, and K_m = 21.5 ± 2.6 μM. The (ZCGdmapa)$_2$ concentrations were 240, 120, 60, and 30 μM, respectively. C. Replot of the slopes of the Dixon plot. D. Cornish−Bowden plot of [S]/*v* versus inhibitor **6** concentration, [I], at pH 7.25, 25 °C in 0.02 M Hepes buffer containing 0.15 M KCl, 1 mM EDTA, and 0.1 mM NADPH. Points are experimental; lines are theoretical for linear competitive inhibition with K_i = 0.12 ± 0.01 μM, V_{max} = 0.154 ΔA/min, and K_m = 21.5 ± 2.6 μM. The (ZCGdmapa)$_2$ concentrations were 240, 120, 60, and 30 μM, respectively.

Relative Contributions of the Positive Charge (N^+) and Hydrophobicity to Binding. It has been suggested that a positive charge on the ligand helps in the specificity of interaction with TR over host GR, as TR has an overall negatively charged active site, whereas GR has a positively charged active site.[17] Simple charge characteristics, rather than differences in hydrophobicity, were proposed to account for a significant portion of the selectivity of this series of these inhibitors. Electrostatic analysis of the structures of TR and human GR provided a rationale for these results and was supported by an in vivo inhibition study of *C. fasciculata*.[17]

The data for the present quaternary compounds provide clear evidence of the improvement in the inhibition of TR by quaternization and/or incorporation of a hydrophobic group. The similar increases in potency for methyl-quaternized and benzyl-quaternized compounds (each ~10-fold relative to chlorpromazine) indicate that the unsubstituted benzyl group is not making a binding contribution relative to a methyl group and that it does not cause steric conflicts. It is not possible to ascribe the 10-fold effect of methyl or benzyl quaternization to solely a charge effect as it is not unlikely that the parent chlorpromazine binds in its ω-N-protonated state, as the pK_a of the tertiary amine group of the free ligand is ~8.6.[18] Thus the binding enhancement of 10-fold is probably an underestimate for the charge contribution.

The ether analogue (**21**) of the most potent quaternary chlorpromazine (**6,** 3,4-dichlorobenzyl), with all the hydrophobic moieties present but the N^+ atom replaced by an O atom, showed no detectable inhibition at 50 μM concentration. Assuming conservatively that a rate decrease of 2% would have been readily detected, a lower limit of >400 μM can be estimated for the K_i. Thus there is a difference of at least 3300-fold on going from the N^+ to the O analogue of the 3,4-dichlorobenzyl quaternary. Linear competitive inhibition is most easily explained by direct binding of the inhibitor to the free enzyme active site, in which situation the reciprocal of K_i is the binding constant for the inhibitor, and can be converted to a binding energy using $\Delta G = -RT \ln(1/K_i)$. Thus, for a ratio of K_i values, the change in binding energy for two inhibitors is $\Delta\Delta G = RT\Delta pK_i$. Using the observed K_i value for **6** and the estimate for **21** gives a value at 298 K of 5.6 kcal·mol^{-1} for the loss in binding

Table 1. Inhibition by Quaternary Alkylammonium Chlorpromazines (Chart 1) of *T. cruzi* TR and Human Erythrocyte GR[a]

	TR inhibition		ED_{50} against *T. brucei*	
compd	IC_{50} (μM)	K_i (μM)	μg/mL (95% confidence)	μM
chlorpromazine[b]		10.8 ± 1.1[c]		
chlorpromazine methiodide (**20**)	28 ± 6.3	1.1 (calcd)[c]		
OFK000 (**1**)	8.02 ± 0.56	1.3 ± 0.16		
OFK001 (**2**)	10.8 ± 1.38	1.8 (calcd)	0.205 (0.224)	0.41
OFK002 (**3**)	12.7 ± 1.05	1.7 ± 0.3[d]	0.675 (0.676)	1.41
OFK003 (**14**)	10.5 ± 3.03	2.3 ± 0.3[e]	0.159 (0.213)	0.32
OFK004 (**4**)	6.6 ± 0.5	2.4 ± 0.1	0.245 (0.396)	0.47
OFK005 (**5**)	11.9 ± 1.76	1.5 ± 0.1	0.508 (0.631)	0.90
OFK006 (**6**)	0.78 ± 0.04	0.12 ± 0.01	0.546 (0.734)	1.07
OFK007 (**7**)	17.9 ± 2.25	2.98 (calcd)	0.348 (0.799)	0.65
OFK008 (**15**)	1.2 ±1.20	0.71 ± 0.12	0.035 (0.058)	0.062
OFK009 (**16**)	9.3 ± 1.44	1.55 (calcd)	0.068 (0.112)	0.12
OFK010 (**17**)	12.8 ± 1.4	1.23 ± 0.17		
OFK011 (**8**)	3.9 ± 0.27	0.47 ± 0.1		
OFK012 (**9**)	5.4 ± 1.1	0.77 ± 0.12		
OFK014 (**18**)	2.95 ± 0.67	0.56 ± 0.07	3.11 (4.69)	5.28
OFK018 (**10**)	10.0 ± 2.20	1.67 (calcd)	1.48 (1.89)	2.55
OFK019 (**19**)	8.6 ± 1.47	1.43 (calcd)	0.159 (0.210)	0.28
OFK025 (**12**)	10.4 ± 1.42	0.47 ± 0.01	0.191 (0.221)	0.35
OFK027 (**13**)	4.1 ± 0.1	0.68 ± 0.08	0.023 (0.047)[f]	0.042

[a] K_i values were determined at 25 °C as linear competitive with respect to $(ZCGdmapa)_2$ in 0.02 M HEPES buffer, pH 7.25, 0.15 M KCl, 1 mM EDTA, 0.1 mM NADPH, 0.24, 0.12, 0.06, or 0.03 mM $(ZCGdmapa)_2$ and 3 μg/mL enzyme. To determine IC_{50} values 0.12 mM substrate was used: for compounds indicated by (calcd), K_i values were calculated using $IC_{50} = K_i(1 + [S_o]/K_m)$, with $(ZCGdmapa)_2$, $K_m = 24$ μM, except for chlorpromazine methiodide for which $T[S]_2$ was substrate, $K_m = 49$ μM. For **22** the ED_{50} value was 0.475 μg/mL (0.74 μM), but TR inhibition was not studied for solubility reasons. [b] From literature.[6] [c] No detected inhibition of GR at 1.0 mM. [d] K_i for human GR was 2.20 ± 0.19 mM. [e] K_i for GR was 2.00 ± 0.35 mM. [f] Activity against *T. cruzi* was 66.2% and 59.2% at 3 and 1 μM, respectively, giving an ED_{50} of <1 μM.

energy on switching from the N^+ to an ether O atom. However, the ablation of this charge interaction not only means a diminution in the electrostatic contribution to binding but may also mean that **21** may be unable to orient correctly in the active site to allow optimal hydrophobic interaction, leading to additional loss of binding energy. The quantitative docking studies described below indicated a 10 kcal·mol^{-1} loss of binding energy on going from **21** to **6** in agreement with this interpretation. Although net charge helps in selectivity, and might also provide some orientation in the active site, the present results support the view that hydrophobicity, combined with this particular positive charge, is important for overall improvement of inhibition, possibly by occupying the Z site of the enzyme active site. The hydrophobic binding options of the component regions of the quaternary inhibitors are further analyzed now in terms of log *P* and quantitative docking analysis.

The hydrophobicity (π or log *P* values) did not correlate the TR inhibitory potency of the quaternary chlorpromazines. For simple mono- or disubstituted aromatic nuclei (**1**−**9**, **12**, **13**) the least-squares linear regression correlation coefficient for $\log(1/K_i)$ versus π was 0.550 (intercept 5.80 ± 0.16, slope 0.29 ± 0.15). With **6** omitted as a 7-fold positive outlier, the correlation coefficient was 0.542 (slope 0.19 ± 0.11, intercept 5.81 ± 0.11). The small overall sensitivity of binding strength to π is reasonable, as the π value for the substituent is only a small fraction of the total hydrophobicity of a tricyclic ligand. The electronic nature (σ)

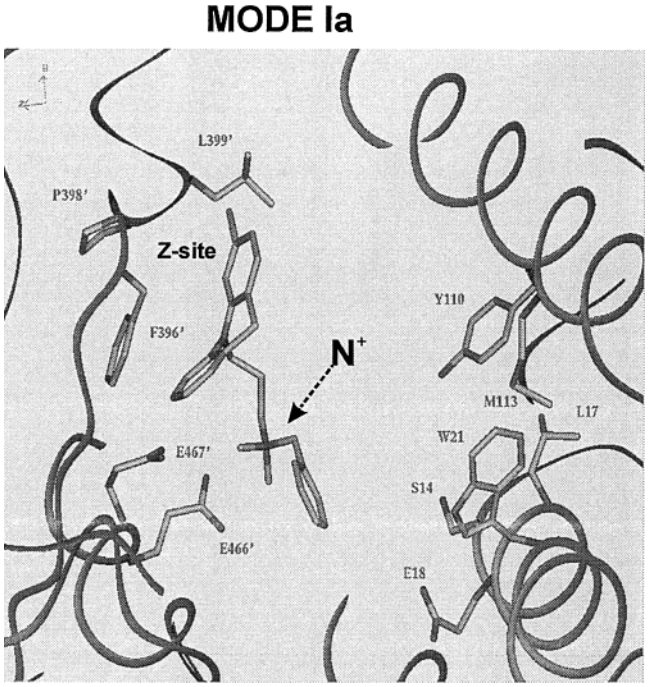


Figure 2. Binding mode Ia for the docked structure of ligand **1**, the benzyl-quaternized derivative of chlorpromazine. There is electrostatic interaction between the quaternary nitrogen atom and the carboxylate side chains of E466′ and E467′, while the tricyclic nucleus occupies the Z site.

of the substituents also showed no significant effect on the sensitivity of binding strength (intercept 6.0 ± 0.14, slope 0.03 ± 0.39, $r = 0.025$ for all data as in the π correlation; if **6** is omitted: intercept 6.0 ± 0.08, slope −0.44 ± 0.25, $r = 0.532$).

Quantitative Docking. Analysis of the results of docking studies shows that although the clustering was poor, two definite general families of preferred binding modes were detected, comparable to each other in binding energy. A group of 23 phenothiazine ligands was studied, 22 quaternary compounds plus chlorpromazine as parent. Studying the top 20 clusters for each ligand showed that for a number of ligands (**2**, **4**, **8**, **11**) approximately one-half of the final docked structures were in either of two conformations (Ia or II) defined below.

Mode Ia: The quaternary nitrogen of the ligand (N^+) interacts electrostatically with E466′ (or sometimes E467′) and the tricyclic moiety resides in the hydrophobic Z site (F396′/P398′/L399′).

Mode Ib: The quaternary nitrogen (N^+) interacts electrostatically with E466′ (or sometimes E467′), with the hydrophobic quaternary aromatic group in the Z site.

Mode Ic: The quaternary nitrogen (N^+) interacts electrostatically with E466′ (or sometimes E467′), with the hydrophobic quaternary aromatic groups apparently not interacting with TR.

Mode II: The quaternary nitrogen (N^+) interacts electrostatically with S14 (usually showing no hydrophobic interactions, but sometimes with the tricyclic or substituted-benzyl group near the hydrophobic region of L17/W21/M113). Modes Ia, Ib, and II are shown in Figures 2−4, respectively, which indicate typical docked structures, together with important side chains and hydrophobic regions of the active site. Compounds **2**, **4**,

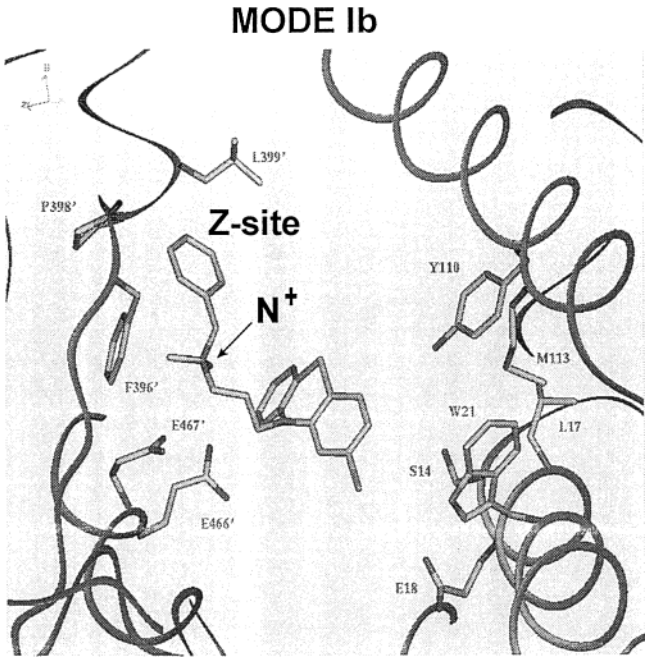


Figure 3. Binding mode Ib for the docked structure of ligand **1**, the benzyl-quaternized derivative of chlorpromazine. There is electrostatic interaction between the quaternary nitrogen atom and the carboxylate side chains of E466′ and E467′, while the hydrophobic benzyl group on the N^+ atom occupies the Z site.

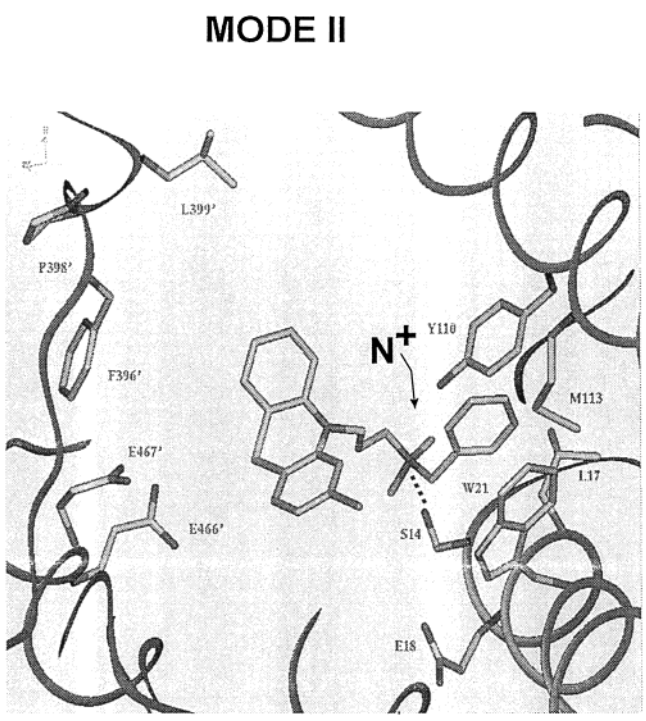


Figure 4. Binding mode II for the docked structure of ligand **1**, the benzyl-quaternized derivative of chlorpromazine. There is electrostatic interaction between the quaternary nitrogen atom and the side chain oxygen atom of S14, with in some cases an interaction between the hydrophobic benzyl group on the N^+ atom and the region L17, W21, and M113.

and **8** have close to equal contributions from modes Ia and II, which are approximately equal in energy, neither being favored. Both modes allow a stabilizing electrostatic interaction of the (N^+) with either a charged acid group (E466′/467′) or a polar hydroxyl group (S14) and both allow the hydrophobic tricyclic moiety or substituted-benzyl group to interact with neighboring hydrophobic amino acid residues. For **3** and **4**, mode II allows a chlorine atom on the benzyl group to form a potential dipolar interaction with E18, if the latter is protonated. For some ligands (e.g. **2**, **8**, **13**) the substituents on the benzyl group appear to make little difference to docking preferences or binding energies. Analysis of these data shows that docking energies and K_i values do not correlate. For many ligands (e.g. **4**−**6**, **8**−**10**, **14**, **17**, **19**, **21**) mode Ib is not detected.

Other ligands do show a preference for one or other mode. Chlorpromazine showed the strongest clustering of all the ligands, with 33 out of the final 100 docked structures in the top cluster alone. This cluster, together with 11 other conformations from separate clusters, had the ligand in mode Ia. One-quarter of this number has the N^+−S14 interaction of mode II.

Of the docked conformations for **14** in the top 20 clusters, almost one-half had the N^+−E466′/467′ interaction. Of these, most had the tricyclic group in the Z site, and only approximately one-third of all the conformations in the final 20 clusters showed the N^+−S14 interaction. For **6** and **7** approximately 3 times as many final conformations were found with the N^+−S14 interaction (mode II) as with N^+−E466′/467′ (mode I). For **6**, in most cases a chlorine atom on the benzyl group interacted with E18 and for **7** some conformations allowed the nitro group to interact with E18. For **16** 3 times as many conformations had N^+ interacting with E465′ as with S14. This preference may be because the E466′ side chain is more exposed to the active site cavity, whereas S14 is less accessible, the large naphthalene substituent sterically hindering binding to S14. With the N^+−E466′ interaction, either the tricyclic or the naphthalene substituent interacts in the Z -pocket, but in the S14 case, there appears to be no equivalent hydrophobic interactions**.** For **11** approximately twice as many of the top 20 clusters resulted in mode Ia/c as were in mode II. Four other ligands showed a complete preference for mode I. For **17** only two runs in the top 20 clusters resulted in N^+ interacting with S14: all others had the N^+−E466′ interaction with many in conformation Ib. A single cluster contained 15 of the final 100 docked structures with the N^+−E466′ interaction, the hydrophobic benzylic group stretching toward the Z site and the tricyclic part toward M113. In neither case are hydrophobic parts of the ligand close enough to the protein side chains for optimum interaction. Many (minor variant) conformations of ligand were also detected in mode Ia. For **15** most of the resulting structures had N^+ interacting with E466′ or E467′, in the most part leaving the tricyclic region to interact in the Z site. Approximately one-sixth had N^+ interacting with E18, but many other conformations did not seem to form any strong electrostatic or hydrophobic interactions and the ligand was sited in the center of the cavity making no apparent interaction. Three ligands (**15**, **17**, **22**) have bulky substituents, which may sterically prevent the N^+−S14 interaction, as S14 is less accessible in the active site than E466′ and E467′.

While it is preferable to use experimental coordinates of a target:inhibitor complex in rational lead design, so far no X-ray data have been reported for TR:phenothi-

azine or TR:imipramine complexes. A structure has been reported for TR complexed to mepacrine, but this showed only low occupancy of the available active sites[9] and kinetic evidence shows that tricyclic inhibitors can bind to TR in more than one micro-orientation.[10,19] Such multiple binding options, with implied rapid dynamics of interchange between orientations, may provide part of the reason for difficulty in obtaining good diffraction data for such systems, perhaps even contributing to the occupancy reported for mepacrine. Our docking studies of mepacrine indicated that of the most favored clusters, one-third, were in mode Ia. Other structures satisfied either the tricyclic region interacting near the Z site, or N^+ with E466′, but not both. None of these docked conformations resembled the X-ray derived site for mepacrine with the tricyclic nucleus near W21/M113. The mepacrine docking studies were carried out with the tricyclic nucleus as neutral, but it is has been pointed out that the tricyclic moiety here may be N-protonated.[9] It is clear from our calculations that mepacrine does not dock to a unique locus and that multiple families also occur for it. Detailed kinetic studies of TR inhibition by acridines indicate that they can inhibit TR by different kinetic models for only rather small structural change and that in the case of 9-aminoacridines more than one inhibitor molecule a can bind to the enzyme.[20]

Thus there is now considerable evidence that multiple binding orientations can occur for tricyclic compounds in general with TR based on kinetic studies[10,20] and on the computational results of the present study. In addition, more than one inhibitor molecule may bind based on kinetics[20] or the photoincorporation of tricyclics intoTR studied by electrospray mass spectrometry.[21]

Antiparasite Activity. The activities against *T. brucei* are given in Table 1. Published data on phenothiazines against leishmanias and trypanosomes indicate that some such compounds can achieve reasonable levels of activity[6] with several compounds having ED_{50} values below 5 μM against the rapidly dividing *T. brucei* bloodstream form trypomastigotes, although the intracellular amastigote stages of both *L. donovani* and *T. cruzi* are less susceptible to phenothiazines in general than *T. brucei*.

The present study provides clear improvements from the quaternized family analogues of phenothiazines against *T. brucei* bloodstream form if chlorpromazine is compared (ED_{50} = 3.7 μM).[6] For the data in Table 1 as a whole, there is no simple, direct correlation of the data sets for K_i values and the ED_{50} values. This is in agreement with structure−activity analyses of antiparasitic activities of the parent nonquaternized tricyclics, which indicated that TR inhibition cannot be the only determining factor for the biological activities of those compounds.[6] The strongest antiparasitic activity against *T. brucei* growth based on ED_{50} values for the parent families of phenothiazines was around 1 μM,[6] and the strongest activity with the presently reported quaternized derivatives of 2-chlorophenothiazine is 40−60 nM, approximately a 20-fold improvement. In terms of actual dose to achieve 50% growth inhibition, this corresponds to ∼30 ng/mL for the quaternaries, as opposed to ∼400 ng/mL for the nonquaternized parents.

Compound **18**, with a coumarinic substitution, is a poor inhibitor of *T. brucei* growth (ED_{50} = 5.28 μM), and comparison with **16**, a naphthyl species with grossly similar steric demands, hints that the rather polar electron distribution in **18** is deleterious to activity. This is consistent with a view that a major determinant is the hydrophobicity of the N-quaternizing group in gross terms. This view is supported by analysis of N-benzylated derivatives with only monosubstitution of the benzene ring (**2**−**5**, **7**, **12**). Apart from the 4-*tert*-butyl (**13**), the mean ED_{50} value is 0.725 ± 0.431, indicating that electronic effects of the substituents are unimportant to anti-parasite activity. In contrast, the 4-*tert*-butyl (**13**) compound has a greatly improved ED_{50} value (23 ng/mL, 42 μM). However, there are very specific factors also operating beyond mere gross hydrophobicity, as the activity of the derivative bearing a pyrenyl group (**22**) is relatively weak compared to **19** (also with a ketomethylene link) or to the naphthyl derivative **16**. Use of the large and hydrophobic benzhydryl substitution leads to a derivative which shows good antiparasitic activity, with an ED_{50} of 35 ng/mL (62 nM).

None of the compounds showed any improvement of potency relative to the chlorpromazine nucleus against *L. donovani*. However, while **15** inhibited *L.donovani* by 20% at 3 μM, its therapeutic window would be small as it showed toxicity toward the macrophages at 10 μM. Compound **13** was the only member of the quaternized series which showed activity against *T. cruzi*, inhibiting by 66.2% at 3 μM and by 59.2% at 1 μM, giving an ED_{50} of <1 μM. This exceptional behavior against *T. cruzi* relative to its analogues is noteworthy.

For a broad range of phenothiazine structures studied no correlation has been found between antiparasitic activity and inhibitory strength versus TR,[6] and this has been proposed to indicate that the biological activity of such materials is reflecting also drug delivery aspects such as cell penetration. The data in this extended structure:in vitro antitrypanosomal activity study for phenothiazines does show that there is considerable sensitivity in biological efficacy to even relatively minor structural alterations. This larger body of data is still consistent with the conclusions of previous studies of tricyclic antidepressant structures and their antitrypanosomal/antileishmanial activities that there appears to be no single structural feature which controls activity.[22,23] However, the effect of structure on biological effectiveness against the parasite can be large, and certainly strong antiparasitic action can be achieved.

Permanently charged phenothiazines have altered biological activities in general compared to the parent structures but are often less active against the dopamine receptor.[24,25] It is known chlorpromazine is more potent than methyl-quaternized chlorpromazine in inducing depressant effects on the spontaneous activity of mice, the forced motor activity of rats, the continuous shock avoidance of rats, and the potentiation of hexobarbital sleeping time in mice. Neither compound significantly altered the threshold of convulsive seizures induced by either pentylenetetrazol or strychnine. Grossly both produce a sedative profile in doses ranging from 1−25 mg/kg, and the LD_{50} for chlorpromazine is much higher than for the quaternary chlorpromazine. From the standpoint of the CNS activity of the quaternary compounds, the ED_{50} and doses producing minimal depression are close to the lethal dose range, so that

the theoretical therapeutic index is much smaller than that of chlorpromazine. Thus, quaternization of the chlorpromazine molecule by methylation of the side chain terminal nitrogen decreases CNS activity but increases toxicity.[26] Such factors will have to be borne in mind in developing the quaternaries as potential antiparasitics, and alternative parent structures may be useful from which to build on the new binding features for TR exposed in the present study.

Conclusions

Binding strength of the tricyclic phenothiazine lead for TR has been improved, with no loss of specificity over host GR, by enlisting a second[6] hydrophobic pocket (the Z site near F396′) in addition to the original design-target[8] hydrophobic wall (around W21, also detected in the X-ray structure of TR-bound mepacrine)[9] and vectoring the inhibitor's interaction by means of a third, electrostatic site (E266′ and E267′). This three-point inhibitor attachment concept has allowed up to 30-fold improvement of the K_i values against TR and is the inhibitor analogue of the "three-point attachment" explanation of Ogston to explain chiral specificity of enzymes.[27] Clearly the "three-point attachment" approach has value also in inhibitor design. Some of the compounds developed here show strong antiparasite activity, and analysis of the antiparasite data demonstrates that features in the molecules in addition to anti-TR-directing aspects were also important in obtaining activity as drug leads.

Experimental Section

Chlorpromazine hydrochloride was purchased from Aldrich Chemical Co., as were 4-methylbenzyl bromide, 4-chlorobenzyl chloride, cyclohexylmethyl bromide, 3-chlorobenzyl bromide, 4-bromobenzyl bromide, 3,4-dichlorobenzyl chloride, 4-nitrobenzyl bromide, bromodiphenylmethane, 2-bromomethylnaphthalene, and 4-(bromomethyl)-7-methoxycoumarin. Lancaster Synthesis supplied 2,3,4,5,6-pentafluorobenzyl bromide and 4-chloro-3-methylphenacyl bromide. Trypanothione disulfide was from Bachem, GSSG and NADPH were from Boehringer, and *N*-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide disulfide was synthesized.[12] Syntheses were monitored by thin-layer chromatography on plastic sheets precoated to 0.2 mm with aluminum oxide (N/UV_{254}) (Merck, Darmstadt). The TLC solvent mixture used was chloroform:ethanol:acetic acid (65:32:3). Spots were made visible by UV illumination or by means of iodine vapor. Melting points were determined on a Reichert (Austria) melting point apparatus supplied by Shandon Scientific Co. Ltd., London.

NMR spectra were recorded on a JEOL JNM EX 270 spectrometer operating at 270 MHz for ^{1}H NMR. Chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si (0.00 ppm) as internal reference. Data are reported using the following convention: chemical shift (integrated intensity, splitting patterns, assignment). Splitting patterns are abbreviated as: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, unresolved multiplet.

Fast-atom bombardment mass spectra (FAB-MS) were taken by means of a Kratos-Concept instrument operating in the FAB mode (Xe beam bombardment) using *m*-nitrobenzyl alcohol (Aldrich Chemical Co.) as a matrix in the Department of Chemistry, University of Manchester. Elemental analyses were recorded on an EA 1108 elemental analyzer (Carlo Erba Instruments) in the Department of Chemistry, University of Manchester.

Enzyme Isolation, Assay, and Inhibition. TR from *T. cruzi* was isolated by means of overexpression of the gene in *E. coli* JM109 cells bearing the expression vector PBSTNAV[28] as previously described.[8] Enzyme activity was assayed at 25 °C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM TSST and 0.1 mM NADPH[4] at an enzyme concentration of approximately 0.3 $\mu g \cdot mL^{-1}$. Human GR was isolated from an overexpression system for GR in *E. coli* SG5 cells[29] and assayed following literature conditions[30] in 0.1 M potassium phosphate buffer, pH 7.00, containing 0.2 M KCl and 1 mM EDTA in the presence of 1 mM GSSG and 0.1 mM NADPH using approximately 15 μg/mL of GR per assay. Enzyme rate assays were conducted spectrophotometrically at 340 nm using a Peltier thermostated cuvette holder maintained at 30.0 °C in a Cary 1E UV−vis spectrophotometer and using the Cary Enzyme Kinetics Software.

Inhibition kinetics were determined using $(ZCGdmapa)_2$ as substrate.[12] Values of IC_{50}, the concentration required to give 50% inhibition under the assay conditions described above, were determined by interpolation from a plot of assay velocity versus inhibitor concentration, fitting to the equation corresponding to linear competitive inhibition by nonlinear regression analysis written into Grafit. Inhibition type was assessed by analyzing the patterns of three diagnostic classes of plot: $1/v_o$ versus **1**/[S_o] for various [**1**]; $1/v_o$ versus [**1**] for various [S_o]; [S_o]/v_o versus [**1**] at various [S_o]. Values of K_i for competitive inhibition were determined by direct weighted ($1/v_o^2$ for weighting) least-squares nonlinear regression analysis of the raw data using the equation for linear competitive inhibition ($v = V_{max}[S_o]/([S_o] + K_m(1 + [\mathbf{1}]/K_i))$) using the Grafit program (Erithacus Software, distributed by Sigma Chemical Co.). Values of V_{max} and K_m were obtained by least-squares nonlinear regression analysis using Grafit.

Synthesis. The Menschutkin direct alkylation was used to quaternize the tertiary amine group of the side chain of chlorpromazine.[31] A typical synthetic procedure was as follows. Aqueous chlorpromazine hydrochloride (0.5 g, 1.407 mmol in 10 mL H_2O) was adjusted to pH > 10 by adding solid potassium carbonate. The oily precipitate, free chlorpromazine base, was extracted with chloroform (5 × 15 mL), the organic extracts were combined and dried over anhydrous sodium sulfate, and solvent was removed under reduced pressure to yield ~0.45 g of chlorpromazine free base as an oily residue. This was dissolved in acetone (5 mL), an equivalent of alkyl halide in acetone (5 mL) added and the mixture stirred overnight (about 18 h) at room temperature. The product precipitated (in some cases induced by adding ether), was collected by filtration, dried and recrystallized from acetone−ether (yields were typically 80−85%). All quaternary compounds were characterized by melting points, TLC R_f values, ^{1}H NMR spectra and FAB-MS. The physical constants of the compounds are presented in Appendix Table 1 and their ^{1}H NMR spectra in Appendix Table 2 (see Supporting Information).

The 3,4-dichlorobenzylchlorpromazine ether analogue (**21**) of **6** was synthesized from chlorpromazine by N-acylation with 3-bromopropionyl chloride to give 2-chloro-*N*-(3-bromopropionyl)amidophenothiazine, followed by reduction of the amide with BH_3/THF, to give 2-chloro-*N*-(3-bromopropyl)aminophenothiazine, which was used to alkylate 3,4-dichlorobenzyl alcohol, forming **21**.

2-Chloro-*N*-(3-bromopropionyl)amidophenothiazine. 2-Chlorophenothiazine (2.25 g, 10 mmol) and 3-bromopropionyl chloride (1.77 g, 10 mmol) in dry chloroform (6 mL) were refluxed for until the evolution of HCl gas ceased (40 min.), filtered and dried in vacuo. The residue was crystallized from ethanol to yield the desired product (3.3 g, yield 90%): TLC (Et_2O:petroleum ether 40−60, 1:1, v/v) R_f 0.45; mp 113−115 °C; *m*/*z* (FAB-MS) 367, 368 (M + 1), 369 (M + 2); ^{1}H NMR ($CDCl_3$) δ_H 7.6−7.2 (7 H, m, Ph), 3.6 (2 H, m, CH_2−Br), 3.0 (2 H, m, CH_2−CO). Anal. Calcd for $C_{15}H_{11}NOBrClS$: C, 48.9; H, 3.0; N, 3.8. Found: C, 49.1; H, 3.0; N, 4.0.

2-Chloro-*N*-(3-bromopropyl)aminophenothiazine. To a cooled solution under N_2 of 2-chloro-*N*-(3-bromopropionyl)-amidophenothiazine (2.5 g, 7 mmol) in anhydrous THF (7 mL) was added dropwise over 1 h commercial 1 M BH_3/THF solution (60 mL, 60 mmol). The mixture was stirred for 30 min,

allowed to react for 2 h at room temperature, then refluxed for 2 h. Excess BH_3 was neutralized by addition of methanol at 0 °C and the solution treated with a mixture of CH_2Cl_2 and brine. The organic layer was separated and evaporated to dryness to yield the desired product (2.4 g, 99%) as a colorless gummy material, which was used without further purification: TLC (Et_2O:petroleum ether 40−60, 1:1, v/v) R_f 0.7; *m/z* (ES-MS) 353; ^{1}H NMR ($CDCl_3$) δ_H 7.2−6.85 (7 H, m, Ph), 4.05 (2 H, t, CH_2−N), 3.5 (2 H, t, CH_2−Br), 2.3 (2 H, m, −CH_2−).

3,4-Dichlorobenzyl Ether Analogue of Chlorpromazine (21). To a solution of 2-chloro-*N*-(3-bromopropyl)-aminophenothiazine (1.27 g, 5 mmol) in toluene (25 mL) was added KOH (2.8 g, 50 mmol) and 3,4-dichlorobenzyl alcohol (0.885 g, 5 mmol) and the mixture refluxed for 40 h. The reaction mixture was then filtered, the filtrate evaporated to dryness, the residue dissolved in a small amount of ether and flash-chromatographed on a silica gel column using Et_2O:40−60 petroleum ether (1:9) to give the crude product as an oily material after evaporating appropriate fractions (detected by TLC). The product **21** was dissolved in methanol and crystallized as a colorless solid: mp 71.5−72 °C; TLC (Et_2O:40−60 petroleum ether, 2:8, v/v) R_f 0.37; *m/z* (ES-MS) 449; ^{1}H NMR ($CDCl_3$) δ_H 7.3−6.85 (10 H, m, Ph), 4.4 (2 H, s, CH_2−Bz), 4.0 (2 H, t, CH_2−N), 3.6 (2 H, t, CH_2−O), 2.05 (2 H, m, −CH_2−). Anal. Calcd for $C_{22}H_{18}NOCl_3S$: C, 58.6; H, 4.0; N, 3.1. Found: C, 58.8, H, 3.9; N, 3.3.

Molecular Modeling and Quantitative Docking. Potential inhibitors were designed to bind via specific interactions: hydrogen-bond charge−charge, and hydrophobic. The published *C. fasciculata*[32−34] and *T. cruzi*[35] X-ray diffraction coordinates were obtained from the Brookhaven database. Molecular modeling was carried out using Sybyl 6.4.2 software[36] and an R10000 or R4000 Silicon Graphics machine.

Docking searches, performed on a Silicon Graphics Origin 2000, took 12−76 h CPU time for each ligand using the program AUTODOCK 2.4,[37] which uses a Metropolis Monte Carlo simulated-annealing for positional and configurational exploration with grids of molecular affinity potentials[38] based on the AMBER force field.[39] This approach of energy calculation allows the inclusion of terms for the van der Waals, electrostatic, and hydrogen-bonding interactions. The X-ray structure of TR complexed with mepacrine[9] was used for docking studies. Mepacrine and all water molecules were removed, charged amines and carboxyl groups capped and essential hydrogens added using the Kollman united-atom library. The X-ray structure was very briefly relaxed (25 steps Simplex) to remove any bad geometry and short contacts present, but not to allow any heavy atoms to move significantly from the calculated crystal structure positions. All ligands to be docked (Table 1) were built in SYBYL 6.4.2[36] with a formal charge of +1 added to the quaternary nitrogen atom. Partial atom-centered charges were calculated using the semiempirical PM3 method implemented in MOPAC,[40] and after an initial Simplex optimization, ligands were minimized to convergence (Powell method, gradient 0.05 kcal/mol). To check the validity of this minimization technique, five phenothiazine stuctures in the Cambridge Crystallographic Database were minimized (SYBYL) as described; rms differences between all atoms measured varied only between 0.68 and 1.44 Å, justifying the method.

The number of rotatable bonds of the ligands, defined in Table 1, varies from 4 to 9; nonpolar hydrogens were united automatically using AUTOTORS. Using AUTOGRID a grid (29.625 Å × 29.625 Å × 30.375 Å, with grid spacing 0.375 Å) was centered on the active site for each test case, with 40, 40, and 41 grid points in each Cartesian direction (80 × 80 × 82 points total per grid). Appropriate Lennard−Jones 12-10 parameters for H atoms bonded to O, N, or S in the ligand, and to O or S in the protein, were specified in the AUTOGRID parameter file (N atoms of TR were all assumed to participate in backbone amide bonds, making their lone pairs unavailable for hydrogen bonds).

As 50 docking runs of AUTODOCK performed for each ligand gave practically no clustering, 100 docking runs were carried out per docking. At the beginning of each simulated annealing run, the ligand was positioned randomly within the grid and movement began with a random translation, random rigid body rotation, and random torsions. Each run consisted of 100 annealing cycles, a cycle terminating if the ligand made either 30 000 sequentially accepted or rejected moves. The annealing temperature (310 K in the first cycle) was reduced linearly at the end of each cycle by 0.95-fold. The minimum energy state of the current cycle was used to start the next cycle. All cycles had a maximum translational step size of 0.2 Å and maximum torsional rotation of 5°/step.

Following docking, cluster analysis of all structures generated for a single compound was performed. Once sorted by total energy of interaction, the structures were sequentially assigned to cluster families, represented by top-of-cluster structures. Structures not satisfying a tolerance of 1.5 Å in the rms deviation for all atoms to each top-of-cluster structure found defined new clusters. Ligand internal energy results were referenced to the initial undocked energy, calculated using AMBER. The calculated differences between the undocked and docked energies were used for analyses.

Parasites. *Leishmania donovani* (strain MHOM/ET/67/L82) was maintained routinely in special pathogen-free (SPF) female Golden hamsters (Wright's strain, Charles Rivers Ltd.) by passage every 6−8 weeks.

Trypanosoma cruzi (strain MHOM/BR/OO/Y) trypomastigotes were derived from cultures of MDCK fibroblasts maintained in Dulbecco's modified Eagle medium (Life Technologies Ltd., Paisley, Scotland) with 10% heat-inactivated fetal calf serum (HIFCS) (Harlan Sera-Lab., Crawley, U.K.) at 37 °C in an atmosphere containing a 5% CO_2−air mixture.

Trypanosoma brucei brucei (strain S427) bloodstream form trypomastigotes were maintained in HMI-18 medium[41] supplemented with 20% HIFCS at 37 °C in a 5% CO_2−air mixture.

In Vitro Assays. *L. donovani* and *T. cruzi*: Peritoneal macrophages were harvested from female CD1 mice (Charles River Ltd., Margate U.K.) by peritoneal lavage 24 h after induction by soluble starch (Merk Ltd., Leics, U.K.). After two washes in medium the exudate cells were dispensed into 16-well Lab-tek tissue culture slides (Nunc Inc., IL) at 4×10^4/well in a volume of 200 μL of RPMI-1640 medium (Sigma-Aldrich Company Ltd., Dorset, U.K.) plus 10% HIFCS. After 24 h, macrophages were infected at a ratio of 10:1 (4×10^5/well) with *L. donovani* amastigotes freshly isolated from hamster spleen, or at a ratio of 5:1 (2×10^5/well) with *T. cruzi* trypomastigotes derived from the MDCK fibroblast overlay. Infected macrophages were then maintained in the presence of drug in a 3-fold dilution series, with quadruplicate cultures at each concentration, for 5 days for *L. donovani* cultures and 3 days for *T. cruzi* cultures. After these periods of drug exposure slides were fixed by methanol and Giemsa stained. Drug activity was determined by counting the percentage of macrophages cleared of amastigotes in treated cultures in comparison to untreated cultures.[42] ED_{50} values were determined by linear regression analysis. Sodium stibogluconate ($NaSb^v$) (Glaxo-Wellcome, Dartford, U.K.) and nifurtimox (Bayer, U.K.) were used as the respective control drugs.

***T. brucei* brucei:** Tests were performed in HMI-18 medium as above.[41] Compounds were tested in triplicate in a 3-fold dilution series from the top concentration. Parasites were diluted to 2×10^5/mL and added in equal volumes to the test compounds in 96-well, flat bottom Microtest III tissue culture plates (Becton Dickinson and Co., NJ) with pentamidine isethionate (Rhone-Poulenc-Rorer) as the positive control set up in parallel. Plates were maintained for 3 days at 37 °C in a 5% CO_2−air mixture. Compound activity was determined by the use of Alamar Blue colorimetric assay[43] on day 3. ED_{50} values were determined by linear regression analysis.

Acknowledgment. We are grateful to Professors Alan Fairlamb and Heiner Schirmer for generous gifts of the overproducing clones for TR and human GR, respectively, for the support of the Wellcome Trust (D.M.) and Association of Commonwealth Universities (M.O.F.K.) and for financial support from the UNDP/

World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Supporting Information Available: Appendix Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM000156+