Articles

Phenothiazine Inhibitors of Trypanothione Reductase as Potential Antitrypanosomal and Antileishmanial Drugs†

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Given the role of trypanothione in the redox defenses of pathogenic trypanosomal and leishmanial parasites, in contrast to glutathione for their mammalian hosts, selective inhibitors of trypanothione reductase are potential drug leads against trypanosomiasis and leishmaniasis. In the present study, the rational drug design approach was used to discover tricyclic neuroleptic molecular frameworks as lead structures for the development of inhibitors, selective for trypanothione reductase over host glutathione reductase. From a homology-modeled structure for trypanothione reductase, replaced in the later stages of the study by the X-ray coordinates for the enzyme from *Crithidia fasciculata*, a series of inhibitors based on phenothiazine was designed. These were shown to be reversible inhibitors of trypanothione reductase from *Trypanosoma cruzi*, linearly competitive with trypanothione as substrate and noncompetitive with NADPH, consistent with ping-pong bi bi kinetics. Analogues, synthesized to define structure−activity relationships for the active site, included *N*-acylpromazines, 2-substituted phenothiazines, and trisubstituted promazines. Analysis of K_i and I_{50} data, on the basis of calculated log *P* and molar refractivity values, provided evidence of a specially favored fit of small 2-substituents (especially 2-chloro and 2-trifluoromethyl), with a remote hydrophobic patch on the enzyme accessible for larger, hydrophobic 2-substituents. There was also evidence of an additional hydrophobic enzymic region available to suitable N-substituents of the promazine nucleus. K_i data also indicated that the phenothiazine nucleus can adopt more than one inhibitory orientation in its binding site. Selected compounds were tested for *in vitro* activity against *Trypanosoma brucei*, *T. cruzi*, and *Leishmania donovani*, with selective activities in the micromolar range being determined for a number of them.

Introduction

Trypanosomiasis and leishmaniasis are major third-world diseases, with many millions of new infections presenting annually. Present chemotherapies are inadequate, toxic, or both with current drugs including the arsenicals, nifurtimox, and pentamidine.[1] The trivalent arsenical drug melarsoprol is still widely used against the second stage of African trypanosomiasis (sleeping sickness) in which the parasitic pathogen (*Trypanosoma brucei rhodesiense* or *T. brucei gambiense*) has invaded the central nervous system, a stage refractory to the drugs suramin and pentamidine.[1] As an arsenical it is no surprise that melarsoprol is toxic, the drug itself leading to the deaths of 4−8% of patients treated with it.[1] Nifurtimox and benznidazole are the two major drugs available for Chagas' disease prevalent in South America (caused by *T. cruzi*), and there is continuing debate about their effectiveness and safety. Against *Leishmania* species the main drugs in use are the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) and, as well as toxicity problems, resistance is now reported to these. Pentamidine has been used to treat this family of diseases but lengthy courses are required and toxicity is a problem.[2] Thus, approaches to develop new drugs are sought in many laboratories. A metabolic difference between the pathogen and the mammalian host recently discovered [3] may provide a means of developing a selective antiparasitic drug. Moreover, it may even prove possible to combat all three diseases, African Trypanosomiasis, Chagas' disease, and leishmaniasis, with a single agent.

Glutathione is responsible for many cellular protection activities including those against free radicals and oxygen-derived species. In the course of this action glutathione disulfide (Figure 1) is formed from glu-

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† Abbreviations: GR, glutathione reductase; GSSG, glutathione disulfide; GSH, reduced glutathione; I_{50}, concentration of inhibitor required to reduce velocity by 50% under conditions defined in text or table legend; K_i, inhibition constant; log *P*, logarithm to base 10 of the partition coefficient for octanol−water (calculated as described); $T[S]_2$, trypanothione disulfide; $T[SH]_2$, dihydrotrypanothione; TR, trypanothione reductase.

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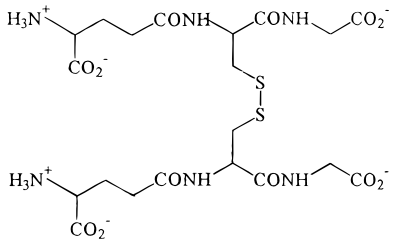
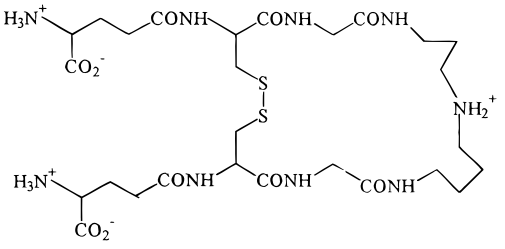


GLUTATHIONE DISULPHIDE(GSSG)



TRYPANOTHIONE DIULPHIDE(TSST)

Figure 1. Structures of glutathione disulfide and trypanothione in its disulfide form.

tathione reductase (GR). Trypanosomes do not contain GR but rather an analogous enzyme, trypanothione reductase (TR), and its substrate is trypanothione disulfide, $T[S]_2$ (Figure 1).[3,4] Parasitic TR differs from host GR in processing $T[S]_2$ but not glutathione disulfide (GSSG). Conversely, host GR does not process $T[S]_2$.[5,6] This mutual substrate exclusivity indicated that selective ligand design should be possible, making TR an important potential target for drug design against parasitic diseases involving trypanosomes and/or leishmanias.[3,6,7] Effective inhibition of TR would compromise the parasites' redox defenses, making them more sensitive to redox-damage drugs, such as nifurtimox. For example, a trypanothione reductase inhibitor might be an effective antitrypanosomal and/or antileishmanial drug in its own right or coadministration with a redox-active drug such as nifurtimox might allow use of lowered doses of the latter. Consequently, we undertook the development of novel drug leads by a priori design of enzyme inhibitors, selective for TR over human GR. Our preliminary results provided strong, selective inhibitors based on either tricyclic heterocyclic nuclei[8] or peptides,[9] neither family being obviously related to the structure of the substrate for TR. We now report in more detail on one family of tricyclic TR inhibitor, the phenothiazines. A further impetus to developing such a group of TR inhibitors was the literature reports that several tricyclic neuroleptics, including the phenothiazines, possess weak, but established, antitrypanosomal and antileishmanial activities.[10−20]

Approach

Our initial approach to inhibitor design was guided by a computer model of TR, constructed by modification of GR using homology modeling,[8] judged successful based initially on its power to rationalize substrate selectivities of GR and TR and to predict selective inhibitors.[8] The crystal structures of TR from *Crithidia fasciculata*[21−23] and recently *T. cruzi*,[24,25] including the TR:mepacrine complex,[26] have been solved. Comparing modeled and experimental coordinates showed a close

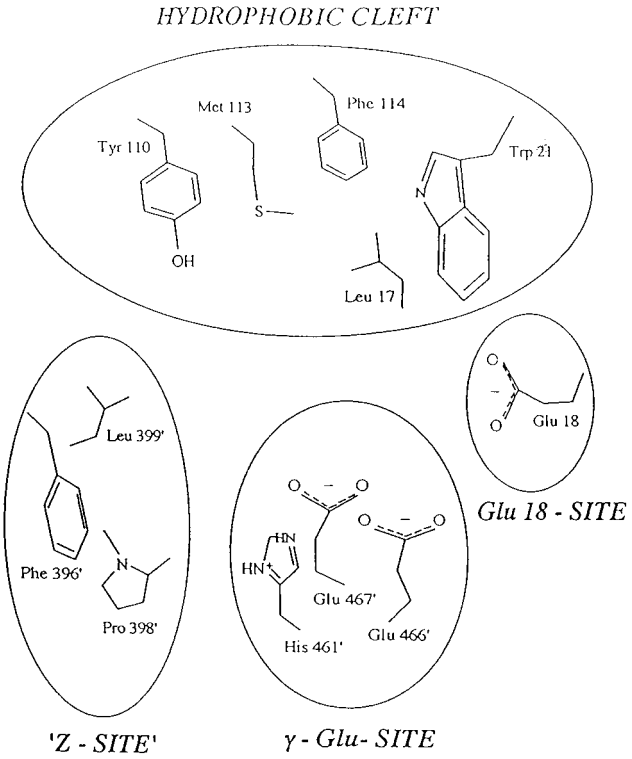


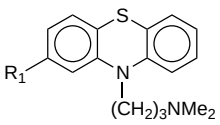
Figure 2. Schematic summary of regions of the active-site of trypanothione reductase used in the design of the phenothiazine-based inhibitors (not to scale).

correspondence between the two structures, especially for the core and active site: two regions of dissimilarity were found in surface loops, not in the active site. The active sites of human GR and TR differ in the hydrophobic pocket of the latter[22] which complements the spermidine portion of the substrate (Figure 2). In GR this region has polar, positively charged side chains to neutralize the carboxylate ion sites of GSSG. In addition, there is a second hydrophobic pocket (not used by trypanothione as substrate), discovered for TR when *N,N*-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-(dimethylamino)propylamide disulfide was found to be a good alternative substrate.[27] This second hydrophobic pocket (called the Z-site), tentatively located from molecular graphics analysis of the region of F396 (Figure 2), was used to help design a TR-specific peptide inhibitor.[9] Peptides make poor leads as drugs, and we needed to develop alternative lead structures. Initially we docked bicyclic (naphthalene) and tricyclic (anthracene) aromatic anchors in the spermidine hydrophobic pocket formed by F114, W21, etc., but these were soon redeveloped to resemble well-known tricyclic neuroleptics, the imipramines and the phenothiazine family.[8] In view of their easier synthetic access compared to that of the imipramines, the phenothiazines were chosen to probe structure−activity effects on TR inhibition and for testing against the parasites.

Results

We have already shown[8] that with the disulfide substrate ($T[S]_2$ or *N,N′*-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-(dimethylamino)propylamide disulfide[27]) as variable substrate, trifluoperazine is a linear, competitive inhibitor with a K_i value of $21.9 \pm 1.7\ \mu$M. This pattern of inhibition was confirmed by detailed analysis

Table 1. Inhibition by 2-Substituted Promazines of Recombinant *T. cruzi* Trypanothione Reductase (Approximately 0.3 $\mu g \cdot mL^{-1}$) in 0.02 M HEPES Buffer Containing 0.15 M KCl, 1 mM EDTA, 0.1 mM NADPH, and 0.12 mM Trypanothione Disulfide at 25 °C[a]



no.	R_1	I_{50} (μM)	reference to material
1	H	*108 ± 14	Sigma-Aldrich
2	CF_3	*110 ± 18	Sigma-Aldrich
3	Cl	*35.4 ± 7.7	Sigma-Aldrich
4	$COCH_3$	502 ± 20	*b*
5	$CCH_3{=}NOCH_3$	351 ± 95	Scheme 1
6	$COCH_2CH_3$	357 ± 30	*b*
7	$CO(CH_2)_2CH_3$	301 ± 86	*b*
8	$CCH_3{=}NOBz$	204 ± 16	Scheme 1
9	$CO(CH_2)_4CH_3$	171 ± 50	Scheme 1
10	$CH_2OC_6H_4$-*p*-$C(CH_3)_3$	*161 ± 29	Scheme 1
11	COOH	*3911 ± 306	Scheme 2
12	CH_2OH	*1187 ± 221	*c*
13	$CONH_2$	*458 ± 96	*d*
14	$COOCH_3$	*348 ± 62	*d*
15	CH_2NH_2	219 ± 13	*d*
16	CH=NOH	145 ± 12	Scheme 2

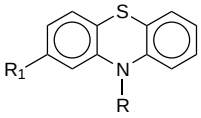
[a] Compounds marked with an asterisk in the I_{50} column were shown to not inhibit human erythrocyte glutathione reductase. K_i values, where determined, were of a linear competitive type. For **1** the K_i value was 59.1 ± 5.9 μM (coefficient of variation 7.9%), for **2**, 30.2 ± 2.6 μM (coefficient of variation 8.5%), and for **3**, 10.8 ± 1.1 μM (coefficient of variation 10.2%). [b] Schnitt,J. ; Boitard, J.; Comoy, P.; Hallot, A.; Suquet, M. *Bull. Soc. Chim. Fr.* **1957**, 938; *Chem. Abstr.* **1962**, *56*, 478. Rhone Poulenc, FR 70, 804 addition to FR 1173133. [c] *Chem. Abstr.* **1960**, *54*, 2372i. Rhone Poulenc GB 816538, 1959. [d] *Chem. Abstr.* **1964**, *60*, 5515b. Cusic, J. W.; Lowrie, H. S. (G. D. Searle & Co.) U.S. Pat. 3112310, 1962.

for **1**, **2, 3**, **22, 23, 24, 25**, and **41** (data not shown, but K_i values are given in Table 2). As TR is a two-substrate enzyme, with NADPH as the second substrate, we also determined the patterns of inhibition for NADPH as the variable substrate, with the results shown in Figure 3a−c (Supporting Information). The pattern of inhibition of TR by trifluoperazine from the diagnostic set of plots in Figure 3 is uncompetitive against NADPH as variable substrate.[28] The value of K'_i determined using the uncompetitive model was 31 μM.

Values of I_{50} are recorded in Table 1 for a range of different 2-substituents. In Table 2 values of I_{50} (and in some cases K_i) for the effects of multiple substitution of the tricyclic nucleus are collected for both ω-(*N*,*N*-dimethylamino)propyl and acetyl substitutions on the tricyclic bridging nitrogen. Table 3 collects inhibition data for the effect of altering the nature of the tricyclic bridging nitrogen for a series with fixed 2-substituents in the tricyclic nucleus. Compounds indicated by asterisks in Tables 1−3 gave no detectable inhibition of human glutathione reductase up to concentrations of 1 mM (0.1 mM for a few compounds of limited solubility).

In Figure 4 (Supporting Information) $\log(1/I_{50})$ is plotted against log *P* values calculated for the 2-substituents in Table 1. Figure 5 (Supporting Information) shows a similar plot of the inhibition data for molecular refractivity values calculated for these 2-substituents. There was no significant correlation of $\log(1/I_{50})$ with log *P* or the molar refractivities for the substituents in

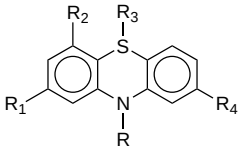
Table 2. Inhibition by N-Substituted Phenothiazines, 2-Chlorophenothiazines, and 2-(Trifluoromethyl)phenothiazines of *T. cruzi* Trypanothione Reductase under the Conditions used in Table 1[a]



no.	substitution pattern	I_{50} (μM)	reference to material
	R_1 = H, R=		
17	H	NI at 1000	Sigma-Aldrich
18	$(CH_2)_3NH_2$	*1412 ± 305	*b*
19	$CH_2C(CH_3)_2N(CH_3)_2$	*1176 ± 319	*c*
20	$(CH_2)_3NHCH_3$	*1028 ± 186	*d*
21	$(CH_2)_2C(NH_2){=}NH$	992 ± 112	*b*
22	$CH_2CHCH_3N(CH_3)_2$	*271 ± 48	Sigma-Aldrich
23	$CH_2CHCH_3CH_2N(CH_3)_2$	*249 ± 70	Sigma-Aldrich
	$R_1 = CF_3$, R =		
24	$(CH_2)_3N$ ⌐ $N(CH_2)_2OH$	*73.7 ± 16.7	Sigma-Aldrich
25	$(CH_2)_3N$ ⌐ NCH_3	*72.0 ± 13.9	Sigma-Aldrich
	R_1 = Cl, R =		
26	$CO(CH_2)_2COOH$	1484 ± 340	Scheme 3
27	$COCH_2NEt_2$	829 ± 219	*e*
28	$COCH_2NHPh$	256 ± 88	*e*
29	$CO(CH_2)_3NEt_2$	320 ± 79	*f*
30	$COCH_2Cl$	862 ± 186	*g*
31	$CO(CH_2)_5NEt_2$	457 ± 57	*h*
32	$CO(CH_2)_2NEt_2$	802 ± 328	*i*
33	$COCH_2Br$	251 ± 80	*j*
34	$CO(CH_2)_2CONHNHPh$	151 ± 53	Scheme 3
35	$CO(CH_2)_2CONHCH_2Ph$	188 ± 24	Scheme 3
36	$CO(CH_2)_2CH_3$	95	Scheme 3
37	$(CH_2)_2NMe_2$	*150 ± 11	*k*
38	$(CH_2)_2C(NH_2){=}NH$	307 ± 65	*l*
39	$(CH_2)_3NH_2$	*139 ± 7	*m*
40	$(CH_2)_3NHCH_3$	*126 ± 4	*n*
41	$(CH_2)_3N{=}N(CH_2)_2CH_3$	*95.5 ± 25.5	*o*
42	$(CH_2)_3N$	*80.8 ± 13.2	*d*
43	$(CH_2)_2NEt_2$	*81.3 ± 15.6	*p*

[a] NI = no inhibition. K_i values were of the linear competitive type for the following compounds: **22**, K_i = 216 ± 33 μM (coefficient of variation 15.1%); **23**, K_i = 127 ± 11 μM (coefficient of variation 8.6%); **24**, K_i = 21.2 ± 3.3 μM (coefficient of variation 15.6%); **25**, K_i = 23.0 ± 1.8 μM (coefficient of variation 7.9%); **41**, K_i = 18.7 ± 2.1 (coefficient of variation 11.2%). [b] Godefroi, E. F.; Wittle, E. L. *J. Org. Chem.* **1956**, *21*, 1163. Rhone-Poulenc, Patent DE 1012915, 1953. [c] G. D. Searle & Co. U.S. Pat. 2590125, 1947; U.S. Pat. 2687414, 1948. [d] *Chem. Abstr.* **1964**, *60*, 5515b. G. D. Searle & Co., U.S. Pat. 3112310, 1962. [e] *Chem. Abstr.* **1957**, *51*, 501a. Gailliot, P.; Robert, J. Rhone Poulenc U.S. Pat. 2738399, 1956. [f] Yale, H. L. *J. Am. Chem. Soc.* **1955**, *77*, 2270. [g] *Chem. Abstr.* **1990**, *113*, 59071. El-Ezbawy, S. R.; Alshaikh, M. A. *J. Chem. Technol. Biotechnol.* **1990**, *47*, 209−18; *Chem. Abstr.* **1967**, *67*, 90823. G. D. Searle & Co. U.S. Pat. 332046, 670516, 650118. [h] *Chem. Abstr.* **1961**, *55*, 6484c. Gritsenko, A. N.; Zhuralev, S. V.; Skorodumov, V. A. *Uch. Zap. Inst. Farmakol. Khimioter. Akad. Med. Nauk. USSR* **1958**, No.1, 13-26. [i] Kano,H.; Makisumi, Y. *Shionogi Kenkyusho Nempo* **1957**, *7*, 511−516; *Chem. Abstr.* **1958**, *52*, 10094. Shurawlew, S. V.; Grizenko, A. N. *Zh. Obshch. Khim.* **1956**, *26*, 3385; *Engl. Trans.* 3769. [j] *Chem. Abstr.* **1968**, *68*, 49530. Yarmukhametova, D. Kh.; Kudryavstev, B. V.; Buzlama, V. S. *Izv. Akad. Nauk. SSSR, Ser. Khim.* **1967**, *7*, 1506. [k] *Chem. Abstr.* **1996**, *64*, 11220b. Rhone-Poulenc U.S. Pat. 2645640, 1951; BASF DE 1011887, 1955; SK and F NL 6505736, 1969. [l] *Chem. Abstr.* **1963**, *59*, 11518h. Yoshitomi Pharm. Pat. JP 6783, 1958. [m] Sawizkaya, N. V.; Czizin, Y. S.; Stchvkina, M. N. *Zh. Obshch. Khim.* **1956**, *26*, 2900−2905. Engl. Transl. S 3227. [n] Rhone-Poulenc U.S. Pat. 2830987, 1956. [o] Rhone-Poulenc GB Pat. 780193, 1955. [p] Morisawa, K.; Terai, Y.; Kawahara, S.; Ichikawa, K.; Oka, Y. *Yakugaku Kenkyu* **1957**, *29*, 161−3; *Chem. Abstr.* **1959**, *53*, 538145.

Table 3. Inhibition by Polysubstituted Phenothiazines of *T. cruzi* Trypanothione Reductase under Conditions Used in Table 1



no.	R_1	R_2	R_3	R_4	R	I_{50} (μM)	reference to material
44	H	Cl		H	$(CH_2)_3NMe_2$	*765 ± 57	*a*
45	$COCH_3$	H		$COCH_3$	$(CH_2)_3NMe_2$	472 ± 156	C, H, N by HRMS, *f*
46	Cl	H		$COCH_3$	$(CH_2)_3NMe_2$	175 ± 60	C, H, N by HRMS, *f*
47	Cl	H		CCH_3=NOBz	$(CH_2)_3NMe_2$	199 ± 78	C, H, N by HRMS, *f*
48	Cl	H	O	H	$(CH_2)_3NMe_2$	*76.6 ± 63	chlorpromazine sulfoxide
49	$COCH_2Cl$	H		H	$COCH_3$	436 ± 37	*b*
50	$COCH_3$	H		H	$COCH_3$	*2811 ± 855	*c*
51	Cl	H		$COCH_3$	$COCH_3$	402 ± 40	*d*
52	$COCH_3$	H		$COCH_3$	$COCH_3$	49.1 ± 8.7	*e*

[a] *Chem. Abstr.* **1955**, *49*, 3268i. Rhone Poulenc U.S. Pat. 2645640. [b] Mousseron, M.; Kamenka, J. M.; Legendre, P.; Stenger, A. *Chim. Ther.* **1996**, *7*, 397−402. [c] Zupancic, B. G. *Kem. Ind.* **1976**, *25* (4), 223−8; *Chem. Abstr.* **1977**, *86*, 55367. [d] *Chem. Abstr.* **1991**, *115*, 232258. Merck U.S. Pat. 5036067. [e] *Chem. Abstr.* **1986**, *104*, 15081. Merck Frosst Eur. Pat. EP 306639.

Table 4. Sensitivity of *T. brucei* Bloodstream Form Trypomastigotes and *L. donovani* Amastigotes and *T. cruzi* Trypomastigotes in Mouse Peritoneal Macrophages to Phenothiazines

	ED_{50} (μM)[a]		
compound (code)	*T. brucei*	*L. donovani*	*T. cruzi*
2 (triflupromazine)	3.2		
3 (chlorpromazine)	3.69	4.90	>10
4 (77009/CC64)	6.42	T	T
5 (CC111)	10−30	>30	>30
6 (CC62)	10.1	>30	>30
7 (CC70)	3.26	10−30	>30
8 (CC112)	1.56	16.5	>30
9 (CC87)	>30		
10 (RJ 15)	>30	>30	>30
15 (79009/CC201)	0.37	T	T
16 (75009/CC196)	7.35	>30	10−30
44 (BA 21752)	3.01	48.4	29.5
45 (CC35)	10−30	>30	>30
46 (CC124)	1.29	12.1	>30
47 (CC126)	3.5	6.78	>30
51 (CC80)	>30	>30	>30
52 (CC22)	>30	>30	>30
33 (CC127)	0.97	5.97	10−30
34 (CC104)	15.2	34.3	T
35 (CC100)	>30	>30	>30
36 (CC90)	>30	>30	>30
18 (BA 18971)	3.83	3.30	54.9
24 (fluphenazine)	>30	>30	>30
39 (GP 38889)	<3, >1	10	5.9

[a] T indicates toxic to the macrophages.

the 2-position if the whole data set were included. For the compounds referred to in region II of Figure 5 (Supporting Information), the data were correlated by the following equation:

$$\log(1/I_{50}) = 2.89 + 0.02\text{MR} \qquad (r = 0.865, F = 17.86)$$

The activities of a number of the compounds in Tables 1−3 against *T. brucei*, *L. donovani*, and *T. cruzi* are given in Table 4.

Discussion

The reversible linear competitive inhibition kinetics observed with this family of structures is consistent with the design process which located the lead nucleus in the hydrophobic pocket of the active site formed by residues M113, A343, and W21.[8] The pattern of inhibition of linear competitive versus $T[S]_2$ and uncompetitive versus NADPH is consistent[29] with a ping-pong bi bi mechanism for TR, a model which has been suggested from initial velocity substrate plots for $T[S]_2$[30−32] and the NADPH-analogue inhibitor 2′,3′-cyclic NADPH.[31] Relative to compounds **1**, **2**, and **3** (with H, CF_3, or C1 in the 2-position, respectively), almost any substitution weakens binding. We return to this point later. Replacing a 2-acetyl group **4** progressively by 2-propionyl **6**, 2-butanoyl **7**, and 2-(*p*-*tert*-butylphenoxy)methyl **10** substituents increases the inhibition strength steadily with **10** binding approximately 3 times as tightly as **4**. The simplest explanation of this is that there is a hydrophobic site available to interact with such 2-substituents. This is not evident merely by inspecting **1**, **2**, and **3** where the major changes are polarity and, to a lesser extent, steric effects. The slightly better inhibition by **8** compared to **5** may also indicate a hydrophobic region, which can be used selectively by 2-substituents.

Figure 4 (Supporting Information) shows that if clear outliers (CO_2H, CH_2OH, and Cl) are omitted, there is a weak trend to increased inhibitory strength for higher values of *P*, but I_{50} decreases only 1.2-fold for a 5-fold change in log *P* (based on the correlation equation for the data in Figure 4). The outliers in Figure 4 can be further examined using the molar refractivity plot of Figure 5 (Supporting Information). (Essentially similar plots are obtained for surface area, volume, polarizability, etc. for the 2-substituent, but as these and the molar refractivity are not independent parameters; the latter has been chosen simply to illustrate the point.) Two major regions are evident in Figure 5. For molar refractivities of less than approximately 15 Å^3, the data are collected into a box labeled region I. Although there is a very small increase in inhibition strength as the molar refractivity increases, the value of $\log(1/I_{50})$ is almost independent of molar refractivity. For molar refractivities greater than approximately 15 Å^3 (box labeled region II). The major distinction between substituents in regions I and II is size (easily determined if the size of the longest axis of the lowest energy conformation of the 2-substituents involved is plotted (data not shown) instead of molar refractivity). For

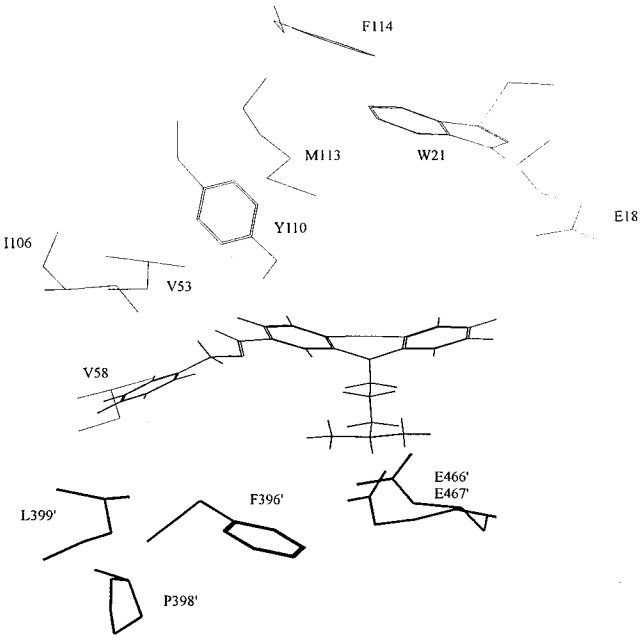


Figure 6. View of the active site of trypanothione reductase showing 2-(*N*-(benzyloxy)methylimino)promazine (**8**) with its benzyloxymo function located in the general region of the Z-site (around F396′, V58, and V53) and showing a retained interaction of the ω-ammonio function of the tricyclic nitrogen atom's side chain with the Glu pincers.

substituents in region I (H, Cl, CF_3, CH=NOH, CH_2NH_2, CO_2Me, $CONH_2$, $COCH_3$, CH_2OH, and CO_2H), inhibitory strength varies by almost 2 orders of magnitude despite the small differences in substituent size. The very small change in inhibitory power (region II) for a considerable variation in substituent (and for a wide range of chemical functionalities) means that larger substituents can be easily accommodated. The most straightforward explanation of this is that these substituents point toward bulk solution. The small but perceptible increase in inhibition with molar refractivity (size, Figure 5, Supporting Information) and log *P* (Figure 4, Supporting Information) for these substituents indicates that some relatively nonspecific interactions are possible with a surface hydrophobic patch of TR for these larger groups. For smaller 2-substituents of region II (Figure 5), there is an accessible hydrophobic region, defined in general terms by M113 and Y110. For larger substituents of region II (e.g. $PhCH_2ON{=}C$), modeling indicates that if the tricyclic aromatic nucleus is sited in the general region of F114/W21[8] then the 2-substituent can access the Z-site (see Figure 2), but this necessitates a compromise with poorer contact available to the tricyclic nucleus (see Figure 6).

For the smaller substituents in region I some very specific interactions clearly take place. The stronger inhibition by the free 2-oxime (compared to **5**) indicates that a bonding feature additional to hydrophobic is available to appropriate 2-substituents. One possibility considered early was the use of E18′ to bind part of the ligand (see Figure 2). To try to test this we introduced a range of substituents. Data in Table 1 are consistent with E18′ involvement of the inhibitors. For example, if the 2-carboxylic acid (**11**) binds as its CO_2^- form, its poor inhibition (I_{50} = 3911 μM and 8-fold weaker than the amide, **13**) would be explicable if E18′ were also ionized to CO_2^- at pH 7.25. This is in accord also with the stronger inhibition by the 2-CH_2NH_2 (**15**) than the 2-CH_2OH (**12**) substituent. If the aminomethyl substituent had bound in its protonated form (reasonable as the pK_a of $RCH_2NH_3^+$ is approximately 10 in free solution), a stronger interaction would have been expected if E18′ were in the CO_2^- form. Other groups with potential to hydrogen bond with E18′ (e.g. **16**, **5**), as donors and as acceptors, also failed to give the increase in binding strength expected of such interactions. Thus the 2-substituents may be directed to the face of the hydrophobic pocket away from E18′ (i.e. toward the Met-113) or not large enough to access E18′. The strength of binding of 2-chloro, and to a lesser extent 2-CF_3, relative to the other small 2-substituents indicates that a particularly favorable interaction/molecular fit is possible for them. This interaction may be provided, at least in part, by the side chain of M113. We discuss later the X-ray data for the TR−mepacrine complex.[26]

The bridging nitrogen can tolerate many substituents (Table 2). However, within this broad statement are covered several separate influences on binding for detailed substitution differences. Relative to the ω-(*N*,*N*-dimethylamino)propyl group (**1**, **2**, and **3**), almost any change is deleterious to inhibition (compare **1** with **17**−**23** or **3** with **38**−**43**). A tertiary amine is important as inhibition weakens by 1 order of magnitude on replacing an ω-NMe_2 group (as in **1** or **3**) by $NHCH_3$ (as in **20** or **40**) or NH_2 (as in **18** and **39**). One substitution which maintains inhibitory strength is the introduction of a substituted piperazine ring (**24** and **25** each bind approximately 1.5-fold more strongly than **2**, based on K_i values). The piperazine is a specially favored case as replacement of the terminal dimethylamino group of **3** by a piperidine (**42**) weakens inhibition. It is possible that for **24** and **25** the extension of the distal nitrogen (further from the tricyclic nucleus) by CH_2CH_2OH and CH_3, respectively, enables a remote hydrophobic region to be accessed. Going from *N*-alkyl to *N*-acyl substitution of the nuclear nitrogen adversely affects inhibition. Thus, amide **27** is almost 1 order of magnitude poorer than its alkylamino counterpart, **43**. The terminal amino compound **32** inhibits 6-fold more strongly than the terminal carboxylic species **26**. The terminal methyl derivative **36** is better still, and its alkyl tail may reach the Z-site (see Figure 2) avoiding the Glu pincers, with which the $(CH_2)_3NR_2$ groups were originally designed[8] to interact in their protonated form. For either binding mode a terminal CO_2^- group would be disfavored. Despite inhibition by **30** and **33** (potential alkylating agents toward TR), there was no evidence of irreversible inhibition of TR by **30** even on extended incubation at concentrations up to millimolar. Thus, there can be no nucleophilic side chains in the immediate vicinity of the reactive α-ketoalkyl site in **30** and **33**, consistent with the design-hypothesis model (Figure 2). The only potentially nucleophilic residues being serines, but with no obvious activation mechanism such as the charge-relay system of the serine proteases.

There are indications that the phenothiazine nucleus can take up more than one orientation in the active-site hydrophobic pocket which involves W21 and M113. Thus, changing the substituent on the bridging tricyclic nitrogen atom from $(CH_2)_3NMe_2$ to $(CH_2)_3N(C_2H_4)_2N^nPr$ weakens binding slightly (2-fold) for a 2-chloro

nucleus (compare K_i values of **3** (10.8 μM) and **41** (18.7 μM)) but strengthens it slightly (by 30%) for 2-trifluoromethyl (compare **2** (30.2 μM) with **25** (23.0 μM)). This type of substitution effect on binding strength ($1/K_i$) is readily accommodated if the precise alignments of the two families (Cl and CF_3) of inhibitors in the active site do not correspond exactly.

In an attempt to see if the strong binding properties of the 2-chloro group could be combined with access to a hydrophobic region near Tyr-110, derivative (**47**) (Table 3) was prepared. With only a 2-Cl group in the phenothiazine nucleus (**3**), the I_{50} value was 35 μM, and with only a 2-*O*-benzyloximo function (**8**) it was 204 μM. Combining these functions (**47**) resulted in an I_{50} value of 199 μM. Clearly, the favored 2-chloro type of interaction is not possible in the presence of a bulky 8-*O*-benzyloximo group. If the 2-Cl group "directs" binding (e.g. by interaction with M113), then modeling indicates that the 2-benzyloximo group is unable to optimally interact with the hydrophobic patch formed by residues including F396′, L399′, Y110. The large TR active site allows inhibitors as small as the currently studied tricyclics considerable scope. An alternative location for one of the tricyclics (**8**) is shown in Figure 6, which also shows how far from the original docking site used for lead generation[8] such a molecule can be located, at least by molecular modeling, and still provide a theoretical rationale for binding. In the X-ray structure to 2.9Å resolution for TR from *T. cruzi* with mepacrine, an acridine-based inhibitor,[26] of the four TR active sites per crystal asymmetric unit only one showed electron density ascribable to the mepacrine inhibitor. The inhibitor lies in the active site, its tricyclic nucleus in the region around M113 and W21, and the chloro group stabilized by interaction with the indolyl NH. The acridine ring nitrogen atom (N10) may be protonated.[26] The alkylamino side chain of mepacrine is extended toward E18′ and not in the same sense as modeled in our original design process.[8] The phenothiazine ring system (unprotonated at neutral pH) may bind with a different orientation to the acridine nucleus in the large hydrophobic cavity of the active site considering the kinetic evidence above of more than one family of binding location even for inhibitors all sharing the phenothiazine nucleus. The opportunity for the tricyclics to adopt more than one bound position within the general confines of the large TR active site may contribute to a low level of X-ray occupancy for mepacrine.

Supplementing a 2-acetyl group already in place (**4**) by introducing a second acetyl moiety at the 8-position (**45**) makes little difference to the inhibitory strength if the substituent on the heterocyclic nitrogen atom is ω-(*N*,*N*-dimethylamino)propyl. However, derivatives with an acetyl group on the heterocyclic nitrogen atom behave sufficiently differently to indicate membership of a different class of inhibitors with respect to the details of their active-site positioning. In the promazine family (*N*-$(CH_2)_3NMe_2$) with an unsubstituted 8-position, introduction of 2-Cl (**3** cf. **2**) improved inhibition by 3-fold and a 2-$COCH_3$ group (**4**) weakened inhibition by 4.6-fold. However, with an *N*-$COCH_3$ group and an 8-$COCH_3$ both present, substitution of the 2-hydrogen (of **50**) by 2-Cl (**51**) improved inhibition 7-fold and a 2-$COCH_3$ group (**52**) increased inhibitory strength still further (57-fold relative to **50**), giving an I_{50} of 49 μM for the triacetyl compound **52**.

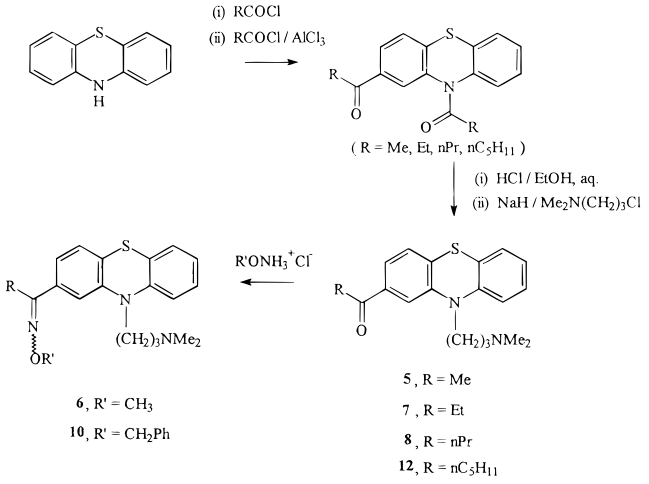
In summary, the binding of phenothiazine tricyclic inhibitors of TR provides evidence of multiple modes of inhibitor acceptance. In addition, the effective area of the hydrophobic pocket available to inhibitors is not limited to the hydrophobic wall formed by M113, F114, and W21 used to recognize the spermidinyl sector of the TR substrate[23] and made use of in the original inhibitor design paper.[8] This region of the active site discriminates $T[S]_2$ and GSSG[23] and is contiguous with another hydrophobic region (e.g. F296′, P398′, L399′, V58, V53), not used to recognize the natural substrate, but which may bind the present tricyclics, some peptide inhibitors[9] and a number of alternative substrates.[27,33]

T. brucei bloodstream form trypomastigotes showed the highest in vitro sensitivity to the phenothiazines with several compounds having ED_{50} values below 5 μM, **8**, **15**, and **33** being most notable. These rapidly dividing extracellular culture forms have a doubling time of around 6 h. In comparison pentamidine, the positive control drug used, has an ED_{50} value of ~0.01 μM. Pentamidine (a polyamine) is not a structural analogue of the tricyclic family under study but is the drug currently most effective against *T. brucei* and used as a test of relative efficacy of new compounds. The intracellular amastigote stages of both *L. donovani* and *T. cruzi* had lower sensitivity to the phenothiazines than *T. brucei*, with *T. cruzi* being the least sensitive. Both of these parasites, grown in murine peritoneal macrophages, have a slower rate of division than extracellular forms. Interpretation of these assays is more complex as at higher concentrations the compounds killed both parasites and host cells (T). Chlorpromazines **3**, **47**, and **33** showed interesting selective activity against *L. donovani* with ED_{50} values of <10 μM. In the same model the ED_{50} value of the standard drug sodium stibogluconate is around 5 μg Sb^V/mL. Only **39** showed any promising activity against *T. cruzi* with an ED_{50} value close to that of the standard drug nifurtimox (around 3 μM). There was no correlation between ED_{50} values for in vitro antiparasitic activities from Table 4 and TR inhibitory strength, even using a log−log plot. Thus if the antiparasitic action of these compunds arises from TR inhibition, molecular features other than TR binding are dominant, e.g parasitic cellular penetration, metabolism. It has been concluded from previous data for antitrypanosomal/antileishmanial activities of tricyclic compounds that no one structural feature controls activity.[11,19] While the data in this study are consistent with such a view, the range of activities (Table 4) is large for any given parasite, and strong antiparasitic action can certainly be achieved (**15** improves on **3** by 1 order of magnitude against *T. brucei*). Thus structural optimization of these leads against parasites, while still to be acheived, is a reasonable goal.

Experimental Section

Promazine, trifluoperazine, and chlorpromazine were purchased from Sigma Chemical Co., Poole, Dorset. Derivatives **4**, **8**, **17**−**20,** and **37**−**44** were gifts from Ciba-Geigy, Basel (additional quantities of **4** were synthesized as described below), and **48** from Dr. D. Attwood. Other derivatives were synthesized as described below or as in the original literature given at the appropriate entry in the tables of kinetic data.

Scheme 1



Purity was established by TLC in three solvent systems, and compounds were characterized by high-resolution mass spectrometry and NMR spectroscopy.

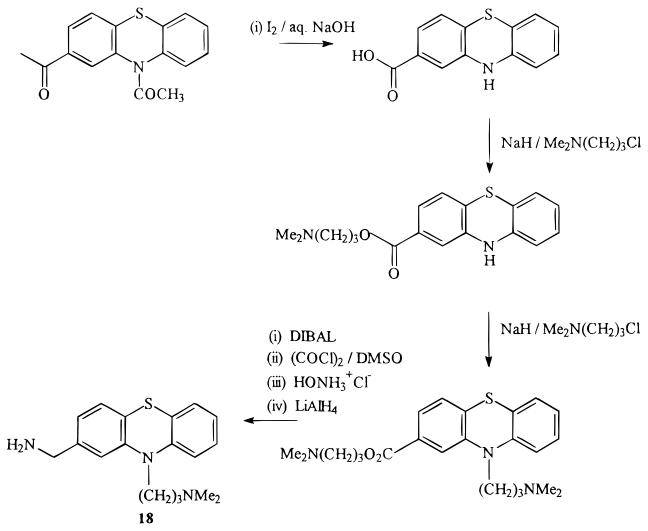
Enzyme Isolation, Assay, and Inhibition Studies. Trypanothione reductase from *T. cruzi* was isolated by means of overexpression of the gene in *Escherichia coli* JM109 cells bearing the expression vection pBSTNAV[34] as previously described.[8] The enzyme was homogeneous by SDS−PAGE and had a specific activity identical with that of wild-type TR.[5] 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 T[S]$_2$, and 0.1 mM NADPH[5] at an enzyme concentration of approximately 0.3 μg·mL^{-1}. Human erythrocyte GR was isolated from human erythrocytes as described[35] and assayed following literature conditions.[36] Assays were conducted spectrophotometrically at 340 nm using a Peltier-thermostated cuvette holder in a Cary 1E UV−visible spectrophotometer and the Cary Enzyme Kinetics Software or using a Perkin-Elmer Lambda 3B instrument thermostated to ±0.02 °C by means of water circulated from a Haake GH Model D8 pump-thermostating system.

Inhibition type was assessed by analyzing the patterns of three diagnostic classes of plot: $1/v_o$ versus $1/[S_o]$ for various [I]; $1/v_o$ versus [I] for various $[S_o]$; and $[S_o]/v_o$ versus [I] at various $[S_o]$. Values of K_i for competitive inhibition were then 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 ($v = V_{max}[S_o]/([S_o] + K_m(1 + [I]/K_i))$) using the Grafit program (Erithacus Software). Values of V_{max} and K_m were obtained by least-squares nonlinear regression analysis using Grafit. Values of I_{50}, the concentration of inhibitor required to give 50% inhibition under the assay conditions described above, were determined by nonlinear least-squares fitting of assay velocity versus inhibitor concentration using the equation derived from linear competitive inhibition. Reversibility of enzyme inhibition was assessed using dialysis at 4 °C overnight against assay buffer (with two changes).

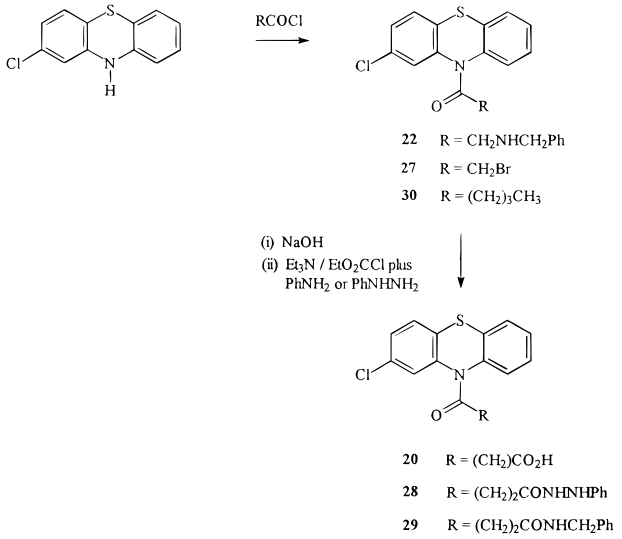
Inhibitor design used a molecular graphics homology model of *T. congolese* TR constructed in this laboratory by modification of the published coordinates of human erythrocyte GR.[37] Toward the end of the project, the published *C. fasciculata*[21−23] X-ray diffraction coordinates were used to replace the modeled coordinates and rationalize the results obtained, although now the *T. cruzi* coordinates are on the Brookhaven database.

Synthesis. 2-Acylpromazines were prepared (Scheme 1) by standard acylation of phenothiazine with acyl chlorides under reflux (2 h) in toluene, followed by Friedel−Crafts acylation ($AlCl_3$/anhydrous CS_2, reflux for 6 h) of the *N*-acyl compounds under literature conditions.[38,39] N-Deacylation followed by alkylation of the 2-acylphenothiazines with sodium hydride and 3-(dimethylamino)propyl chloride afforded the desired substituted-promazines (**4**, **6**, **7**, **9**). Oximes of 2-acetylpromazine (**5**, **8**) were prepared under standard conditions using the appropriate alkoxyamine hydrochlorides in pyridine. 2-(Aminomethyl)promazine was synthesized by Scheme 2. *N*,2-Diacetylphenothiazine was converted to 2-carboxyphenothiazine[38] and alkylated with 1 equiv of sodium hydride and 3-(dimethylamino)propyl chloride to give the corresponding ester. Reduction of the ester function using diisobutylaluminum hydride gave 2-(hydroxymethyl)promazine (**12**). Oxidation of the primary alcohol using Swern's conditions yielded the aldehyde, which was converted to the oxime (**18**). Lithium aluminum hydride reduction of this oxime afforded the desired 2-aminomethyl compound (**17**). 2,8-Diacetylphenothiazine[39] and 2-acetyl-8-chlorophenothiazine (similarly prepared) were N-alkylated to the corresponding promazines (**45**, **46**) and the oxime prepared from the 2-acetyl compound.

Scheme 2



Scheme 3



N-Acyl-2-chlorophenothiazines were prepared (Scheme 3) by acylation of the appropriate 2-chlorophenothiazine. *N*-(3-Carbomethoxypropionyl)-2-chlorophenothiazine was de-esterified using sodium hydroxide and the resulting carboxylic acid (**26**) converted to the amide (**13**) and hydrazide (**34**) by the ethyl chloroformate method.

Molecular Modeling. The design of inhibitors was guided by visualization of particular inhibitor target structures with the active site of TR using QUANTA 4.1 and CHARMm 4.2 (Molecular Simulations Inc., Burlington, MA, 1995). Calculations of log *P* (not determined experimentally) and other molecular parameters were carried out using the ChemPlus

module of Hyperchem Version 4.0 (Hypercube Inc., Waterloo, Ont., Canada, 1995),[40] which uses an additive approach based on atomic fragment contributions to the overall molecular property. Partial least squares analyses were performed using the QSAR facility of SYBYL 6.2 (Tripos Inc., St. Louis, MO, 1995).

Biological Testing: *in Vitro* Assays. *Trypanosoma brucei* brucei (strain S427). Bloodstream form trypomastigotes, cultured in HMI-9 medium,[41] were diluted to 10^5 cells/mL and aliquots (100 μL) dispensed into wells in a 96-well microtiter plate. Test compounds diluted in HMI-9 medium were added in another 100 μL volume to give a final drug concentration. Each drug concentration was set up in triplicate in a 3-fold dilution series, and cultures were incubated at 37 °C in 5% CO_2/air mixture. After 48 h cells were counted in a ZM Coulter Counter with a 70 μm aperture or by Neubauer haemocytometer. ED_{50} values were calculated by linear regression analysis from dose−percent inhibition curves.

***Leishmania donovani* (MHOM/ET/69/L82).** Mouse peritoneal macrophages were isolated and maintained in Labtek 8 chamber tissue culture slides in RPMI 1640 medium with 10% heat-inactivated foetal calf serum at 37 °C in a 5% CO_2 mixture. *L. donovani* amastigotes were isolated from spleens of infected hamsters were used to infect cultures. Twenty-four hours after infection, aliquots of fresh medium containing test compounds were added in 3-fold dilution series in quadruplicate and maintained for 5 days following previously described methods.[42] After 5 days slides were methanol-fixed and Giemsa-stained, and the proportion of infected macrophages was determined in each culture. ED_{50} values were calculated by linear regression analysis.

***Trypanosoma cruzi* (MHOM/BR/00/Y).** Trypomastigotes, derived from infected cultures of L6 rat myoblast cells, were used to infect mouse peritoneal macrophages at a ratio of 5 parasites:1 macrophage and incubated at 37 °C in 5% CO_2/air mixture. Twenty-four hours after infection, cultures were exposed to fresh medium containing test compounds. Each compound was tested in a 3-fold dilution series in quadruplicate. After 3 days, cultures were fixed, stained, and analyzed as described above for *L. donovani*.

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Supporting Information Available: Figures 3−5 (4 pages). Ordering information is given on any current masthead page.

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