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2-Amino diphenylsulfides as new inhibitors of trypanothione reductase

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Abstract

Trypanothione reductase (TR) is the primary enzyme responsible for the reduction of trypanothione, the analog of glutathione found in trypanosomatidae. We have discovered a series of diphenylsulfides which are potent inhibitors of TR and have no activity on mammalian glutathione reductase. These compounds are also active in vitro on various stages of the parasite. Although structurally related to phenothiazines, which are known to be TR inhibitors, these compounds are devoided of any neuroleptic activity, making them attractive leads to develop specific and non toxic anti-chagasic drugs.

Keywords: *Trypanosoma cruzi*; Trypanosomatidae; Trypanothione reductase; 2-Amino-diphenylsulfide; Enzyme inhibitors

1. Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a major health problem in South and Central America. Actually, two nitroheterocycles: nifurtimox (3-methyl-4-(5′-nitrofurfurylideneamino) tetahydro-4H-1,4thiazine-1,1-dioxyde) and benznidazole (N-benzyl-2-nitroimidazole acetamide) are used to cure the disease. They eliminate acute phase symptoms, shortening the course of infection but their efficiency in the treatment of chronic patients is controversed. Due to their lack of specificity, both derivatives are responsible for serious side-effects, a major drawback since a large number of doses must be administrated for long periods of time [1–6]. The need for more rationally designed molecules has led to the search for significant distinctions between the metabolic pathways of the host and its parasite [7]. A major breakthrough occurred with the discovery by Fairlamb et al. that the mammalian redox defense system based on glutathione and the associated enzyme, glutathione reductase (GR) is replaced in trypanosomes by an analogous system in which glutathione is replaced by a spermidine conjugate named trypanothione (N^1,N^8-bis(glutathionyl) spermidine, $T(S)_2$ which is maintained in its reduced dithiol form, $T(SH)_2$, by trypanothione reductase (TR) [8]. Despite 41% homology in their primary structure, human GR and *T. cruzi* TR show almost total mutual discrimination toward their respective substrates. Therefore it seems possible to design selective TR inhibitors likely to compromise the natural defenses of the parasite [9]. As part of a random screening effort to identify novel compounds capable of inhibiting TR [10], we have discovered a series of 2-amino-diphenylsulfide derivatives which are specific inhibitors of TR and devoided of any activity on GR. These compounds are active in vitro against epimastigote, trypomastigote and amastigote forms of *T. cruzi*.

2. Materials and methods

2.1. Materials

Biochemicals and their sources were: glutathione (Janssen); NADPH (Bohringer); trypanothione (Bachem); bovine intestinal mucosa glutathione reduc-

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tase (Sigma); recombinant trypanothione reductase was obtained from the SG5 *E. coli* strain with the overproducing expression vector pIBIT cz TR (gift from Dr. C. Walsh and K. Nadeau, Harvard Medical School) and was purified as previously described [11]. Chemicals were purchased from Janssen. Melting points were determined on a melting point Büchi apparatus and were uncorrected. All reactions were monitored by thin layer chromatography (acetone/NH_4OH 28%, 9/1) or (CH_2Cl_2/CH_3OH, 9/1) carried out on 0.2 mm E. Merck silica gel plates (60F-254) using UV light as visualizing agent and 10% ninhydrin in acetone or Reindel Hoppe (R.H.) as developing agents. Purity of final compounds was checked by HPLC (Nucleosil cyanopropyl) before preparing oxalate salts. ^1H spectra were obtained using a Brucker 300 MHz spectrometer; mass spectra were recorded on a time of flight plasma desorption spectrometer using a Californium source. Microanalyses for all compounds (or their oxalate salts) were obtained from CNRS (France); they were indicated by the symbols of the elements and were always within 0.4% of theoretical values.

2.2. Chemistry

The different steps are presented in Fig. 1.

General procedure for the preparation of 4 or 4'-chloro-2-nitro-diphenylsulfides. To a solution of the appropriate thiophenol (0.1 mole) in absolute ethanol (20 mL), at 0°C, were slowly added some lumps of sodium (2.3 g, 0.1 mole). After dissolution of the sodium, a solution of the appropriate 2-nitro-chlorobenzene (0.1 mole) in absolute ethanol (80 mL) was added and the mixture was refluxed for 30 min. NaCl was then eliminated by filtration and the nitro derivative was precipitated by addition of water. It was recrystallized from ethanol (150 mL), yield: 85%.

General procedure for the preparation of 2-amino-4 or 4'-chloro-diphenylsulfides. In the presence of Raney nickel, at 80°C, for 5 h the nitro derivative was reduced by hydrogen under pressure (100 bars). The corresponding amino derivative was recrystallized from petroleum ether, yield: 75%.

General procedure for the preparation of 4 or 4'-chloro-2-(3-chloro)acetyl or propionylamido diphenylsulfides. To an ice-cooled solution of 2-amino-4 or 4'-chlorodiphenylsulfide (2.5 g, 10.6 mmol) in ether (44 mL) and pyridine (1.7 mL) was added dropwise either chloroacetyl or chloropionyl chloride (12.7 mmol). The reaction mixture was allowed to reach room temperature and was successively washed with aqueous NaCl (saturated solution), 1 M HCl and 2.5 M NaOH. The organic solution was then separated, dried and evaporated to yield quantitatively the corresponding chloro acetyl or propionylamides.

General procedure for the substitution of 4 or 4'-chloro-2-(3-chloro)acetyl or propionylamido diphenylsulfides by secondary amines. A solution of the chloro acetyl or propionylamide derivative (8.5 mmol), triethylamine (8.5 mmol) and secondary amine (dimethylamine, pyrrolidine or N-methyl piperazine, 9.4 mmol) in benzene (40 mL) was refluxed for 16 h. The organic phase was washed with water then 1 M HCl. The aqueous phase was then alcalinized and extracted with ether. The ethered phase was dried and evaporated to dryness to yield quantitatively the corresponding amines.

*General procedure for the reduction of the amidodiphenylsulfides into amines: compounds **1a–c, 2a–c** and **3a–c**.* To a solution of dimethylamino, pyrrolidino or piperazinoamide (75 mmol) in THF (10 mL), was slowly added $LiAlH_4$ (375 mmol) and the mixture was refluxed for 2 h. After cooling, water was added dropwise to eliminate the excess of $LiAlH_4$. The organic phase was dried and evaporated to dryness. Amines **1a–c**, **2a–c** and **3 a–c** were isolated asoxalate salts according to the following procedure: to a saturated solution of amine in ethyl acetate was added dropwise a saturated solution of oxalic acid in ethyl acetate and the mixture was kept at 4°C for 3 h. Oxalates were isolated by filtration and successively washed with ice cold ethyl acetate and ether. All data concerning purity of the tested compounds are given in Table 1.

2.3. Assays of TR and GR activity

TR activity was measured at 28°C in a 0.02 M Hepes buffer, pH 7.25 containing 0.15 M KCl, 1 mM EDTA and 0.2 mM NADPH with an enzyme concentration of 0.02 U ml^{-1}. The reaction was started by addition of the enzyme and the absorbance decrease was followed at 340 nm. Kinetic constants were determined either according to Lineweaver-Burk with four different inhibitor concentrations or in Dixon plots with four different substrate concentrations.

Inhibitory potencies of the compounds were also tested toward bovine intestinal mucosa GR in the same conditions as TR assays.

2.4. Neuroleptic activity

Fresh rat striatum was homogenized with an ultraturax homogenizer in 40 volumes of ice-cold buffer I (50 mM Tris-HCl, pH 7.7 at 20°C) for 10 s, three times successively. Homogenate was centrifuged at 35 000 *g* and 4°C for 10 min (Centrifuge L8-55M Beckman Rotor 50T). Pellet was resuspended two times in the same buffer and centrifuged at 35 000 *g* for 10 min. The final

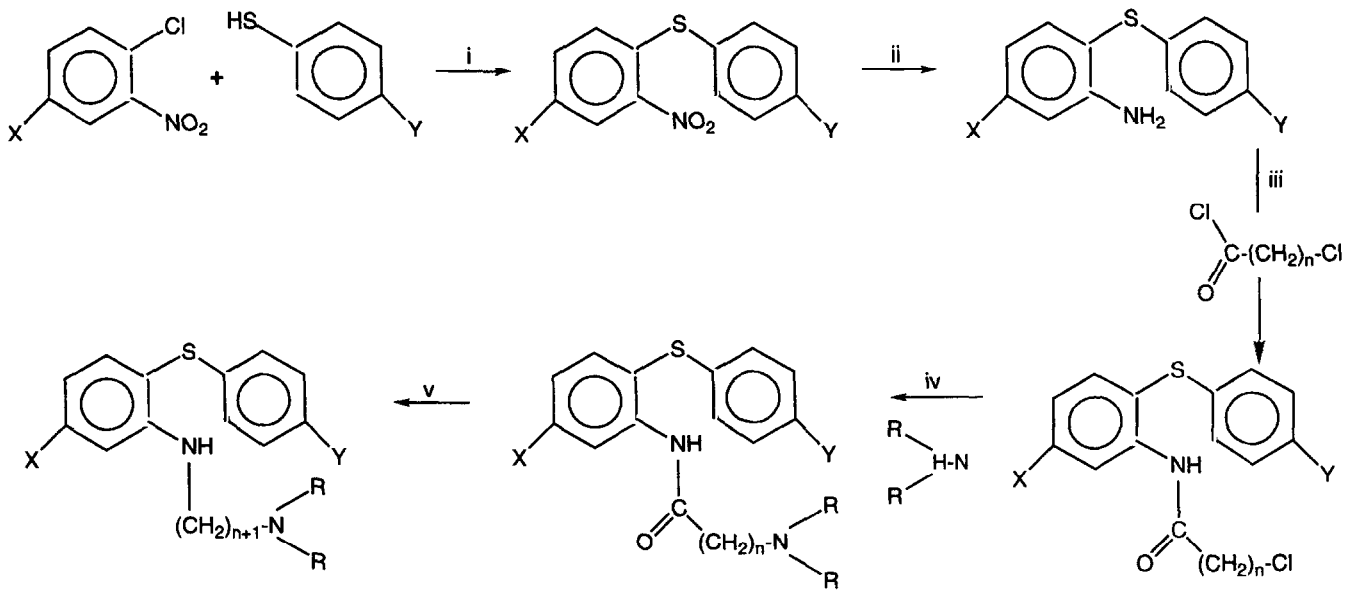


Fig. 1. Preparation of 2-amino-diphenylsulfides **1a–c**, **2a–c** and **3a–c**: **1a–c**, X = Cl, Y = H, n = 2; **2a–c**, X = Cl, Y = H, n = 1; **3a–c**, X = H, Y = Cl, n = 1. Reagents and conditions: (i): Na, ethanol, 2 h reflux; (ii): H_2, 100 bars, Raney Ni, 5 h; (iii): ether-pyridine, 0°C; (iv): triethylamine, $NH(R)_2$, benzene, 16 h reflux; (v): $LiALH_4$, 5 eq., THF, 2 h reflux.

pellet was resuspended in 200 volumes of buffer II containing 50 mM Tris-HCl, 120 mM NaCl, 5 mM EDTA, 0.1% ascorbic acid and 0.01 mM pargyline (IMAO), pH 7.6 at 20°C. The incubation mixture consisted of 100 mL of ^3H spiperone (20 Ci/mmol, 0.4 nM final concentration), 100 mL of the compound solution and 800 mL of freshly prepared tissue suspension in a total volume of 1 mL. The samples were incubated in triplicate for ten minutes at 37°C and then immediately filtered under reduced pressure. Radioactivity of the GFC filtrate was determined using 5 mL of liquid scintillation (Ready Protein Beckman). All the steps were carried out in the dark at 4°C.

2.5. Parasites

The Y strain of *T. cruzi* was used throughout the study. Trypomastigotes were obtained in tissue culture by weekly infection of 3T3 fibroblasts as described elsewhere [12]. The amastigotes forms were obtained by differential centrifugation on metrizamide gradient [13]. *T. cruzi* epimastigotes were cultured at 28°C in glucose-

Table 1
Physico-chemical characteristics of compounds **1a–c**, **2a–c** and **3a–c**

Compound	MP (°C)	Anal C, H, N, S, Cl	Mol. weight	NMR: δ (ppm, $CDCl_3$)
1a	218	$C_{24}H_{30}N_3O_8SCl$	556	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (13H, CH_2 + CH_3); 1.70 (m, 2H, CH_2)
1b	153	$C_{21}H_{25}N_2O_4SCl$	437	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (6H, CH_2); 1.70 (m, 2H, CH_2); 1.60 (m 4H,CH_2)
1c	150	$C_{19}H_{23}N_2O_4SCl$	411	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (8H, CH_2); 1.70 (m, 2H, CH_2)
2a	216	$C_{23}H_{28}N_3O_8SCl$	542	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (13H, CH_2)
2b	195	$C_{20}H_{23}N_2O_4SCl$	423	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (6H, CH_2); 1.60 (m, 4H, CH_2)
2c	165	$C_{18}H_{21}N_2O_4SCl$	397	7.45–7 (7H, aniline + phenyl); 6.60 (d, 1H, H_6); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (7H, CH_2)
3a	215	$C_{23}H_{28}N_3O_8SCl$	542	7.40 (1H, H_6); 7.30 (1H, H_4); 7.15 (2H, Cl meta); 6.95 (2H, Cl ortho); 6.75–6.70 (2H, H_{3-5}); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (13H, CH_2); 1.70 (m, 2H, CH_2)
3b	163	$C_{20}H_{23}N_2O_4SCl$	423	7.40 (1H, H_6); 7.30 (1H, H_4); 7.15 (2H, Cl meta); 6.95 (2H, Cl ortho); 6.75–6.70 (2H, H_{3-5}); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (6H, CH_2); 1.60 (m, 4H, CH_2)
3c	153	$C_{18}H_{21}N_2O_4SCl$	397	7.40 (1H, H_6); 7.30 (1H, H_4); 7.15 (2H, Cl meta); 6.95 (2H, Cl ortho); 6.75–6.70 (2H, H_{3-5}); 5.35 (s, 1H, NH); 3.15 (m, 2H, CH_2); 2.40–2.15 (8H, CH_2)

lactalbumin serum-haemoglobin medium (GLSH) supplemented with 10% (v/v) heat-inactivated fetal calf serum (HFCS) and antibiotic [14].

2.6. Parasite in vitro assay for drug activity

Epimastigotes forms were washed and aliquots (5×10^5) were transferred to 96-well microplates with the drug in a total volume of 200 μl and pulsed with ^{3}H-thymidine (0.5 μl was added to give 0.5 μCi/well). In the case of amastigotes and trypomastigotes forms, the cultures were collected and washed in RPMI 1640 containing 5% HFCS, then they were aliquoted (5×10^6) and pulsed with ^{3}H-uridine (0.5 μl was added to give 0.5 μCi/ml). Control cultures consisted of parasites incubated in the presence of culture medium alone. After different incubation times, parasites were harvested in an automated sample harvester. The filter discs were dried, placed in scintillation fluid and counted in a scintillation counter. Results are expressed as the radioactivity incorporated. All cultures were set up in triplicate. The results were compared with the Student's t-test as described elsewhere [15].

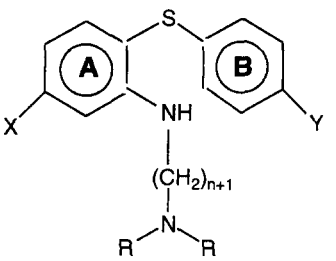
3. Chemistry

The substituted 2-amino-diphenylsulfides given in Fig. 2 were prepared by the general method shown in Fig. 1 and described in Material and Methods [16]. Condensation of the appropriate thiophenol and 2-nitro-chlorobenzene gave the 2-nitro-diphenylsulfide which upon catalytic reduction of the nitro group afforded the 2-amino-diphenylsulfide. Acylation of the amino group with either 2-chloro-acetyl chloride or 3-chloro-propionyl chloride followed by condensation with various amines and reduction of the resulting amide group by $LiAlH_4$ afforded compounds **1a–c**, **2a–c** and **3a–c**.

4. Results

4.1 In vitro TR inhibition

The inhibiting capacity of the various compounds submitted to the screening was measured by their ability to inhibit the TR mediated disappearance of NADPH measured at 340 nm (K_i determination for **1a** is given as an example in Fig. 3). Simultaneously, the inhibiting activity of the selected compounds on GR isolated from bovine intestinal mucosa was tested in order to establish their specificity. This allowed to discover a series of 2-amino-diphenylsulfides shown in Fig. 2 [16]. Although the inhibiting capacity of these compounds remain in the micromolar range, the most active **1a**, is comparable with clomipramine, the most potent TR inhibitor



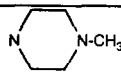
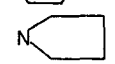
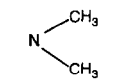
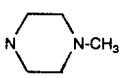
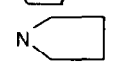
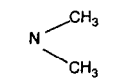
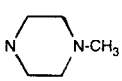
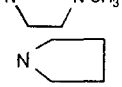
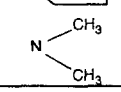
	X	Y	n	R, N, R	Ki(μM)
1a	Cl	H	2		27±2
1b	Cl	H	2		44±6
1c	Cl	H	2		66±6
2a	Cl	H	1		46±4
2b	Cl	H	1		86±6
2c	Cl	H	1		90±10
3a	H	Cl	1		80±10
3b	H	Cl	1		160±20
3c	H	Cl	1		280±30
Clomipramine					12±1

Fig. 2. Structures and measured K_i-values of compounds **1a–c**, **2a–c** and **3a–c**.

reported to date [17]. None of these compounds was active on GR.

The general structure of these diphenylsulfide derivatives offers an attractive lead since it possesses several sites with potential for structural modifications. Even with the limited number of derivatives available in this study, several structure-activity relationships for the inhibition of TR could be established:

The presence of a chlorine substituent is more efficient on cycle A than on cycle B as shown by the higher activity of compounds **2a–2c** compared to compounds **3a–c**.

A spacer consisting in 3 methylene located between the two amino groups is better than 2 methylene as shown by the higher activity of compounds **1a–c** compared to compounds **2a–c**.

The nature of the amino group N-(R)-R also plays a role, in all cases, the bulkier substituents being endowed of the higher inhibiting capacity: N-methyl-piperazine > pyrrolidine > dimethylamine.

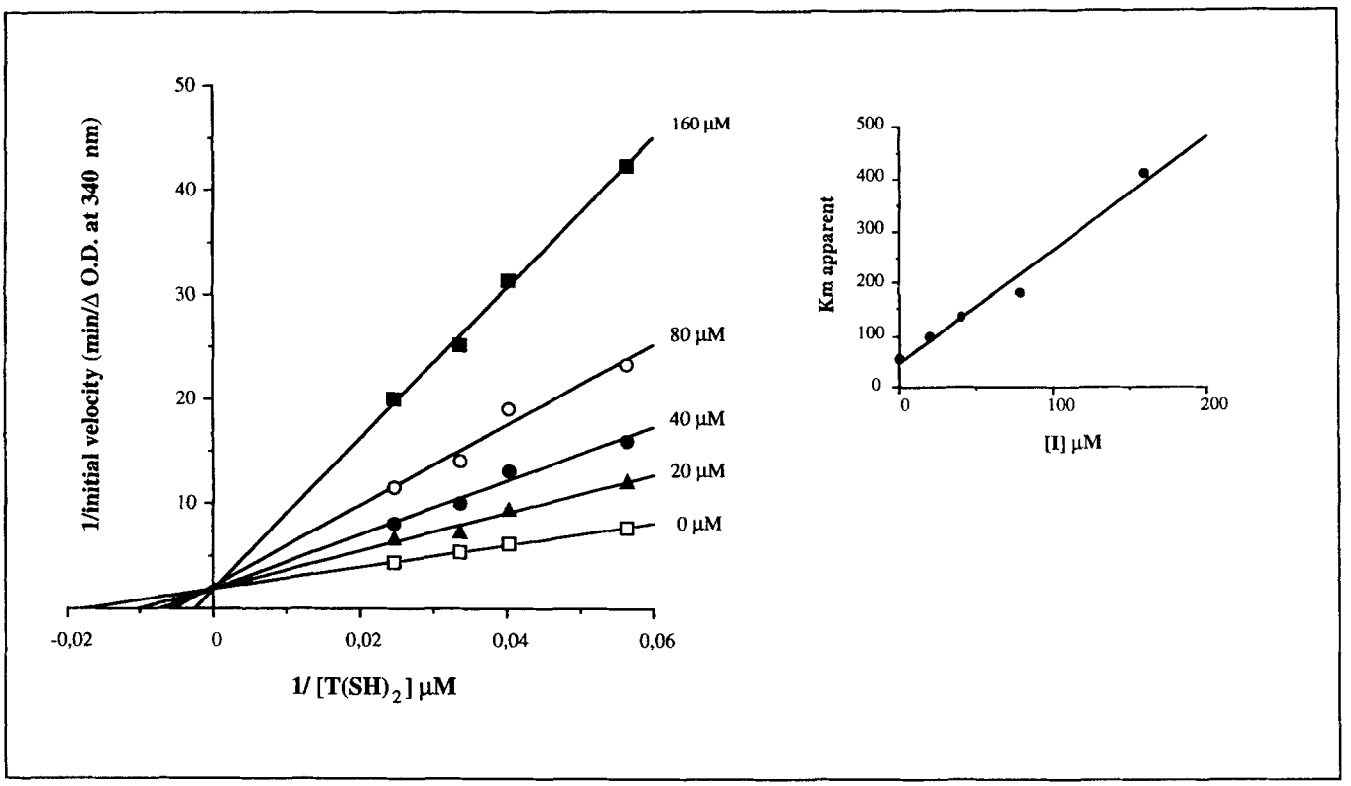


Fig. 3. Lineweaver-Burk plot for the inhibition of TR from *T. cruzi* by compound **1a**.

4.2. *In vitro effect on* T. cruzi

Considering the fact that these compounds exhibit a tenfold range of inhibiting capacity from 27 μM for **1a** to 280 μM for **3c**, we were interested to test whether such differences were correlated with their effect on the parasite. Three compounds **1a** (K_i = 27 μM), **2b** (K_i = 86 μM) and **3c** (K_i = 280 μM) were selected in order to cover the maximum range of TR inhibiting capacity. They were tested for their capacity to inhibit *T. cruzi* epimastigotes proliferation in an axenic medium, as determined by ^{3}H-thymidine incorporation. In these experiments, benznidazole at a concentration of 380 μM was used as a reference. Fig. 4 shows the inhibitory effect of the three compounds at 10 μM: **1a**, the most active inhibitor of TR displays an effect comparable with benznidazole at 380 μM while **2b** and **3c** which are less potent inhibitors are also less active towards the parasite. This was confirmed in Fig. 5 when increasing doses of **1a** and **3c** were tested, **1a** is significantly more active than 3c.

In order to determine whether the observed effect was due to a trypanostatic or to a trypanocidal activity, parasites were incubated for 12 hours with either **1a** (27 μM) or benznidazole or nifurtimox (380 μM). Viable epimastigotes were counted at 3 h intervals during 12 h. As can be seen from Fig. 6, in the presence of **1a**, the number of parasites decreases sharply and after 12 h, no surviving parasite can be detected. By contrary, neither nifurtimox nor benznidazole were able to achieve a similar effect, being only endowed with a cytostatic effect.

The study of the activity of **1a** against the vertebrate forms of *T. cruzi* (e.g. amastigotes and trypomastigotes)

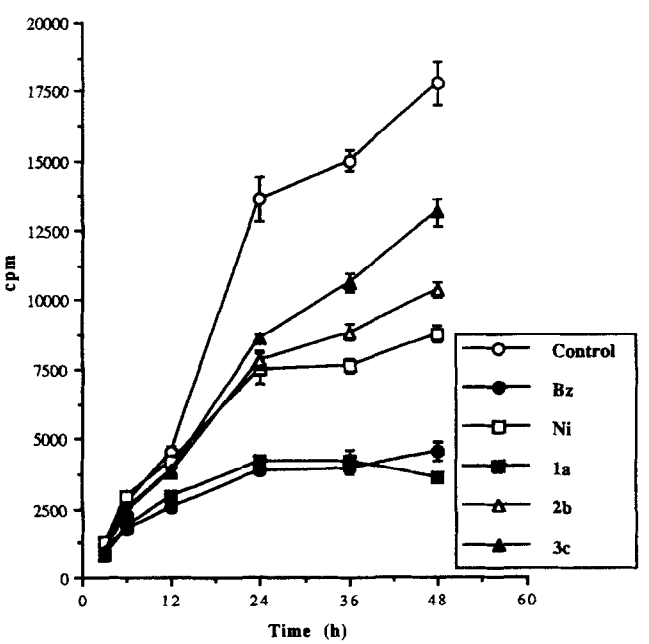


Fig. 4. Effect of compounds **1a**, **2b** and **3c**, at 10 μM concentration on *T. cruzi* epimastigotes growth.

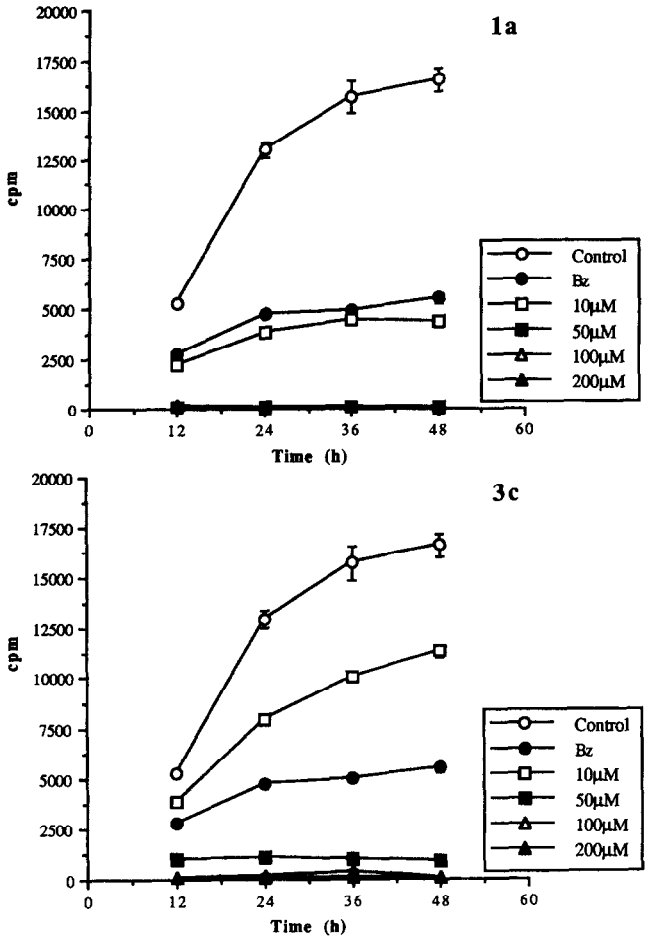


Fig. 5. Effect of different concentrations of compounds **1a** and **3c** on *T. cruzi* epimastigotes growth.

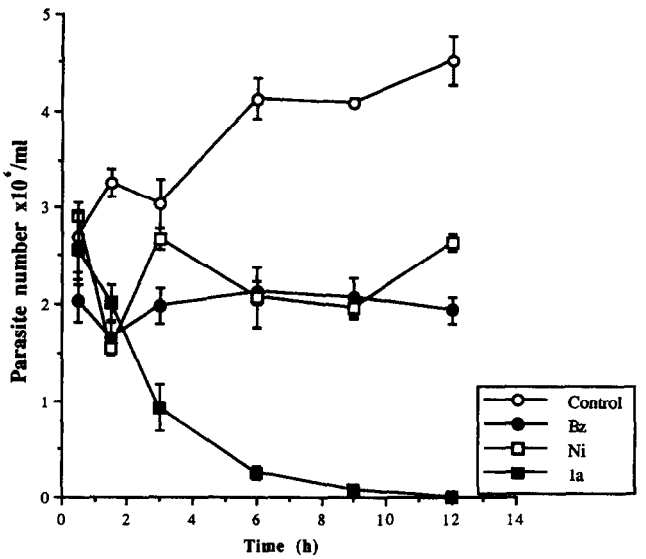


Fig. 6. Effect of benznidazole (**Bz**), nifurtimox (**Ni**) and compound **1a** on *T. cruzi* epimastigotes growth.

was also performed. In this case, as shown in Fig. 7, we obtained a significant inhibition of the RNA precursor incorporation by these two parasite stages (significant differences between control cultures and drug-treated parasites: $P < 5.10^{-2}$ and $P < 10^{-3}$ for *T. cruzi* amastigotes and trypomastigotes, respectively).

4.3. In vitro neuroleptic activity

Neuroleptic activity of compounds **1a–c**, **2a–c** and **3a–c** was evaluated in vitro according to the protocol described in Material and methods. Specific binding was determined by subtracting non-specific binding (in the presence of 0.1 mM domperidone) from the total binding (in the absence of domperidone). Neuroleptic activity was correlated to the percentage of displacement for the specific binding spiperone ^{3}H-D2 receptor. Compared to control: Prochlorperazine (81% displacement), results at 10^{-8} M were as follows: **1a**: 6%; **1b**: 0%; **1c**: 0%; **2a**: 18%; **2b**: 9%; **2c**: 6%; **3a**: 26%; **3b**: 22%; **3c**: 0%.

5. Discussion

Several independent screening and molecular modelling studies have led to the discovery that phenothiazines and tricyclic antidepressant drugs are effective against epimastigote, trypomastigote and amastigote forms of *T. cruzi* [18–23]. Molecular modelling studies performed by docking fragments into the binding pocket of TR led to the hypothesis that these drugs could be accommodated at the hydrophobic wall site which represents the main difference between TR and GR. This was confirmed by subsequent assay against the enzyme in which typical neuroleptics, clomipramine, an imipramine and trifluoroperazine a phenothiazine (Fig. 8) were found to be linear competitive inhibitors of TR with reported K_i of respectively 7 μM and 22 μM [17]. These findings were confirmed in our random screening program by the discovery that three other phenothiazine derivatives were endowed with significant TR activity (unpublished results). Unfortunately, due to their intrinsic neuroleptic activity, these compounds could not be considered for clinical use as the doses required to affect *T. cruzi* are evaluated for instance at least about four times higher than those tolerated by humans in the case of chlorpromazine or trifluoroperazine [24]. Interinstingly, the 2-amino-diphenylsulfide derivatives **1a** to **3c** among which some are endowed with inhibiting capacity comparable to the best tricyclic neuroleptics cited above, had been synthesized almost thirty years ago in our laboratory as an attempt to apply Dodd's theory of open cycles to phenothiazines. According to this theory, 2-amino-diphenylsulfides could be considered as phenothiazine structural analogs in which the constraint imposed by the central ring had been suppressed. The fact

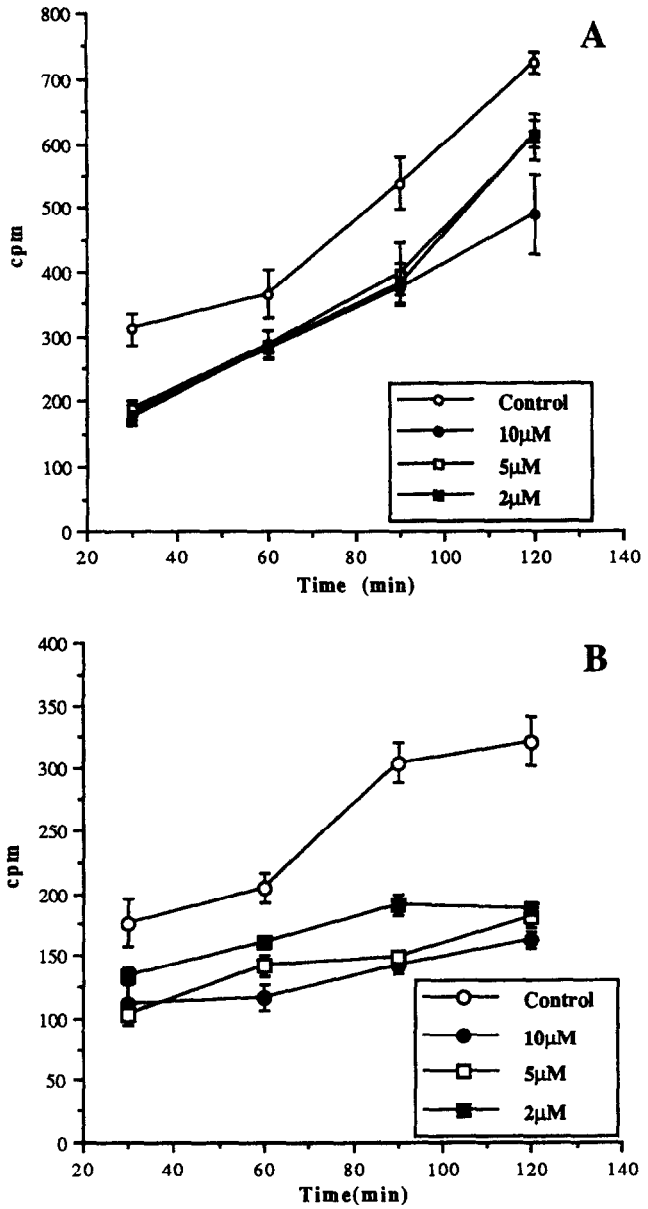


Fig. 7. Dose-dependent inhibition of ^{3}H-uridine incorporation by *T. cruzi* amastigotes (A) and trypomastigotes (B), caused by the compound **1a**.

that none of these compounds had shown any significant neuroleptic activity at the time in the pharmacological tests used (mobility, curiosity, balance) becomes for our purpose, a clear advantage. Moreover, this was confirmed by binding experiments on the D2 receptor using prochlorperazine as control in which our derivatives at 10^{-8}M led to a weak or non significant displacement. This in vitro assay correlates with in vivo models like inhibition of apomorphine stereotypy in rats. However, the metabolism of the drug may influence this correlation. The maximal value observed for the displacement of the radioligand is 22–26%; it is close to the limit of sensibility (estimated at 20%) in the binding assay and

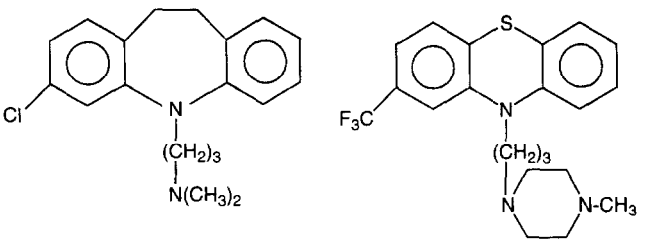


Fig. 8. Structures of clomipramine and trifluoroperazine.

therefore does not lead to predict some side-effects in an animal model.

Together, these results indicate that, although the angle formed by the two benzenic rings in phenothiazines has been shown to be of considerable importance for their neuroleptic activity, this constrain is not an absolute requirement for TR inhibition so that both activities can be easily dissociated. By contrary, the presence of the tertiary amine group in the alkyl side chain appears to be a common requirement to all these derivatives probably extending backwards from the hydrophobic wall toward the general direction of the active site. These observations make 2-amino-diphenylsulfides as well as other phenothiazine analogues which show low neuroleptic activities attractive candidates as TR inhibitors and antichagasic drugs.

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