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STRUCTURE-ACTIVITY RELATIONSHIPS IN 2-AMINODIPHENYLSULFIDES AGAINST TRYPANOTHIONE REDUCTASE FROM *Trypanosoma cruzi*

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Abstract : In order to establish structural elements responsible for inhibition of trypanothione reductase (TR) from *Trypanosoma cruzi* by 2-aminodiphenylsulfides, a series of dissymmetrical derivatives, corresponding to the replacement of one aromatic moiety by different amines, was synthesized. TR inhibition studies revealed the importance of the aromatic rings and of the amino groups in the side chains for potent inhibition. Quinonic moities were also introduced with the aim of acting as TR redox-cycling substrates.

Introduction

Chagas' disease, which is caused by the flagellate protozoan *Trypanosoma cruzi*, represents an important medico-social problem in South and Central America.[1] The two nitro compounds, used presently as drugs, are non-specific, fairly toxic and have become inactive due to resistances developed by the parasite.[2] *T. cruzi* possesses an original redox defence system, based upon trypanothione $T(SH)_2$, a bis(glutathionyl)-spermidine conjugate, and trypanothione reductase (TR), a NADPH-dependent flavoprotein, which regenerates trypanothione from its oxidized form.[3-5]

Despite 40 % identity in their primary sequences, TR and human glutathione reductase (GR) are exclusive towards their respective substrates ($T(S)_2$ and GSSG).[6] Therefore TR is a potential rational drug design target for use during chemotherapy against Chagas' disease.

We developed, in our laboratory, a high-throughput assay[7] to screen for new TR inhibitors, that allowed us to isolate, among other potential lead drugs, three structurally-related TR inhibitors : the 2-amino-diphenylsulfide derivative **A** and the phenothiazine derivatives **B** and **C** (Figure 1).

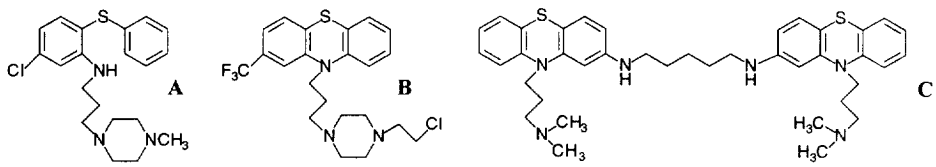

Figure 1. Structures of TR inhibitors **A**, **B** and **C** selected from a high-throughput assay

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Despite a structural similarity to phenothiazines, compound **A** has the advantage of being a specific competitive inhibitor of TR *versus* human GR with only residual neuroleptic activity.[8]

Structure-activity relationship studies were previously established in the **A** series.[9] Therefore **A** was selected as a lead drug to design TR inhibitors. The position and the conformation of **A** in the catalytic site of TR from *Crithidia fasciculata* (2.4 Å resolution)[10] were studied by molecular dynamics simulation. According to these results, **A** is bound, in the TR active site, to two carboxylate groups of glutamic residues through its *N*-methylpiperazine extremity and with a hydrophobic pocket through the two aromatic rings.[9]

As certain hydrophobic residues of the catalytic site of TR (Trp 21 and Met 113) were absent in human GR and some acidic residues (Glu 18, Asp 116) were replaced by basic residues, we synthesized a series of bis(2-aminodiphenylsulfides) **D**, analogues of **C**, to increase the number of proton-accepting amino groups (Figure 2).[9,11]

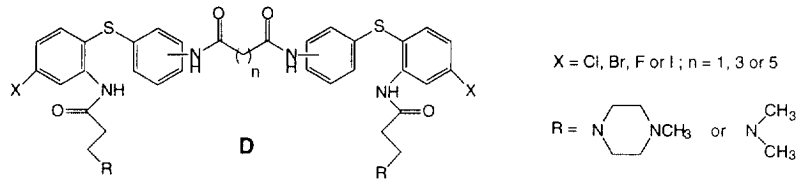


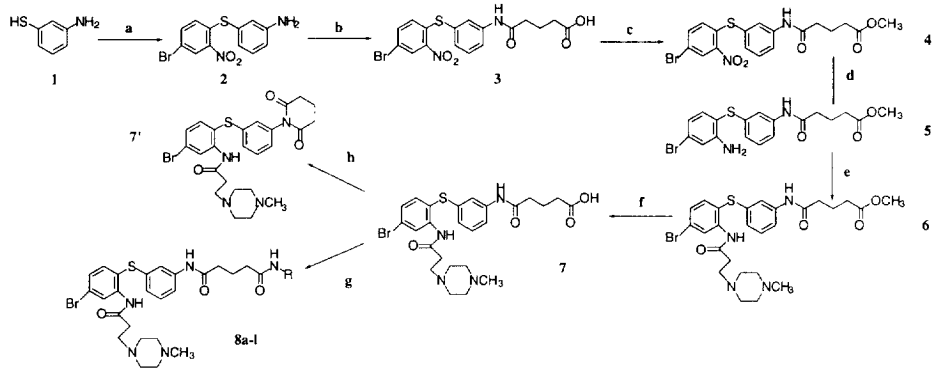
Figure 2. Structures of Bis(2-aminodiphenylsulfides) **D**

In this series, the most potent and specific TR inhibitor corresponded to n = 3, X = Br, R = *N*-methylpiperazine and displayed an IC_{50} of 0.55 μM for $[T(S)_2]$ = 57 μM.[11] In order to study the position and the role of each aromatic moiety in TR recognition, we designed a series of dissymmetrical compounds in which one of the two diphenylsulfide moieties was replaced by an aliphatic, an aromatic or an alicyclic entity.

These new entities were introduced as primary or secondary amines, reacting with the appropriate aminodiphenylsulfide linker *via* its terminal carboxylic acid group. Morever, since it is known that quinone compounds are reduced by TR *via* a single-electron mechanism to generate free radicals with concomitant production of superoxide,[12-16] numerous amino-quinones were also linked with the aim of conjugating two specific actions onto the enzyme : the inhibition of the disulfide reduction and, in the presence of oxygen, the production of superoxide anion *via* TR-catalyzed reduction of the quinone.

Chemistry

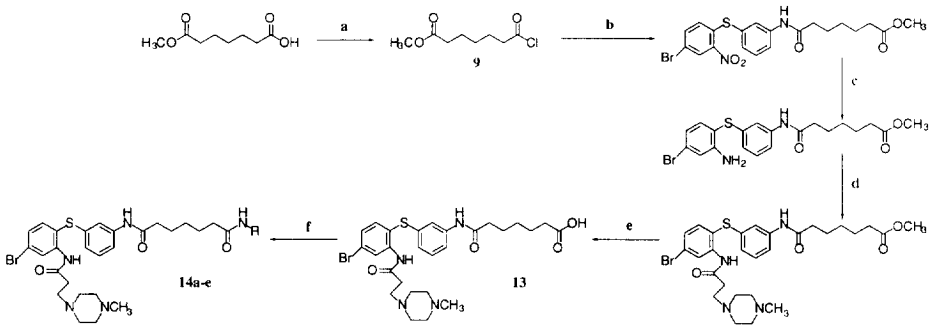
The dissymmetrical compounds were prepared from carboxylic acid **7**, associating a glutaric acid linker with the 2-aminodiphenylsulfide moiety of the most potent TR inhibitor in the **D** series, corresponding to n = 3, X = Br, R = *N*-methylpiperazine. Derivatives **8a-l**[17] were synthesized as shown in Scheme 1, starting from an aromatic nucleophilic substitution of 3-aminothiophenol **1** upon 2,5-dibromonitrobenzene.[18]



Scheme 1. Reagents and conditions : a) 0.67 eq. 2,5-dibromonitrobenzene, 3.33 eq. anhydrous CH_3COONa, absolute EtOH, reflux, 36h, 84%; b) 2 eq. glutaric anhydride, pyridine, reflux, 3h, 97%; c) 0.5 eq. HCl 12 N, MeOH, reflux, 1h, 81%; d) 6 eq. Fe, 2.5 eq. HCl 12 N, EtOH 95°, reflux, 1h, 100%; e) 2.5 eq. 3-chloropropionylchloride, 4.5 eq. 1-ethylpiperidine, anhydrous THF, 0°C, 3h, then 15 eq. 1-methylpiperazine, anhydrous THF, reflux, 4h, 78%; f) 2 eq. Na_2CO_3, MeOH, H_2O, rt, 24h, 100%; g) 1.2 eq. amine, 1 eq. PyBroP, 3 eq. DIEA, DMF, rt, 63-89%; h) 5 eq. $SOCl_2$, CH_2Cl_2, reflux, 1h.

Reaction of amine **2** with glutaric anhydride produced acid **3**, which was then esterified to yield methyl ester **4**. Sequential reduction of the nitro group, fixation of the side chain and of the amino group,[11] hydrolysis of the ester function[19] and finally coupling with aliphatic, alicyclic or "benzylic-type" amine using PyBroP reagent (bromotris(pyrrolidino)phosphonium hexafluorophosphate)[20] led to the desired target molecules **8a-l**.

PyBroP did not allow coupling of aromatic amines, therefore other activated forms of the carboxylic acid were considered. Acyl chloride or acyl fluoride led to **7'** by fast intramolecular cyclisation. Aniline or benzylamine was introduced to a solution of methyl ester **6,** either directly or in presence of boron tribromide, methylmagnesium iodide or hydrazine, but, in all cases, no reaction resulted. Thus, the acid analogue of **7** with two additional carbon atoms (n = 5) was synthesized, on the basis that, in the **D** series, the analogues n = 3 and n = 5 exhibited similar inhibition values.[11] Aminodiphenylsulfide derivatives **14a-e**[17] were obtained as shown in Scheme 2.



Scheme 2. Reagents and conditions : a) 5 eq. $SOCl_2$, CH_2Cl_2, reflux, 2h, 100%; b) 1.1 eq. **2**, 1.5 eq. TEA, CH_2Cl_2, rt, 12h, 83%; c) 6 eq. Fe, 2.5 eq. HCl 12 N, EtOH 95°, reflux, 1h, 100%; d) 2.5 eq. 3-chloropropionylchloride, 4.5 eq. 1-ethylpiperidine, anhydrous THF, 0°C, 3h, then 20 eq. 1-methylpiperazine, anhydrous THF, reflux, 4h, 86%; e) 2 eq. Na_2CO_3, MeOH, H_2O, rt, 24h, 100%; f) 2 eq. cyanuric fluoride, 1 eq. pyridine, CH_2Cl_2, -10°C, 5h, then 2 eq. aromatic amine, 3 eq. pyridine, CH_2Cl_2, rt, 12h, 10-40%.

Thus, reaction of amine **2** with acyl chloride **9** (obtained from monomethylpimelate) led to ester **10**, which following reduction of its nitro group and subsequent acylation afforded ester **12**. Hydrolysis of this compound and coupling between the acyl fluoride and aromatic amines yielded the desired molecules **14a-e**.[21] No coupling was observed when using the acyl chloride.

Naphthoquinone derivative **14f**[17] was prepared by reaction of acid **13** with the hydroxyl group of 3-bromo-2-hydroxymethyl-1,4-naphthoquinone[22] in the presence of DCC and DMAP.[23]

Results and discussion

The inhibiting potency of the different compounds was evaluated by measuring the IC_{50} against TR in the presence of 57 μM of $T(S)_2$ and increasing concentrations of inhibitor (0-60 μM) (Table 1).[11,24] Comparative enzymatic studies revealed that the different inhibitors were specific towards TR from *T. cruzi versus* human GR.

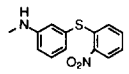
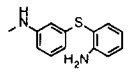
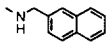
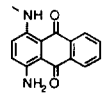
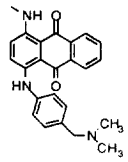
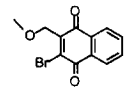
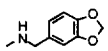
Compound	- NHR	IC_{50} (μM)	Compound	- NHR or - OR	IC_{50} (μM)
8a		35	**14a**		14
8b		47	**14b**		> 60
8c		28	**14c**		16
8d		25	**14d**		> 60
8e		> 60			
8f		20	**14e**		3.3
8g		> 60			
8h		23			
8i		> 60			
8j		> 60	**14f**		41
8k		33			
8l		> 60			

Table 1. Inhibitory activities of compounds **8a-l** and **14a-f** against TR from *T. cruzi*

The substitution of one aromatic portion of the bis(2-aminodiphenylsulfides) by aliphatic, alicyclic or heterocyclic amines strongly decreased the inhibitory action upon TR. This decrease was notably weaker for "benzylic-type" amines or phenethylamine. Inhibitory potencies were higher with aromatic amines as proved

the case with compounds **14a** and **14c**. The presence of a nitro group proved to be unfavourable (**14b**).

The presence of an amino group in the side chain of each aromatic moiety is essential for recognition as revealed by the inhibitory activities of **14c** and of the corresponding non-brominated bis(2-amino-diphenylsulfide) (IC_{50} = 1.8 μM),[11] or of **14d** and **14e**.

In addition to the potent inhibition of the enzymatic reduction of $T(S)_2$, we evaluated the ability of the quinones **14e** and **14f** to act as turncoat inhibitors of TR. When tested as redox-cycling substrates for TR,[25] no quinone-dependent oxidation of NADPH and no quinone-dependent reduction of cytochrome *c* occured with the anthraquinone derivative **14e** suggesting that the amino group of the side chain, which conjugates with the aromatic moiety of the anthraquinone portion, could influence the redox potential of **14e**. To avoid the presence of donor groups directly attached to the quinone portion of the molecule, we envisaged additional functionalization of menadione, already known for its redox-cycling activity against *T. congolense* TR.[14] **14f** was tested as a non-amino derivative of menadione. The presence of the halogen atom was not thought to exert a considerable effect upon the redox potential of the quinone, according to the literature values.[26] With 100 μM **14f** as substrate, TR catalyzed the oxidation of NADPH and the reduction of cytochrome *c*, either directly (42%), or by the $O_2^{\bullet-}$ mechanism (58%). The following kinetic parameters were obtained for the naphthoquinone **14f** (menadione derivative) and compared to the parent molecule, menadione : **14f**, Km = 160 μM, kcat/Km = 8.4 x 10^3 $M^{-1}.s^{-1}$; menadione, Km = 882 μM, kcat/Km = 5.0 x 10^4 $M^{-1}.s^{-1}$. The Km values reveal the importance of the presence of the 2-aminodiphenylsulfide moiety attached to the quinone for TR recognition in NADPH oxidase activity. Nevertheless, the steric hindrance of the 2-aminodiphenylsulfide moiety leads to a less favourable position of the quinone moiety for the single-electronic transfer from the flavin group.

In conclusion, we have shown the importance, for TR recognition, of the presence of two aromatic moities binding to two proton-accepting amino groups. Moreover, bearing in mind that these molecules do not need to be symmetrical in order to bind to the TR active site, we were able to take advantage of this feature by associating a second inhibition mode with the introduction of a quinone moiety. Work is currently in progress to measure the trypanocidal effects of this series upon the parasite to develop new structure-activity relationships for the NADPH oxidase activity of the TR.

Acknowledgments

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23. 1 eq. DCC, 3 eq. DMAP, THF, rt, 24h.
24. The IC_{50} were measured with the oxalate salts of the different synthesized compounds.
25. The quinone reductase activity of TR was assayed by monitoring, at 340 nm, the substrate-dependent oxidation of NADPH ($\varepsilon_{340,\,NADPH} = 6.22\ mM^{-1}.cm^{-1}$). The assays were performed in 0.02 Hepes buffer, 1 mM EDTA, 0.15 M KCl, pH 7.25 at 22°C, in the presence of 200 µM NADPH, 0-100 µM substrate and TR (0.5 units in 120 µl-cuve). Quinones were dissolved in DMSO and the NADPH-oxidase activity was measured at 4 distinct concentrations in the presence of final 1.6% DMSO. For the determination of the turnover number kcat and kcat/Km, the steady-state rates were graphically analyzed by Lineweaver-Burk plot. Alternatively, TR activity was monitored at 550 nm by coupling radical formation to the reduction of 25 µM cytochrome *c* ($\varepsilon_{550,\,cyt\ c\ (Fe2+)} = 18.91\ mM^{-1}.cm^{-1}$) and by measuring absorbance changes in the absence or in the presence of superoxide dismutase (0.5 units in 120 µl-cuve).
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