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Parallel Synthesis of a Library of 1,4-Naphthoquinones and Automated Screening of Potential Inhibitors of Trypanothione Reductase from *Trypanosoma cruzi*

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Abstract—Solid- and solution-phase parallel syntheses of 1,4-naphthoquinones (1,4-NQ) are described. A library of 1360 amides was constructed from the combination of 12 newly synthesised 1,4-NQ carboxylic acid and 120 amines, and was screened for inhibition of trypanothione reductase (TR) from *Trypanosoma cruzi*. The most active hits from a primary screening were re-synthesised and confirmed. This approach proves that it is possible to design potent and highly specific TcTR inhibitors deriving from menadione, juglone and plumbagin.

Introduction

The use of parallel synthesis for the production of large arrays of individual compounds is gaining wide acceptance because of the advantages afforded by its application toward accelerated lead optimisation. Our aim was to prepare a library of amides in the 1,4-NQ series as potential inhibitors of trypanothione reductase from *Trypanosoma cruzi* (TcTR), the causal agent of Chagas' disease. Validity of TR as a target for the search of new trypanocidal drugs has recently been proved by studies with *Leishmania donovani*[1] and *Trypanosoma brucei*[2] TR null mutants and *L. donovani* inactive trans-dominant TR mutant.[3] As TcTR and hGR are exclusive towards their respective substrates (TS_2 and GSSG), selectivity between both enzymes could be expected from rational drug design. Many ammonium functional side-chain-substituted compounds have been described as effective and specific ligands of TR versus human glutathione reductase (hGR). The recognition of these ligands is based upon cation-π bonding and ionic interactions, which are responsible for the discrimination in binding in TcTR and hGR, and include the interaction of Trp21 and Glu18, in the active site of TcTR. Among the numerous 1,4-NQ reported for their antiparasitic activity, menadione and plumbagin are known to play, in the presence of oxygen, the role of subversive substrate for TR.[4–6] They promote both a non-physiological reaction leading to the production of superoxide anions, and they inhibit the physiological disulfide reduction. In this paper, we report the design and synthesis of three series of 1,4-NQ. Using a versatile and high yielding two-step sequence, we prepared, under mild conditions, an array of diverse amino-substituted 1,4-NQ. Both solid-phase and solution-phase methodologies were considered. A broad diversity could be easily accessed by acylation of commercially available amines with the terminal carboxyl group of 12 newly prepared 1,4-NQ intermediates. Thus it was possible to design several series of TR inhibitors, characterized by a redox-cycler component, such as menadione and plumbagin. The library of potential specific inhibitors was screened for rapid selection of potent inhibitors, using an automated assay based on the recombinant TcTR produced in our laboratory.

Chemistry and Library Generation

Strategy and rationale for combinatorial library

Taking into account that the affinity of a ligand for TR disulfide site is governed by the presence of one or two

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aromatic groups bound to polyamine chains, we prepared a large number of 1,4-NQ derivatives corresponding to structures A, B and C, in order to increase the inhibitory activity and to improve the specificity for TcTR binding (over hGR binding) (Fig. 1).

Three series of four carboxylic intermediates ($\mathbf{1}_n$–$\mathbf{3}_n$) bearing spacers of varying length ($n = 2$ to 5) were prepared on a 5-g scale by an oxidative decarboxylation,[7] in the presence of $AgNO_3$ and $(NH_4)_2S_2O_8$ (Fig. 2).

The carboxylic group allows the introduction of different amino-side chains, via an amide bond. For both the A and B series, one hundred commercially available amines were selected according to their diversity, with each one being in possession of a reactive amino function (Fig. 3). These could be linear or branched, functionalized by different aromatic groups with a variety of substituents (halogen, methoxy, piperonyle, nitrophenyle...), or not at all, as well as having other protonable amine groups. Some amines lacking an additional protonable amino group were also introduced in order to increase the diversity.

Besides the monofunctional amines, 20 diamines possessing two reactive amino groups were chosen for the C series (Fig. 4). They were symmetrical or dissymmetrical, and some of these molecules bear an additional site for protonation.

Solid-phase methodology

Since product isolation and purification had proved to be generally difficult in the case of polyamines (tri- or tetramines), solid-phase methodology was considered initially for the preparation of compounds. As coupling reagent for the acylation of *N*-nucleophiles, we used the recently reported polymer-supported HOBt.[8] The synthesis of amides was performed according to the general reaction pathway outlined in Scheme 1. This is a two-step procedure: (i) formation of the polymer bound activated ester of the carboxylic acid using bromotrispyrrolidinophosphonium hexafluorophosphate (PyBrop); (ii) release of the amide in solution by addition of the amine. The polymeric reagent plays the role of leaving group and thus a simple filtration allows the isolation of the desired product. Optimisation of both steps was performed using 50 to 500 mg polymer-supported HOBt (0.63 mmol/g) in order to obtain a solution of amide free of by-products. The best conditions for activation were found to be: 2×40 min activating time step in dimethylformamide (DMF). For non-aromatic monoamines, the coupling step was carried out in 20 h with 0.9 equiv of the amine in a DMF solution. In the case of polyamines, addition of triethylamine (TEA) to DMF was necessary to release compounds from the resin. In the menadione and plumbagin monoacid series, good yields (76 to 99%) and good to high purities (70 to 91%) were obtained with amides. In contrast, juglone diacid derivatives led to mixtures since both acid groups could not be simultaneously activated on the polymer. For high-throughput synthesis, the efficient process described above from 1,4-NQ-monoacids requires to be implemented on an automated synthesizer, to respect washing times of the resin and thus to avoid the decomposition of the polymer-bound esters.

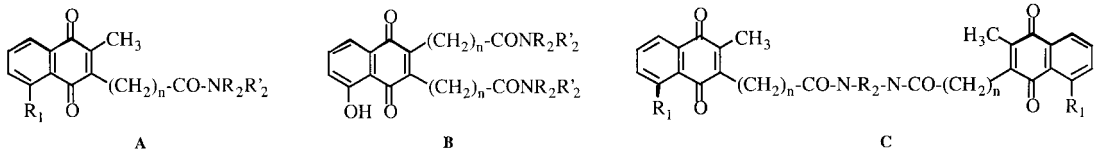


Figure 1. Structures of the three series of 1,4-naphthoquinone derivatives: 3-(polyaminocarbonylalkyl)1,4-NQ (**A**), 2,3-bis(polyaminocarbonylalkyl)-1,4-NQ (**B**) and 3,3′-[polyaminobis(carbonylalkyl)]-bis(1,4-NQ) (**C**). R_1 = H, OH; $NR_2R'_2$,N-R_2-N = substituted amine or polyamine; $n = 2$ to 5.

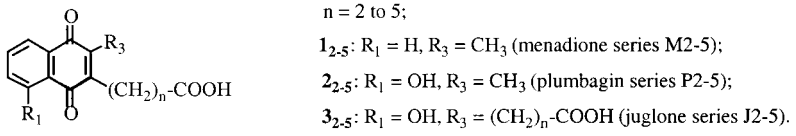


Figure 2. Structure of the 1,4-NQ carboxylic acid intermediates $\mathbf{1}_n$–$\mathbf{3}_n$ that were coupled with 120 amines.

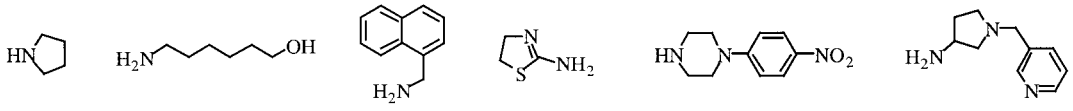


Figure 3. Examples of amines selected in A and B series.

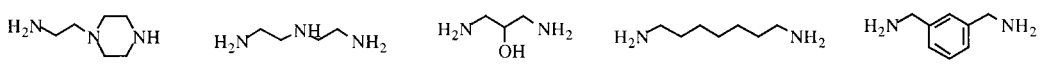
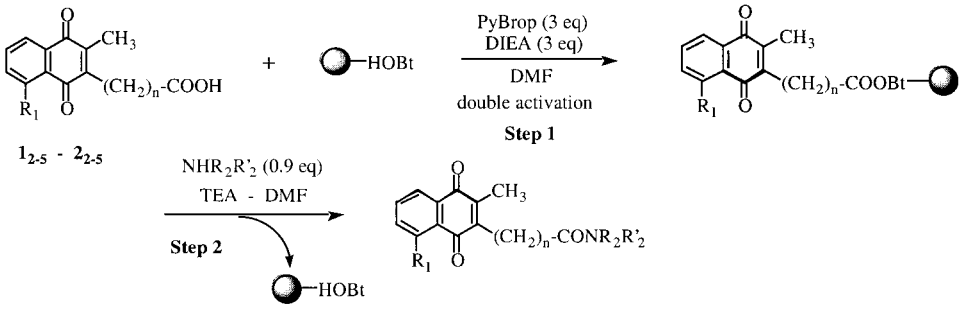


Figure 4. Examples of amines selected in C series.



Scheme 1.

Solution-phase methodology

A solution-phase methodology was eventually used for the preparation of the different mono and bis (1,4-NQ) according to Scheme 2. The 120 selected amines were dissolved in DMF:DCM (0.1 M, 1:0 to 1:1) and quantitatively coupled in parallel for 3 h to the 12 1,4-NQ carboxylic acid intermediates dissolved in DMF:DCM (0.1 M, 1:1) in the presence of DIEA (3 equiv for monoacids and 6 equiv for diacids). HBTU was used as coupling reagent (1 equiv for monoacids and 2 equiv for diacids). The amides obtained in 5 μmol-scale in the microtiter plates were then evaporated under reduced pressure, yielding a library of 1360 members.

Quality Control

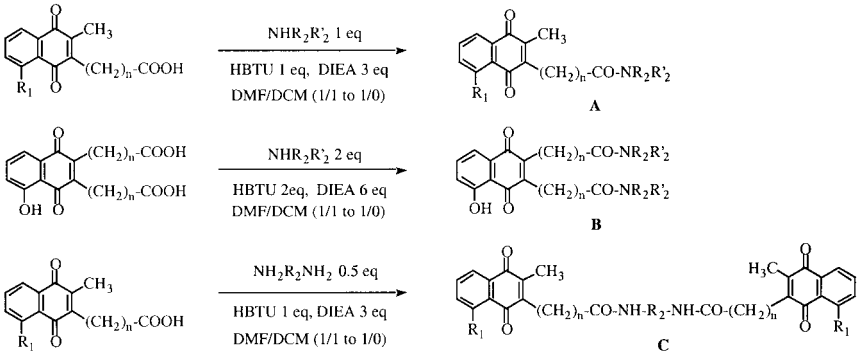
The general reliability of the protocol was demonstrated by the complete analysis of a mini-library of 20 products, structurally representative of the whole. The quality of the final library was then assessed by analyzing 15% of the compounds. For each plate of 80 compounds, 12 were selected for control by mass spectrometry (Time of flight) and HPLC (on a Bioion 20K and a Schimadzu 10A apparatus, respectively). The data indicated that the anticipated products were present, along with HOBt as a by-product in each well. HOBt was demonstrated to be inactive towards TcTR. Therefore, the percentage of purity was calculated excluding HOBt and was found to range from 58 to 100% (average: 78%) for the A series, 51 to 95% (average: 71%) for the B series, and 45 to 75% (average: 59%) for the C series. Hits displaying more than 80% of inhibition were analysed by LC–MS. Mass spectra and HPLC profiles were obtained on a Micromass Platform system composed of a Gilson 215 autosampler, a HP 1050 LC, a simple quadrupole mass analyser and a HP UV–visible diode array detector. The data indicated that in each case the major product formed was the anticipated one.

Enzymic Tests

Automated[9] high-throughput TcTR inhibitor screening (HTS) was performed in microtiter plates, by using the colorimetric assay based upon the alternative substrate for trypanothione disulfide, DTNBA,[10] which had been previously designed and synthesized in our laboratory. The library was diluted to a 250 μM concentration in enzymatic buffer containing 10% DMSO and screened at 25 μM against TcTR, in the presence of 200 μM DTNBA, 500 μM NADPH and 1% DMSO. The non-specific absorbance, assessed by control wells containing 200 μM disulfide and 500 μM NADPH, was substracted from each data point. For each compound tested, the percentage of inhibition was calculated, and compared with those of menadione and plumbagin.

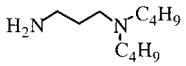
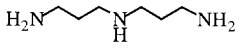
Results and Discussion

From the HTS performed with TcTR as target, 13 amines conferred >70% inhibition upon 33 hits selected as potential TcTR inhibitors. The same hits did not



Scheme 2.

Table 1. Inhibitory potency of the four most potent TR inhibitors from the library

Name	Series	Structure (R_1)	Amine	% Inhibition[a,b] (25 μM)	IC_{50} TcTR[c,d] (μM)	IC_{50} hGR[c,d] (μM)
4	A	P4 (OH)		88	0.3	41
5	A	M4 (H)		86	1.1	>50
6	B	J4		81	0.6	>50
7	C	P4 (OH)		79	0.5	10

[a]Values from primary screening, obtained with crude compounds.
[b]In the presence of 200 μM DTNBA as substrate.
[c]Values obtained with purified compounds.
[d]In the presence of 50 μM TS_2 as substrate for TcTR and GSSG as substrate for hGR.

inhibit the human enzyme hGR at 25 μM to the same extent. For both A and B series, 3-(dibutylamino)propylamine derivatives displayed a high inhibitory potency. The best results were obtained for $n=4$ (compounds **4**, **5** and **6** in Table 1). The selection of this amine was not surprising, since it was homologous to the 3-(diethylamino)propylamine amino side chain of mepacrine,[11] known for its competitive inhibitory activity of TcTR. The bis(3-aminopropyl)amine conferred upon compound **7** the highest percentage of inhibition in the C series, The optimum spacer length was found to be $n=4$, as in A and B series. The other polyamines did not display such a high inhibitory potency and a drastic loss of activity was observed when the amino side-chain did not have a protonable amine at physiological pH, even if the side chain length was the same as in the lead compound **7** (Fig. 4). The data obtained in the three series underline that, as previously observed, protonable amino groups are critical for TR recognition.

These four most potent inhibitors were re-synthesised manually, using the solution phase method conditions described above. They were purified by preparative TLC and characterized by ^1H and ^{13}C NMR, mass spectrometry and HPLC,[12] before enzymatic studies were undertaken. IC_{50} values were determined and inhibition percentages were found to be in approximately the same range as those for a concentration of 25 μM during the primary screening. This result demonstrates that the crude products were pure enough to display an activity approaching that of purified compounds, and that potential by-products did not interfere with the assay.

IC_{50} values of the newly synthesised molecules **4**–**7** were found to be very low when compared with IC_{50} values from the parent molecules, menadione and plumbagin ($IC_{50}=55$ and 28 μM, respectively, in the presence of 50 μM of trypanothione disulfide TS_2). In particular, the most promising inhibitor 3-{4-[3-(dibutylamino)propylaminocarbonyl]butyl}-5-hydroxy-2-methyl-1,4-naphthoquinone **4**[12] ($IC_{50}=0.3$ μM) displayed an activity 93-fold higher when compared with the corresponding parent molecule, plumbagin. In the presence of increasing concentrations of TS_2 (25 to 200 μM) as substrate, and molecules **4** or **7** (0 to 2 μM), an uncompetitive inhibition type was deduced from the Lineweaver–Burk plot $1/V$ versus $1/S$.[13] In order to assess the specificity of the four TcTR inhibitors with the human homologue GR, compounds **4**–**7** were tested in the GSSG reduction assay[14] by measuring IC_{50} values. The results indicated specific inhibition by the compounds **4**–**7** of the trypanosomal enzyme versus the human homologous disulfide oxido-reductase hGR. Moreover, the lead compounds were much more specific than menadione (hGR $IC_{50}=63$ μM) and plumbagin (hGR $IC_{50}=38$ μM). The selectivity of **4** was 100-fold higher (hGR IC_{50}/TcTR $IC_{50}=137$) than plumbagin (hGR IC_{50}/TcTR $IC_{50}=1.36$). However, although the IC_{50} values for TcTR of derivatives **4** and **7** were similar, the selectivity of **4** was 7-fold higher (hGR IC_{50}/TcTR $IC_{50}=137$) than **7** (hGR IC_{50}/TcTR $IC_{50}=20$). Thus, lead structures from the A and B series appear to be more promising than those from the C series.

Conclusion

In conclusion, this 1,4-naphthoquinone-based library provides an interesting new tool to access to QSAR studies. A comprehensive analysis of structure–activity relationships from the primary screening, is currently in progress.[13] Moreover, the ease with which these new potent inhibitors may be synthesized renders possible to obtain large quantities for in vitro and in vivo studies.

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12. Compound **4**: ^{1}H NMR (DMSO, 300 MHz) δ 11.97 (s, 1H), 8.94 (br s, 1H), 7.67 (t, 1H, J = 7.5, 8.5 Hz), 7.50 (dd, 1H, J = 1.1, 7.5 Hz), 7.29 (dd, 1H, J = 1.1, 8.5 Hz), 3.09–3.00 (m, 2H), 3.00–2.89 (m, 6H), 2.53 (t, 2H, J = 7.75 Hz), 2.08 (t, 2H, J = 7.25 Hz), 2.05 (s, 3H), 1.75–1.61 (m, 2H), 1.61–1.43 (m, 6H), 1.43–1.32 (m, 2H), 1.32–1.15 (m, 4H), 0.65 (t, 6H, J = 7.25 Hz); ^{13}C NMR (DMSO, 90 MHz) δ 191.7, 184.3, 173.0, 161.0, 146.7, 145.0, 137.0, 132.5, 124.3, 119.0, 115.3, 52.7, 50.6, 36.3, 35.9, 28.5, 26.5, 26.3, 25.9, 24.6, 20.2, 14.2, 13.5; HMRS m/z ($M^+ + H^+$) calcd 457.3066 for $C_{27}H_{41}N_2O_4$, found 457.3067. Compound **5:** ^{1}H NMR (DMSO, 300 MHz) δ 9.10 (br s, 1H), 8.15–8.12 (m, 2H), 7.98–7.95 (m, 2H), 3.22–3.01 (m, 2H), 3.15 (br s, 6H), 2.72 (t, 1H, J = 7.75 Hz), 2.28–2.23 (m, 2H), 2.25 (s, 3H), 1.89–1.85 (m, 2H), 1.75–1.67 (m, 6H), 1.59–1.51 (m, 2H), 1.47–1.41 (m, 4H), 1.04 (t, 6H, J = 7.25 Hz); ^{13}C NMR (DMSO, 90 MHz) δ 181.3, 180.8, 169.2, 142.9, 139.7, 130.5, 128.4, 122.4, 48.6, 46.5, 32.3, 31.9, 24.5, 22.9, 22.1, 21.9, 20.5, 16.2, 10.2, 9.2; HMRS m/z ($M^+ + H^+$) calcd 441.3117 for $C_{27}H_{41}N_2O_3$, found 441.3123. Compound **6:** ^{1}H NMR (DMSO, 300 MHz) δ 11.94 (s, 1H), 7.79–7.88 (m, 2H), 7.68 (t, 1H, J = 8.0, 1.0 Hz), 7.47 (dd, 1H, J = 8.0, 1.0 Hz), 7.27 (dd, 1H, J = 8.25, 1.0 Hz), 3.10–3.01 (m, 4H), 3.01–2.87 (br s, 12H), 2.54–2.47 (m, 4H), 2.13–2.03 (m, 4H), 1.75–1.62 (m, 4H), 1.62–1.44 (m, 12H), 1.44–1.32 (m, 4H), 1.32–1.16 (m, 8H), 1.77 (t, 12H, J = 7.25 Hz); ^{13}C NMR (DMSO, 90 MHz) δ 190.7, 184.9, 173.0, 161.3, 148.7, 147.0, 137.3, 133.0, 124.7, 119.7, 115.7, 52.5, 50.4, 36.4, 35.9, 29.7, 27.2, 26.5, 26.3, 25.4, 20.2, 14.2; HMRS m/z ($M^+ + H^+$) calcd 711.5424 for $C_{42}H_{71}N_4O_5$, found 711.5436. Compound **7:** ^{1}H NMR (acetone-d_6, 300 MHz) δ 12.01 (br s, 2H), 7.63 (t, 2H, J = 6.0 Hz), 7.56 (dd, 4H, J = 8.50, 7.50 Hz), 7.41 (dd, 4H, J = 7.50, 1.0 Hz), 7.11 (dd, 4H, J = 8.50, 1.0 Hz), 3.32 (t, 4H, J = 5.50 Hz), 3.04 (t, 4H, J = 6.50 Hz), 2.54 (t, 4H, J = 7.50 Hz), 2.24 (t, 4H, J = 7.50 Hz), 2.04 (s, 6H), 1.87 (qt, 4H, J = 6.25 Hz), 1.64 (qt, 4H, J = 7.50 Hz), 1.44 (qt, 4H, J = 8.0 Hz). ^{13}C NMR (acetone-d_6, 90 MHz) δ 190.7, 185.2, 175.8, 161.4, 146.8, 145.7, 136.7, 132.7, 125.6, 123.7, 118.8, 45.3, 35.5, 35.4, 28.3, 27.2, 26.2, 26.0, 12.3. HMRS m/z ($M^+ + H^+$) calcd 672.3285 for $C_{38}H_{46}O_8N_3$, found 672.3277.
13. Complete details of kinetic characterization of the inhibitors (inhibition type, K_I, specificity) together with the analysis of QSAR data will be published elsewhere in a full paper.
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