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Original article

Novel inhibitors of *Trypanosoma cruzi* dihydrofolate reductase

Fabio Zuccotto[a], Marketa Zvelebil[b], Reto Brun[c], Shafinaz F. Chowdhury[a], Raffaella Di Lucrezia[a], Isabel Leal[d], Louis Maes[e], Luis M. Ruiz-Perez[d], Dolores Gonzalez Pacanowska[d], Ian H. Gilbert[a,*]

[a]Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff CF10 3XF, UK
[b]Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK
[c]Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland
[d]Instituto de Parasitologia y Biomedicina, Consejo Superior de Investigaciones Cientificas, C/Ventanilla 11, E-18001 Granada, Spain
[e]Tibotec n.v., Intercity Business Park, Gen. De Wittelaan L11B 3, B-2800 Mechelen, Belgium

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Abstract – There is an urgent need for the development of new drugs to treat Chagas' disease, which is caused by the protozoan parasite *Trypanosoma cruzi*. The enzyme dihydrofolate reductase (DHFR) has been a very successful drug target in a number of diseases and we decided to investigate it as a potential drug target for Chagas' disease. A homology model of the enzyme was used to search the Cambridge Structural Database using the program DOCK 3.5. Compounds were then tested against the enzyme and the whole parasite. Compounds were also screened against the related parasite, *Trypanosoma brucei*. © 2001 Éditions scientifiques et médicales Elsevier SAS

drug design / docking / database searching / parasitic diseases

1. Introduction

Chagas' disease is caused by the parasitic protozoa *Trypanosoma cruzi* and has a wide distribution in Central and South America. It is endemic in 21 countries and according to the World Health Organisation, 16–18 million people are infected and about 100 million are at risk [1]. In about one-third of the cases, a chronic form of infection develops, causing damage to the heart, oesophagus and colon. The current treatment of Chagas' disease is based on nifurtimox or benznidazole, compounds that cause side effects and show poor clinical efficacy [1]. There is, therefore, an urgent need for the development of effective agents acting at key targets in *T. cruzi*. The present study was undertaken to investigate the opportunities that the enzyme dihydrofolate reductase (DHFR) from *T. cruzi* offers for the development of new drugs.

* Correspondence and reprints
E-mail address: gilbertih@cf.ac.uk (I.H. Gilbert).

The role of DHFR is to catalyse the NAPDH dependent reduction of dihydrofolate to give tetrahydrofolate, a central component in the single carbon metabolic pathway. The tetrahydrofolate is methylated to methylene tetrahydrofolate, which is directly involved in thymidine synthesis (assisting the methylation of deoxyuridine monophosphate to give thymidine monophosphate) and indirectly implicated in the metabolism of amino acids and purine nucleotide. Inhibition of DHFR thus prevents biosynthesis of DNA leading to cell death [2].

DHFR has already been used as a drug target for the development of anti-microbial and anti-cancer agents. Methotrexate is used in cancer chemotherapy and compounds like trimethoprim and pyrimethamine are successfully used to treat bacterial infections and malaria, respectively [3, 4]. Recently, there has been a growing interest in the possibility of targeting DHFR in other disease areas such as tuberculosis [5] and infections caused by *Pneumocystis carinii* and *Toxoplasma gondii* [6, 7].

DHFR is a successful drug target as it is an essential enzyme and because the structure of the enzyme varies subtly from species to species allowing selective drug design [2]. However, little has been done to assess DHFR as a target for the development of compounds active against Chagas' disease [8–10]. From modelling studies, the *T. cruzi* enzyme is structurally different from the corresponding human enzyme, opening up the possibility of selective drug design [11].

Most known DHFR inhibitors structurally contain a heterocyclic pyrimidine or triazine ring with amino substituents at the 2- and 4-positions [2]. A huge effort has been made to produce analogues in the attempt to optimise the activity of such compounds [2, 8–10]. However, we were interested to see if we could develop novel DHFR inhibitors with a completely different basic chemical structure as potential therapeutic agents for the treatment of Chagas' disease. To search for lead structures it was decided to computationally screen databases of compounds. The method used an automated docking/scoring procedure to generate and evaluate a set of putative ligand–protein complexes. The programme DOCK 3.5 [12], which includes both a docking algorithm and different scoring functions, was used for this purpose. The crystal structure of *T. cruzi* DHFR has not yet been solved. However, we have developed a homology model using the crystal structure of DHFR from *Leishmania major*, a related organism [11].

It was only possible to acquire and screen a limited number of compounds. Therefore, compounds highlighted during the computational screening were selected for biological testing by visual inspection of the ligand–protein interactions [13].

The selected compounds were then assayed for activity against *T. cruzi* and human DHFR and in vitro against the clinically relevant form (amastigote) of the parasite. Several analogues of active compounds were also acquired and tested. Related to Chagas' disease is African trypanosomiasis, which is also a major health problem and for which there is a similar need for new chemotherapeutic agents [14]. Modelling studies show that this enzyme has a very similar active site to the corresponding *T. cruzi* enzyme [11] and it is likely that a compound that inhibits the enzyme from one species will also inhibit the enzyme from the other species. Compounds were also assayed against the organism *Trypanosoma brucei*. The results of these assays are presented in this publication.

2. Modelling

Today the virtual screen of compound databases is, together with high-throughput screening, one of the most common strategies used in the process of finding novel leads for new targets. The method is based on the use of structural information of the protein (target) to attempt to predict the actual binding mode of a set of ligands (potential leads) in the active site.

The docking program DOCK, originally developed by Kuntz et al. in 1982 [12], was used to screen part of the Cambridge Crystallographic Database (CSD), a compendium of several thousands of molecules whose crystal structures have been experimentally determined. The CSD provides a wide range of small molecules, including several 'drug like' compounds, in conformations likely to be of low energy. In addition, the database is freely distributed. This database has been used with DOCK previously [15, 16].

A set of 56 868 compounds was rigidly docked into the folate active site of the *T. cruzi* DHFR homology model [11]. Initially, the docking results were discriminated and ranked using the DOCK contact score function which is based simply on the heavy atom contacts between the ligand and the receptor in the putative complex.[1]

As DHFR is present in both the protozoan parasites and man, the efficacy of most of the compounds is based on selective inhibition of the parasite enzyme against the human enzyme. In the context of our project, the ideal lead compound should hence display a greater affinity for the *T. cruzi* DHFR than for the human DHFR. Although the problem of selectivity is usually addressed in the later stage of lead optimisation, we tried to gain some information about the potential selectivity of the compounds by repeating the docking/scoring procedure using the human DHFR structure. For each compound, the difference in contact scores obtained for the binding to the two enzymes was taken as indication of selectivity (the larger the difference, the more selective a compound is likely to be).

[1] The program, DOCK3.5, offers other scoring functions based on non-bonded interaction terms of force fields. However, such scoring functions, in addition to the standard 3D coordinates, require point charge calculation for both protein and the ligands and additional information on ligand atom type and atomic charges. Obtaining such data for the entire CSD database was computationally very expensive.

The 100 compounds with the best dock contact score were selected as a working set and with the assistance of molecular graphics were visually examined in the context of the *T. cruzi* DHFR folate active site. Interestingly, the ligand showed a remarkable diversity of molecular architectures and mode of binding to the protein. No 2,4-diaminopyrimidine compounds were present in the working set.

Each compound was examined on the basis of the following criteria:

1. How well the compound might interact with the active site in the putative ligand receptor complex. Particular relevance was given to the space occupied by the ligand in the active site and to the H-bonds, π-stacking and hydrophobic interactions between the protein and the ligand. Compounds that did not show interactions with the active site residues and compounds that did not fit deep in the active site were given a low score.
2. How good a drug candidate a compound is likely to be. Highly charged and highly functionalised compounds were treated as poor drug candidates as they might give problems in cellular permeation. Similarly, poorly functionalised compounds were not considered to be good drug candidates as they could display solubility problems. Rigid compounds were preferred to flexible ligands as the latter may have an entropic barrier to binding. Compounds likely to cause toxicity, such as polycyclic aromatics, were given a low score. Another important factor that was taken into consideration was the synthetic accessibility of a compound. In this project, complex compounds were not considered good possible leads, as it would not have been synthetically easy to produce a series of analogues for future structure–activity studies.

Each compound involved in one of the 100 complexes analysed as above was given a score (Visual Score, VS) ranging from 0 (poor drug candidate) to 6 (good drug candidate). To provide some degree of objectivity, this process was completed independently by two different persons. Only compounds with VS greater than or equal to 4 were further considered (36 in total).

3. Biological assays

Compounds stored in the CSD, with few exceptions, are not commercially available. This considerably limited the compound acquisition process for biological testing. However, a reasonable number of compounds was obtained. Thus compounds **1** [17], **2** [18], **3** [19], **4** [20], **5** [21], **6** [22], **7** [23], **8** [24] (*table I* and *figure 1*) were acquired. In addition, several analogues of these compounds or other compounds in the CSD were obtained and assayed (**9** [17], **10** [25], **11** [20], **12** [23], **13** [23], **14** [26], **15** [27], **16** [27]) (*table II* and *figure 2*). In a few cases, very little material was available for assay and in those cases only the in vitro assays were carried out and not the enzyme assays.

Table I. Compounds selected for assay based on contact score and visual score and enzyme assay data [a].

Compound	CSD code	Visual score	Contact score ranking	Difference in contact score [b]	IC_{50} *T. cruzi* DHFR (μM)	IC_{50} human DHFR (μM)
1	SUBSUD	6	8	160	40%/15	25%/15
2	DORJOJ	5	50	52	80	
3	JONWOY	4	4	81	n.d.	
4	PESYOB	4	9	51	>200	18%/75
5	BZILBY	4	14	85	>100	
6	FIVREH	4	25	26	95	
7	SARXEO	4	69	84	100	
8	GIJPUK	4	93	26	38%/90	
MTX					$K_i = 0.038$ nM	$K_i = 0.179$ nM
PYR					$K_i = 0.098$ μM	$K_i = 0.12$ μM
TMP					$K_i = 1.0$ μM	$K_i = 1.38$ μM

[a] MTX = methotrexate; PYR = pyrimethamine; TMP = trimethoprim. Data for these compounds shown as K_i [8].
[b] Contact score for *T. cruzi* less contact score for human. The larger this number the more selective the compound is predicted to be. Where it has not been possible to calculate the IC_{50} (usually due to solubility problems), a percentage inhibition at a given concentration is indicated.

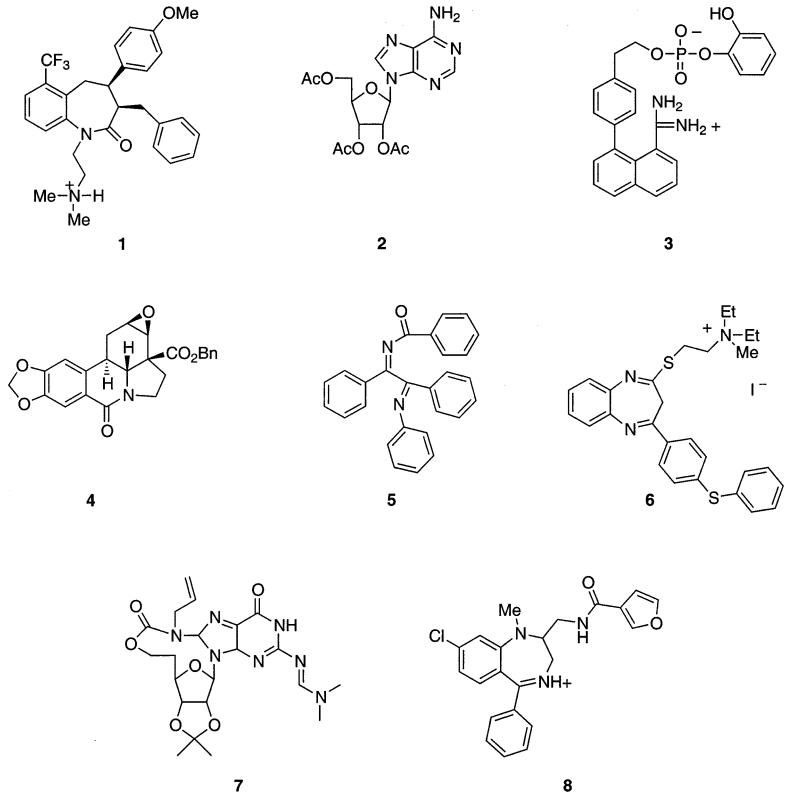

Figure 1. Initial compounds selected from CSD.

3.1. Enzyme assays

Compounds were assayed against recombinant *T. cruzi* DHFR-TS and, in some cases, recombinant human DHFR [28]. For the compounds selected from the CSD database, the assay results are shown in *table I* and the results for their analogues are presented in *table II*. Of the 12 compounds tested against the enzyme, three displayed activity (IC_{50}) of less than 100 μM. Compound **1**, previously investigated as a possible calcium channel-blocker [17], showed the greatest activity (40% enzyme inhibition at 15 μM) and some degree of selectivity for the parasite enzyme (25% inhibition of human DHFR at 15 μM). Interesting results were also obtained for compound **2**, an adenosine derivative (IC_{50} 80 μM), and compound **6**, a benzodiazepine derivative studied for its anti-bacterial activity (IC_{50} 95 μM) [22]. The other molecules showed lower activity against the enzyme (with IC_{50} values equal to or greater than 100 μM). The IC_{50} values were measured using a dihydrofolate concentration of 30 μM in all assays.

3.2. In vitro assays

Compounds were also tested in vitro against the whole parasite. *T. cruzi* has a multistage lifecycle [1, 29]. The vector is a reduviid bug, which releases infective stages in the faeces. The parasite enters the mammalian host and transforms into the intracellular amastigote

Table II. Enzyme assay data for analogues of compounds selected.

Compound	Analogue of	IC_{50} *T. cruzi* DHFR (μM)
9	**1**	n.d.
10	GEGSUG [a]	n.d.
11	**4**	31%/60
12	**7**	34%/90
13	**7**	>100
14	PIKYOZ [b]	n.d.
15	EMCPTP [c]	45%/90
16	EMCPTP [c]	13%/80

[a] Visual score = 4, contact score ranking 76.
[b] Visual score = 4, contact score ranking 69.
[c] Visual score = 6, contact score ranking 45.

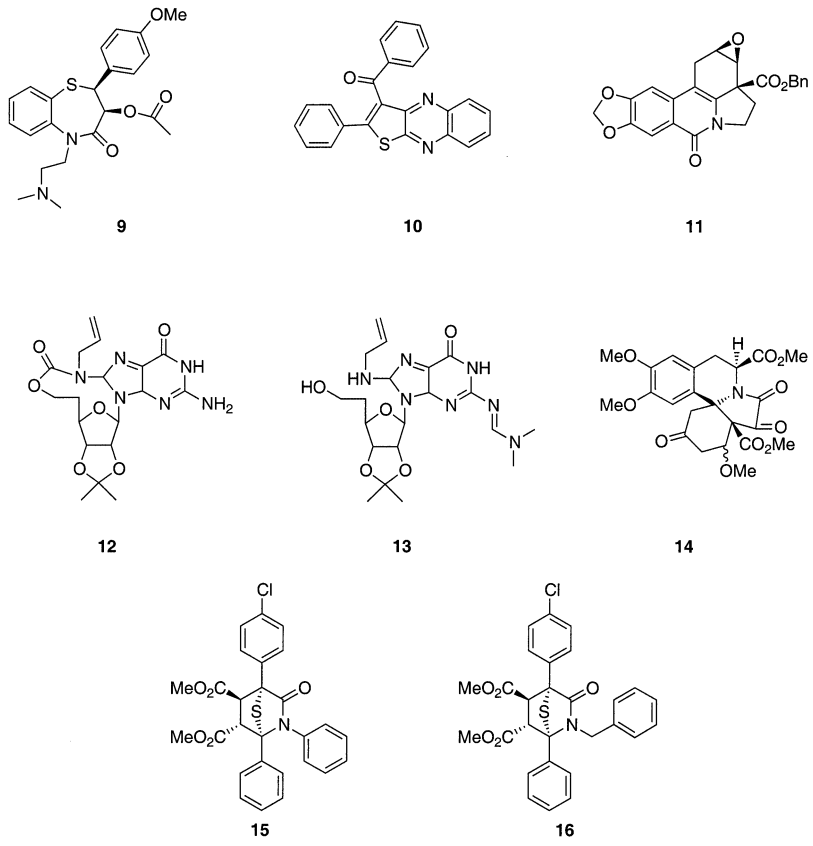

Figure 2. Analogues of compounds selected from CSD.

form. The amastigotes release trypomastigotes into the blood stream, which invade other cells and are transmitted to the vector during a blood meal, completing the lifecycle. Therefore, a drug must be able to destroy the amastigote form and hence the compounds were assayed against the amastigotes cultured in macrophages (*table III*). Compounds were also assayed against the related protozoan parasite *Trypanosoma brucei rhodesiense*. Tests were carried out against L-6 cells (rat skeletal muscle myoblasts) as a measure of cytotoxicity.

3.3. T. cruzi

Most of the compounds tested showed little activity against *T. cruzi* amastigotes (*table III*). The most promising compounds against *T. cruzi* amastigotes were **1** and **6**. The cytotoxicity values on L-6 cells are clearly above the pharmacologically active concentration, suggesting that the effect of **1** and **6** is not just general cytotoxicity, but some specific cellular action.

3.4. T. brucei rhodesiense

Compounds were also assayed in vitro against *T. brucei rhodesiense* as the trypomastigote form (*table III*). Interesting results were obtained for compounds **1** and **6** that showed IC_{50} values below 5 μM.

4. Further investigation of lead compounds

The two most active hits from the enzyme assay data and the parasite screens were **1** (supplied by Bristol Myers Squibb) and **6** (supplied by Recordati). In the case of compound **1**, the in vitro activity against the intact *T. cruzi* (and possibly the other parasites) may be the result of inhibition of DHFR. However, in the case of compound **6**, this is unlikely to be the case, as activity against the intact parasite was stronger than activity against the enzyme.

It was possible to acquire analogues of these compounds for further assays (*figures 3* and *4*). The

results of the tests of analogues of **1** are shown in *table IV* and those of **6** in *table V*.

4.1. Analogues of *1*

All analogues showed a lower enzyme activity than the lead molecule **1** (*table IV*). Against intact parasites, the activity against *T. cruzi* amastigotes and *T. brucei rhodesiense* was broadly similar to that of the lead, **1**. The compounds vary according to substitution at a number of different positions on the seven-membered lactam ring and the aromatic ring (positions a, b, c and d in compound **1**, *figure 3*). Generally there are no major effects on activity with little variation between compounds.

Thus compounds **1** and **17**–**21** show the effect of alteration of the substituent at the a-position (benzyl, acetate, allyl, hydroxyl and methyl). There is little variation on activity against *T. cruzi* or *T. brucei*. However, compound **18** is slightly more active than the others, whilst compounds **19** and **21** are less active.

Inversion of the stereochemistry of **17** at the a-position (**21**), b-position (**23**) or both a- and b-positions (**24**) has small effects, with compound **24** being the most active of all the four isomers against both *T. cruzi* and *T. brucei* and compounds **21** and **23** being slightly less active.

Table III. In vitro activity of compounds against *T. cruzi* and *T. brucei rhodesiense* [a].

Compound	*T. cruzi*	*T. brucei rhodesiense*	L-6 cells
	MIC (μM)	IC_{50} (μM)	MIC (μM)
1	54	3.6	>163
2	n.a.	n.a.	>200
3	n.a.	12.4	>71
4	n.a.	n.a.	>200
5	n.a.	38.6	>85
6	55	1.0	>166
8	n.a.	33.5	>200
9	n.a.	48	>73
10	n.a.	n.a.	>200
12	n.a.	n.a.	>200
13	n.a.	n.a.	>200
14	n.a.	n.a.	>222

[a] Data against *T. cruzi* amastigotes and *T. brucei rhodesiense* trypomastigotes. Cytotoxicity data against L-6 cells, a rat skeletal myoblast cell line, is included; n.a. = not active (MIC against *T. cruzi* >70 μM; IC_{50} against *T. brucei rhodesiense* > 100 μM). Controls *T. cruzi*, benznidazole, MIC = 14.2 μM. *T. brucei rhodesiense*, melarsoprol, IC_{50} = 6.1 nM. Results are the mean of one experiment run in duplicate.

Replacement of the methoxy group with a hydroxy group on the aromatic ring has little effect (compare **20** and **22**).

Compounds **25**, **26** and **27** show variation at the c-position. In **25**, the CF_3 substituent is replaced by a methoxy group and in **26** and **27** the c-substituent has been moved and replaced by chlorine in the case of **27**. These changes cause minor reduction in the activity against *T. brucei*.

The final changes occur on the d-position (compounds **28** and **29**). Removal of one methyl group has little effect (**28**) although removal of the whole side chain (**29**) leads to a decrease in activity against *T. brucei*.

The set of analogues investigated only explore a limited portion of the pharmacophoric space available and more work is needed to have a complete SAR.

It is also interesting to compare with the data for **9**, which is a thio-analogue of **1**. This compound has a very low activity, suggesting that the presence of the thio group may have a detrimental effect on activity.

4.2. Analogues of *6*

Most analogues showed similar activity to the lead, **6** (*table V*). All analogues, except **31**, showed a similar activity against *T. cruzi* DHFR. Compounds not having a large lipophilic group attached to the phenyl substituent in either the *meta* or *para* positions (**30**, **31**, **32**) appeared to have lower in vitro activity against *T. cruzi*. Against *T. brucei rhodesiense*, **32** appeared to have a lower activity, whilst the remaining compounds assayed had similar activities.

4.3. In vivo testing

Compound **20** was shown to be the most active compound against *T. brucei rhodesiense*. An in vivo experiment was undertaken using an acute rodent model of African trypanosomiasis. However, dosing at 40 mg kg^{-1} day^{-1} for 4 days had no effect on the course of the infection and on the mean survival day.

5. Conclusions

The identification of novel non-2,4-diaminopyrimidine lead structures showing activity against the *T. cruzi* DHFR was successfully accomplished by computer-aided screening techniques. Despite the small

Compound	Substituent at a (R^1)	Stereochemistry	Substitutent at b (R^2)	Stereochemistry	Counter-ion
1	CH_2Ph	R	4-MeO-Ph	R	Fumarate
17	OAc	R	4-MeO-Ph	S	Chloride
18	$CH_2CH{=}CH_2$	S	4-MeO-Ph	R	Chloride
19	OH	R	4-MeO-Ph	S	Chloride
20	Me	R	4-MeO-Ph	R	fumarate
21	OAc	S	4-MeO-Ph	S	Chloride
22	Me	S	4-HO-Ph	R	Chloride
23	OAc	R	4-MeO-Ph	R	Chloride
24	OAc	S	4-MeO-Ph	R	chloride

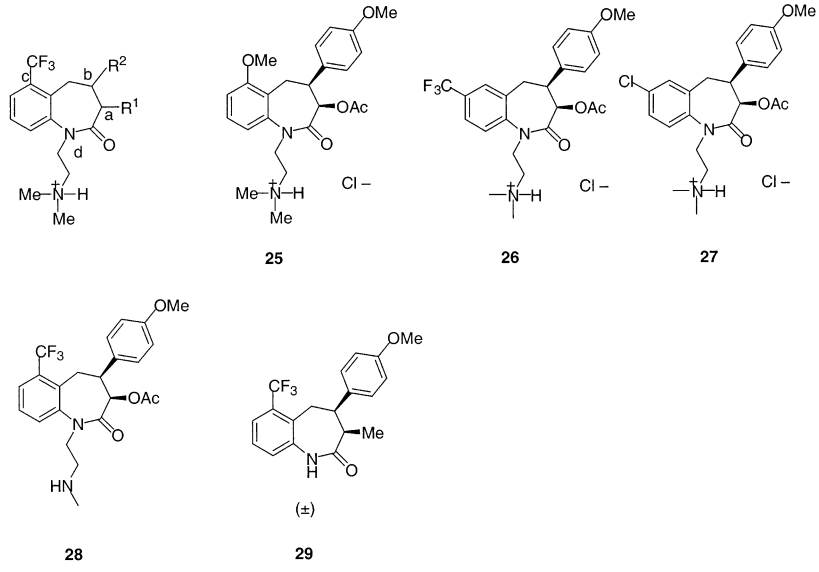

Figure 3. Analogues of compound **1**.

number of compounds tested, three molecules (compounds **1**, **2** and **6**) that showed moderate enzyme inhibition activity ($IC_{50}<100$ μM) were identified. Compound **1** appeared to show selectivity for the parasite enzyme (several times more active against *T. cruzi* than human). The in vitro behaviour of the available compounds was also considered. Interestingly, some in vitro activity against *T. cruzi* was observed for both compounds **1** and **6** (MIC 54 and 55 μM, respectively).

Compounds derived from this type of approach are unlikely to be final drug candidates but rather the starting point for successive lead optimisation programmes. Therefore, we were interested to investigate, where possible, the enzyme/in vitro activity of analogue compounds. A number of analogues of compounds **1** and **6** were obtained and assayed against the enzyme and the intact parasite. None of these compounds showed better enzyme inhibition than the parent compounds although similar in vitro activity against intact parasites was observed for most of the compounds.

Compounds were also assayed against the related organisms *T. brucei rhodesiense* and were found to have higher activity, in particular compounds **1** and **6**, which showed low micromolar activity. This higher activity against the intact parasite is probably due to the easier access of compounds to *T. brucei rhodesiense*. This organism is cultured directly in the medium, whilst the *T. cruzi* is cultured as amastigotes inside macrophages. Again, analogues of **1** and **6** were investigated. Similar in vitro activities were obtained for these analogues. One compound was investigated in vivo in a rodent model of infection, but there was no activity.

Whilst compound **1** inhibits *T. cruzi* DHFR, it is unclear if this is the primary in vitro mode of action of the compound. Compound **6** shows little enzyme inhibition, but shows activity against the organism in vitro. It is probable that the mode of action of this compound is not by inhibition of DHFR.

6. Experimental

6.1. Modelling

Modelling was carried out using Silicon Graphics workstations (Indigo 2 and O2) running Irix.

The starting structure used was the homology model of *T. cruzi* DHFR generated from using the structure of *L. major* DHFR as a template [11]. DOCK3.5 was used to carry out the database search and the October 1995 release of the CSD was used. Only organic compounds were selected from the database, giving a subset of 60 197 compounds that were saved in FDAT format. These were converted into the file format required for DOCK called DOCKDB [30] by the program MKDB [30]. Not all compounds were successfully converted, giving rise to a database of 56 868 structures. In this step, only the largest molecule in each entry was written out; some of the entries in the CSD contain counter-ions and solvent molecules. Only the folate active site was considered in the search. In order to facilitate identification of the active site, the universal DHFR inhibitor and close analogue of the enzyme substrate, methotrexate, was modelled into the active site. This was achieved by least-squares fitting of the backbone atoms of the model of *T. cruzi* DHFR with those of *L. major* using the program PROFIT [31]. The structure of *L. major* DHFR contains methotrexate in the active site, allowing it to be modelled into the active site of the *T. cruzi* enzyme. All

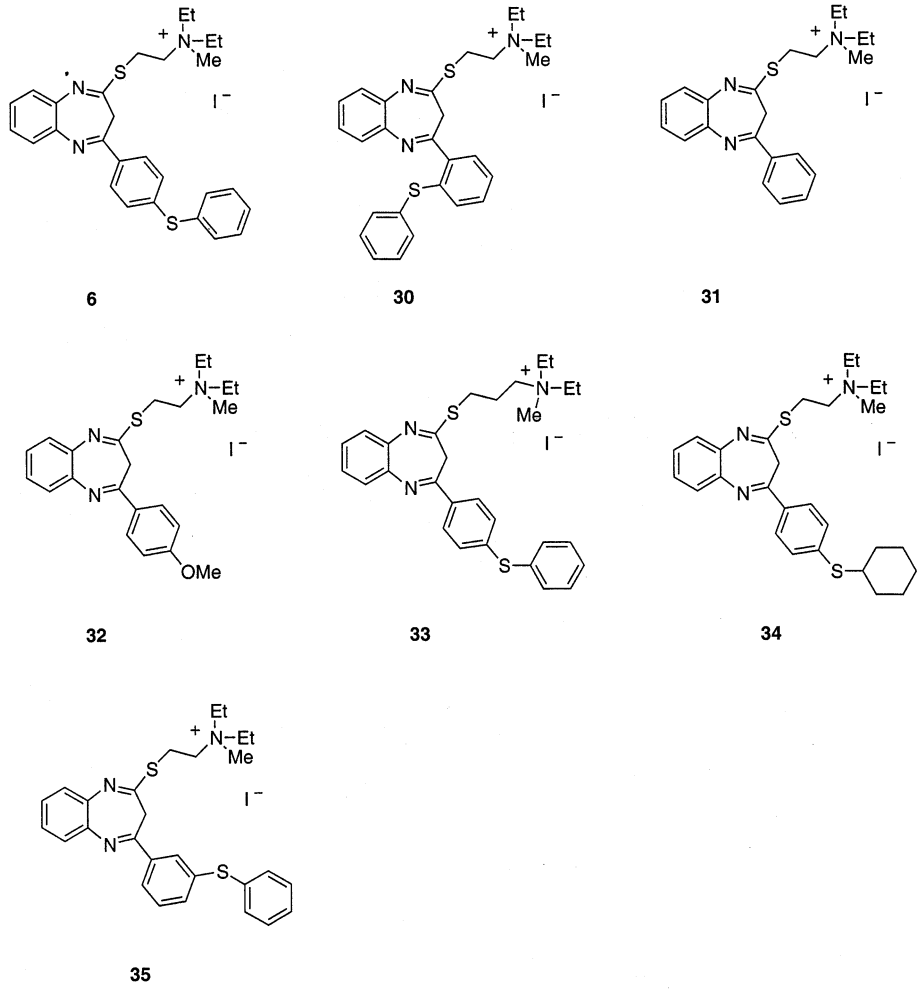

Figure 4. Analogues of compound **6**.

Table IV. Activity of analogues of **1** [a].

Compound	*T. cruzi* DHFR enzyme inhibition (μM)	*T. cruzi* amastigotes IC_{50} (μM)	*T. brucei rhodesiense* trypomastigotes IC_{50} (μM)	L-6 cells MIC (μM)
1	40%/15	n.d.	3.6	>163
17	31%/90	30.1	4.2	180
18	29%/75	9.7	4.2	62
19	32%/75	37.8	12.4	196
20	31%/75	29.8	1.6	56
21	32%/75	45.8	20.4	180
22	36%/75	22.6	2.2	68
23	27%/75	30.0	24.9	180
24	32%/75	16.4	2.9	60
25	31%/75	16.7	10.0	194
26	35%/75	29.8	11.5	180
27	34%/75	43.4	7.6	193
28	28%/68	23.7	1.5	66
29	35%/68	17.7	75.1	86

[a] Data against *T. cruzi* amastigotes and *T. brucei rhodesiense* trypomastigotes. Cytotoxicity data against L-6 cells, a rat skeletal myoblast cell line, is included; n.d. = not determined. Controls *T. cruzi*, benznidazole, $IC_{50} = 2.5$ μM. *T. brucei rhodesiense*, melarsoprol, $IC_{50} = 6.1$ nM. Results are the mean of one experiment run in duplicate.

Table V. Activity of analogues of **6** [a].

Compound	*T. cruzi* DHFR enzyme IC_{50} (μM)	*T. cruzi* amastigotes IC_{50} (μM)	*T. brucei* trypomastigotes IC_{50} (μM)	L-6 cells MIC (μM)
6	95	n.d.	1.0	>166
30	122	63	3.5	150
31		88	n.d.	>182
32	150	50.7	22.7	>172
33	67	15.4	5.1	>16
34	38%/75	23.7	2.2	>16
35	38%/75	22	3.2	>48

[a] Data against *T. cruzi* amastigotes and *T. brucei rhodesiense* trypomastigotes. Cytotoxicity data against L-6 cells, a rat skeletal myoblast cell line, is included; n.d. = not determined. Controls *T. cruzi*, benznidazole, $IC_{50} = 2.5$ μM. *T. brucei rhodesiense*, melarsoprol, $IC_{50} = 6.1$ nM. Results are the mean of one experiment run in duplicate.

residues within 7 Å of methotrexate were selected with the program GET_NEAR_RES [30] and the rest of the protein excluded using INVERTPDB [30]. The molecular surface of the binding site was calculated using the Connolly algorithm [32] as implemented in AUTOMS [30] (using a surface density of three surface points per $Å^2$ and a probe radius of 1.4 Å). The active site shape was characterised by a set of overlapping spheres generated by SPHGEN [30]. Optimisation of the DOCK sphere models was done by manual modification of the first cluster calculated by SPHGEN. DOCK [30] was then used to score the compounds in the database (using contact scoring). Scoring of compounds required a pre-calculated scoring grid [33]. A cube defining the volume for which the grid needed to be calculated was generated and the grid generated by DISTMAP [30]. In general, default settings were used for the DOCK program.

6.2. Compounds

Compounds were supplied as follows: **2** (Sigma–Aldrich); **1** and **17**–**29** (Bristol Myers Squibb — USA); **11** and **4** (Prof. Arthur G. Schultz, Rensselaer Polytechnique Institute, USA); **7**, **12** and **13** (Prof. K. Minamoto, Nagoya University, Japan); **15** (Prof. M. Baudy-Floc'h, University of Rennes 1, France); **8** (Solvay Pharmaceuticals, Germany); **5** (Dr Anthony Woodhouse, IRS, New Zealand); **14** (Dr Shinzo Hosoi, Kanazawa University, Japan); **6** and **30**–**35** (Recordati Pharmaceuticals, Italy); 3 (Dr G. Muller, University of Geneva, Switzerland); **9** (Fluka); and **10** (N. Otto).

6.3. Enzyme assays

Recombinant DHFR-TS from *T. cruzi* was purified according to the method of Reche et al. [28]. Recombinant human DHFR was a gift of Drs R. Ridley and Dietrich Stuber, Roche Pharmaceuticals.

DHFR assays were carried out at 28°C by measuring the decrease in absorbance due to NADPH at 340 nm in a Hewlett–Packard model 8543 spectrophotometer [8, 10]. Standard assay mixtures contained 50 mM TES pH 7.0, 75 mM mercaptoethanol, 1 mM EDTA, 1 mg mL^{-1} BSA, 0.1 mM NADPH and 30 μM dihydrofolate in a final volume of 1 mL. Enzyme concentrations were 0.109 and 10 ng mL^{-1} for the *T. cruzi* and human enzymes, respectively. Typically five to eight different concentrations of inhibitor were used. In each case, a control was carried out which contained all reagents except the inhibitor. Dihydrofolate was prepared from folic acid by reduction with sodium dithionite according to the method of Futterman [34].

6.4. In vitro testing

6.4.1. T. cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells per well per 100 μL in RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) were added in 100 μL per well with two times of a serial drug dilution. The plates were incubated at 37°C in 5% CO_2 for four days. After 96 h, the minimum inhibitory concentration (MIC) was determined microscopically. For measurement of the IC_{50}, the substrate CPRG/Nonidet was added to the wells. The colour reaction which developed during the following 2–4 h was read photometrically at 540 nm. From the sigmoidal inhibition curve, IC_{50} values were calculated. Cytotoxicity was assessed in the same assay using non-infected L-6 cells and the same serial drug dilution. The MIC was determined microscopically after four days. Each experiment was run in duplicate and the result is the mean of these.

6.4.2. T. brucei rhodesiense

Minimum essential medium (50 μL) supplemented according to Baltz et al. [35] with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 50 μL of trypanosome suspension (*T. brucei rhodesiense* STIB 900) was added to each well and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 h. Alamar Blue [36] (10 μL) was then added to each well and incubation continued for a further 2–4 h. The plate was then read with a Millipore Cytofluor 2300 using an excitation wavelength of 530 nm and emission wavelength of 590 nm. Fluorescence development was expressed as percentage of the control, and IC_{50} values determined.

6.5. In vivo testing: T. brucei rhodesiense acute mouse model

Mice were infected i.p. with *T. brucei rhodesiense* STIB 900 (day 0). The mice were treated with 40 mg kg^{-1} i.p. on four consecutive days (days 1–4). Tail blood was examined for parasites twice a week until day 30. A group of untreated mice was used as controls and to determine the mean survival day.

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