Journal of Computer-Aided Molecular Design, **15:** 465–475, 2001.
KLUWER/ESCOM

Selective inhibition of 6-phosphogluconate dehydrogenase from *Trypanosoma brucei*

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Received 21 September 2000; accepted 2 February 2001

Key words: docking, enzyme inhibition, selectivity, structure activity relationships, trypanosomiasis

Summary

A number of triphenylmethane derivatives have been screened against 6-phosphogluconate dehydrogenase from *Trypanosoma brucei* and sheep liver. Some of these compounds show good inhibition of the enzymes and also selectivity towards the parasite enzyme. Modelling was undertaken to dock the compounds into the active sites of both enzymes. Using a combination of DOCK 3.5 and FLEXIDOCK a correlation was obtained between docking score and both activity for the enzymes and selectivity. Visualisation of the docked structures of the inhibitors in the active sites of the enzymes yielded a possible explanation of the selectivity for the parasite enzyme.

Introduction

African trypanosomiasis is a serious disease in Sub-Saharan Africa and has recently become resurgent [1]. There is currently a crisis in trypanosomiasis chemotherapy since commercial investment in new generation drugs against this disease has more or less halted. Chemotherapeutic development has traditionally depended on some inspiration and lots of trial and error. The revolution in molecular and structural biology has led to optimism that much of the trial and error may be removed from drug design [2]. As more information becomes available on both the structure of microbial enzymes, and also the constraints imposed upon small molecule binding, it is hoped that a new generation of rationally devised chemotherapeutically active reagents may emerge. Trypanosome enzymes are now the subject of studies investigating whether structural approaches to chemotherapy will be of practical use. There are a number of different types of computational methodologies used in the design of potential ligands/ inhibitors to an enzyme active site. For example programmes such as DOCK [3] are used to dock potential ligands in the active site and evaluate or "score" how well the ligand binds; programmes such as GRID [4] and XSITE [5] can predict where a particular functional group will bind in the active site and can be used to design new ligands or modify existing ones; and other programmes such as LEAPFROG [6] allow *de novo* ligand design in the active site. Due to our interest in the *T. brucei* 6-PGDH as a potential target for chemotherapy we have used this as a model to try and correlate data from inhibitor studies of this enzyme with predictions about the binding of these reagents.

The enzyme 6-phosphogluconate dehydrogenase (6-PGDH) is the third enzyme in the pentose phosphate pathway [7] that is responsible for generation of NADPH and of ribose 5-phosphate. In particular the enzyme carries out the oxidative decarboxylation of 6-phosphogluconate to give ribulose 5-phosphate. It uses $NADP^+$ as a cofactor that is reduced to NADPH during the reaction.

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It has been speculated that this enzyme is a potential drug target in African trypanosomiasis. There are a number of factors that lead to this reasoning. In eukaryotes deletion of the gene for 6-phosphogluconate dehydrogenase is lethal [8, 9]. This enzyme is part of a key pathway for the parasite as they require a large supply of NADPH as part of the protective system against oxidative damage [10]. In addition, in the host stage of the life-cycle of the parasite, the parasite does not undergo aerobic respiration, but relies exclusively on glycolysis for its energy supply [11]. Inhibition of 6-PGDH will lead to increased levels of 6-phosphogluconate which is a potent inhibitor of glycolysis; 6-phosphogluconate inhibits the enzyme phosphoglucose isomerase [12], leading to increased levels of glucose 6-phosphate and hence increased levels of 6-phosphogluconate which will inhibit phosphoglucose isomerase further initiating a toxic positive feedback loop.

There is also promising data from structural and inhibitor studies. The crystal structure of the *T. brucei* enzyme shows differences to that of the sheep enzyme (the mammalian enzyme for which there is a crystal structure), which it may be possible to exploit for selective inhibitor design [13]. Inhibitors have been found that are selective for the enzyme from *Trypanosoma brucei* (the causative organism of African trypanosomiasis) [14, 15]; in particular 2-deoxy-6-phosphogluconate. In addition various trypanocidal drugs have been shown to be inhibitors of the *T. brucei* enzyme [14], although it is not clear whether this is significant in the effect of the drugs *in vivo*.

We were interested to investigate the activities of various compounds against the *T. brucei* and sheep enzymes and then to try and rationalise the data. In work previously described, a number of anti-trypanosomal drugs and related compounds were investigated as inhibitors of 6-PGDH [14]. Amongst these compounds, were several triphenylmethane derivatives, including bromocresol purple and sulfobromophthalein. Triphenylmethane derivatives were first investigated as potential trypanocides nearly a hundred years ago, and considerable research was carried out into parafuchsin, although unacceptable host toxicity precluded further development of this class of compound [16]. In this paper a more extensive series of these derivatives was investigated in order to build up a structure activity relationship. As crystallographic information is available form both the *T. brucei* [13] and sheep enzymes [17], it was decided to try and use this to rationalise the experimental data, with the aim of being able to design more potent and selective inhibitors.

Materials and methods

Enzyme assays

Chemicals and reagents were purchased from Sigma-Aldrich. The structures of the compounds assayed are shown in Figure 1. Purification of the *T. brucei* and sheep liver 6PGDH were as reported [15]. For the *T. brucei*, enzyme, K_m for 6-phosphogluconate = 3.5 μM; K_m for $NADP^+$ = 1 μM [14]; for the sheep liver enzyme, K_m for 6-phosphogluconate = 19 μM; K_m for $NADP^+$ = 5 μM [18]. The assays were performed at 20 °C and followed spectrophotometrically (measuring the production of NADPH) at 340 nm using a Kontron Uvikon 930 spectrophotometer. Compounds were dissolved in either methanol (triphenylmethane, triphenylmethanol and rosolic acid), acetone (crystal violet, tetrabromophenolphthalein, tetrabromophenolphthalein ethyl ester and bromocresol purple) or water. The assays were carried out by adding 10 μl of the inhibitor solution to 1 ml of the assay mixture at pH 7.5 (50 mM triethanolamine-HCl, 1 mM EDTA, 250 μM NADP and a variable concentration of 6-phosphogluconate [4–60 μM]). The concentration of inhibitors was fixed (between 10 and 200 μM depending on the inhibitor) and the reaction was initiated by the addition of 0.4 μg of enzyme per ml of reaction mixture. Experiments were repeated twice at a number of concentrations. Competitive inhibition was determined using Lineweaver–Burk plots and the inhibition constant, Ki, was obtained from the equation:

$$\text{slope in the presence of inhibitor / slope in the absence of inhibitor} = 1 + [I]/Ki,$$

where $[I]$ is the inhibitor concentration used.

Molecular modelling

Molecular modelling was performed on Silicon Graphics O2 and Indigo 2 workstations running irix 6.5. The structures of the *T. brucei* [13] (at 2.8 Å resolution) and sheep [17] (at 2.5 Å resolution) 6-PGDH enzymes were supplied by Margaret Adams, University of Oxford, but can now be obtained from the Protein Data Bank (1PGJ and 1PGP respectively). A crystal structure of the sheep enzyme with the

substrate 6-phosphogluconate co-crystallised is available. To localize 6-phosphogluconate within the binding site of the *T. brucei* enzyme, the *T. brucei* and sheep structures were aligned, by selecting conserved residues in the active site. The location of substrate in the *T. brucei* enzyme was then extrapolated from its cognate position in the sheep structure. The aligned residues were as follows (RMSD of backbone atoms 0.309 Å):

Sheep	^{127}VSGG//^{183}KMVHNGIEY// ^{287}R//^{446}RDYFGAH,
T. Brucei	^{129}ISGG//^{185}KMYHNSGEY// ^{289}R//^{453}RDVFGRH.

Structures of inhibitors were drawn into the SYBYL package [6] and then minimised using the conjugate gradient method until the gradient was 0.001 kcal/mol, with the TRIPOS forcefield and the Gasteiger-Huckel charge model using a distance-dependent dielectric function. The structures were then subject to geometry optimisation by semi-empirical quantum mechanics calculations (MOPAC within SYBYL) using the PM3 Hamiltonian Method. These structures were then used for the docking experiments.

DOCK 3.5 [3] was used in single compound mode to find the optimal orientation of the compounds in the active sites of both the *T. brucei* and sheep enzymes. All residues that had at least one atom within 7Å of the substrate (6-phosphogluconate) were selected and considered part of the active site. A box identifying that area was selected and used for scoring. The dimensions of the box were 14.349 × 16.998 × 13.038 Å^3 and a grid spacing of 0.3 Å was used. Compounds were docked into the active site using contact scoring and force field scoring. The contact score is based on an empirical function resembling the Van der Waals attractive energy between ligand and enzyme. Force field scores approximate to molecular mechanics interactions energies between ligand and enzyme and consist of Van der Waals and electrostatic components. DOCK generated different orientations for each ligand within the active site; the orientation with the highest score was selected.

The binding pocket for FLEXIDOCK [6], part of the SYBYL package, was defined by selecting all residues within 4 Å of the ligand. The ligand was initially positioned in the active site using the output from DOCK 3.5 in contact scoring mode. FLEXIDOCK was then carried out.

Multiple regression analysis was carried out with TSAR, part of the Oxford Molecular Suite of software [19]. This was carried out in order to measure the correlation between predicted and experimental enzyme selectivity.

Results

Enzyme assays

Compounds were investigated for their potency as competitive inhibitors with respect to binding of substrate against 6-phosphogluconate dehydrogenases of *T. brucei* and sheep liver enzymes respectively (Table 1 and Figure 1).

Some of the compounds showed good affinity for the *T. brucei* enzyme. In particular compounds **1a**, **6, 8** and **9** had inhibition constants of less than 10 μM (see Figure 1 for structures). A series of conclusions regarding the structure activity relationship can be drawn from these data.

Simple triphenylmethanes (compounds **10** and **11**) have low activity, probably due to the lack of functional groups that can bind to the enzyme active-site, since there is little chance for hydrogen bonding or polar interactions. Compounds **12** and **13**, surprisingly, have even lower activity than the unsubstituted analogues. This may be due to the nature of the substituents. For example, the methyl groups meta to the phenolic hydroxyl groups may restrict the conformation of the molecule.

Molecules **1a**, **2**, **3**, **4**, and **9** all inhibit. Differences between these compounds relate to substitution on the phenolic moieties. The precise interpretation of this data is not clear. However, the presence of less electronegative substituents (Me or I) increased activity (compounds **1a** and **9**). More electronegative or no-substituents led to lower activity (compounds **2**, **3** and **4**).

The presence of a sulphonate group was not itself essential for binding affinity (compare **4** and **5**; **3** and **7**; **3** and **8**). Addition of electronegative substituents on the sulphonated aromatic ring did increase affinity (compare **6** and **3**).

Compounds **14** and **15** show similar binding affinity to **4** and **5**, although the functional groups and overall structure are quite different; in particular the former are positively charged at physiological pH, whilst compound **4** is negatively charged and compound **5** is neutral.

Table 1. Inhibition data for compounds against *T. brucei* and sheep 6-phosphogluconate dehydrogenase. Selectivity is defined as K_i (sheep)/ K_i (*T. brucei*)

No.	Name	*T. brucei* K_i (μM)	Sheep K_i (μM)	Selectivity
1	Bromocresol Purple	42.7	367	8.6
1a	Bromocresol Purple, sodium salt	5.7	231	40.5
2	Bromochlorophenol blue, sodium salt	35.4	154	4.3
3	Bromophenol blue, sodium salt	45.2	277	6.1
4	Phenol red, sodium salt	34.1	99.0	2.9
5	Rosolic acid	12.9	44.5	3.4
6	Tetrabromophenol blue, sodium salt	4.5	22.7	5.0
7	Tetrabromophenolphthalein	14.3	127.6	8.9
8	Tetrabromophenolphthalein ethyl ester	2.9	62.6	21.6
9	Tetraiodophenolsulfonephthalein, sodium salt	1.8	39	21
10	Triphenylmethane	80.3	158.4	1.9
11	Triphenolmethanol	117.1	198	1.7
12	Xylenol blue, sodium salt	127.3	724	5.7
13	Xylenol orange	366	1120	3.1
14	Crystal violet lactone	14.9	125.0	8.4
15	Basic Fuchsin	14.4	49	3.4
16	Brilliant green	43	1031	24.0
17	Brilliant blue G250	77	476	6.1

Table 2. DOCK and FLEXIDOCK scores for compounds in the active sites of the *T. brucei* and sheep enzymes.

No.	*T. brucei*				Sheep			
	K_i (μM)	DOCK contact score	DOCK force field score	Flexidock score	K_i (μM)	DOCK contact score	DOCK force field score	Flexidock score
1	42.7	181	ND	−190.23	367	170	ND	−100.3
1a	5.7	161	ND	−198.79	231	166	ND	−95.11
2	35.4	180	−28.5	−188.3	154	171	−23.9	−123.6
3	45.2	174	−26.94	−178.8	277	169	−23.1	−116.1
4	34.1	179	−27.32	−173.91	99.0	175	−19.78	−131.8
5	12.9	175	−29.94	−198.7	44.5	170	−25.81	−150.7
6	4.5	150	ND	−200.1	22.7	147	ND	−139.8
7	14.3	180	−28.13	−190.5	127.6	171	−25.23	−107.4
8	2.9	186	−34	−204.6	62.6	189	−29.71	−119.7
9	1.8	153	ND	−203.26	39	158	ND	−109.56
10	80.3	176	−20.1	−91.3	158.4	175	−19.78	−79.95
11	117.1	154	−24.3	−95.7	198	161	−22.1	−84.43
12	127.3	169	ND	−148.7	724	172	ND	−97.21
13[a]	366	ND	ND	ND	1120	ND	ND	ND
6PG		154	−38.5	−296.4		156	−38.1	−293.7

6PG is 6-phosphogluconate.
[a]Compound **13** did not DOCK into the active site.

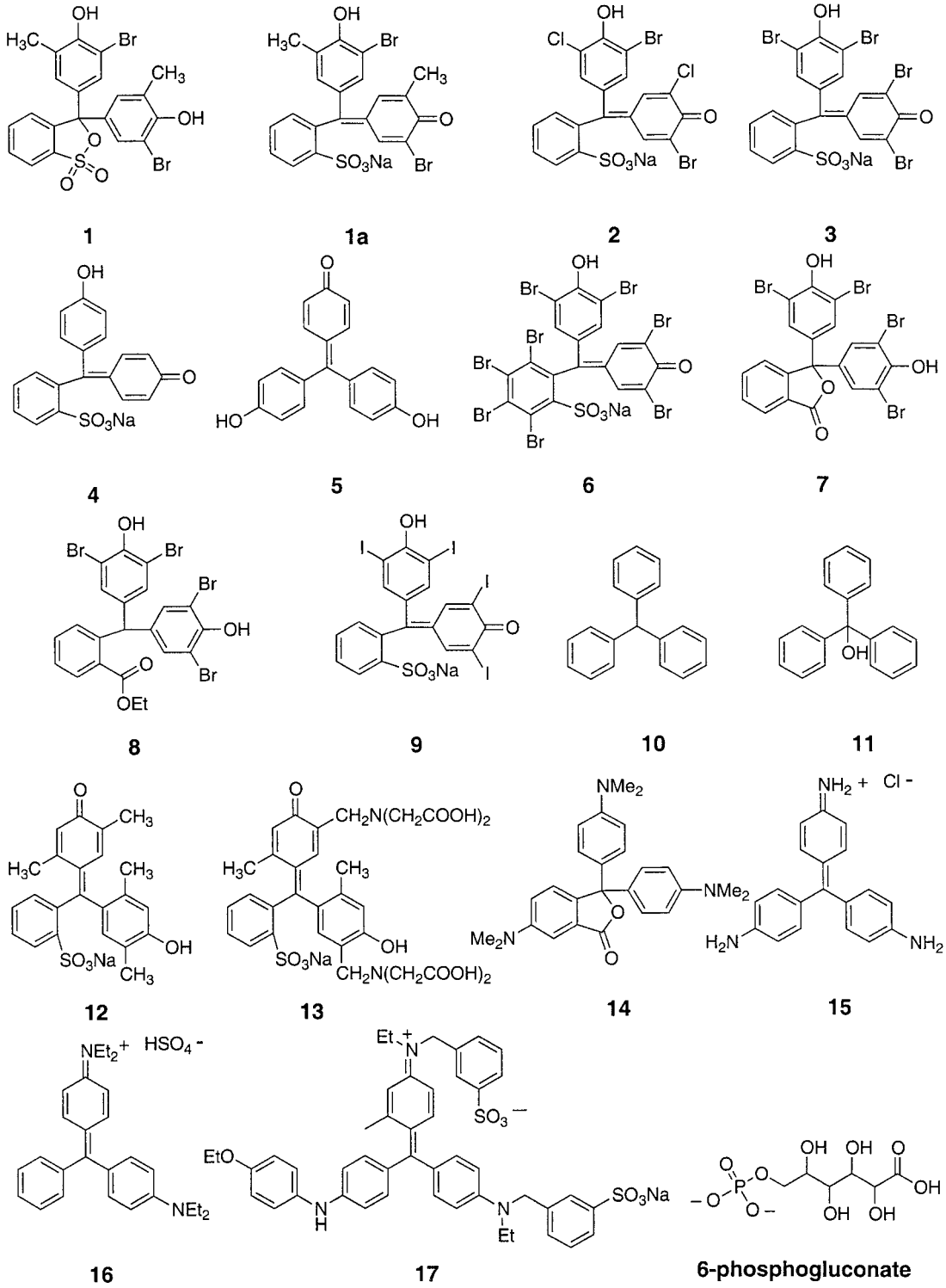

Figure 1. Structures of compounds investigated as inhibitors of 6-phosphogluconate dehydrogenase

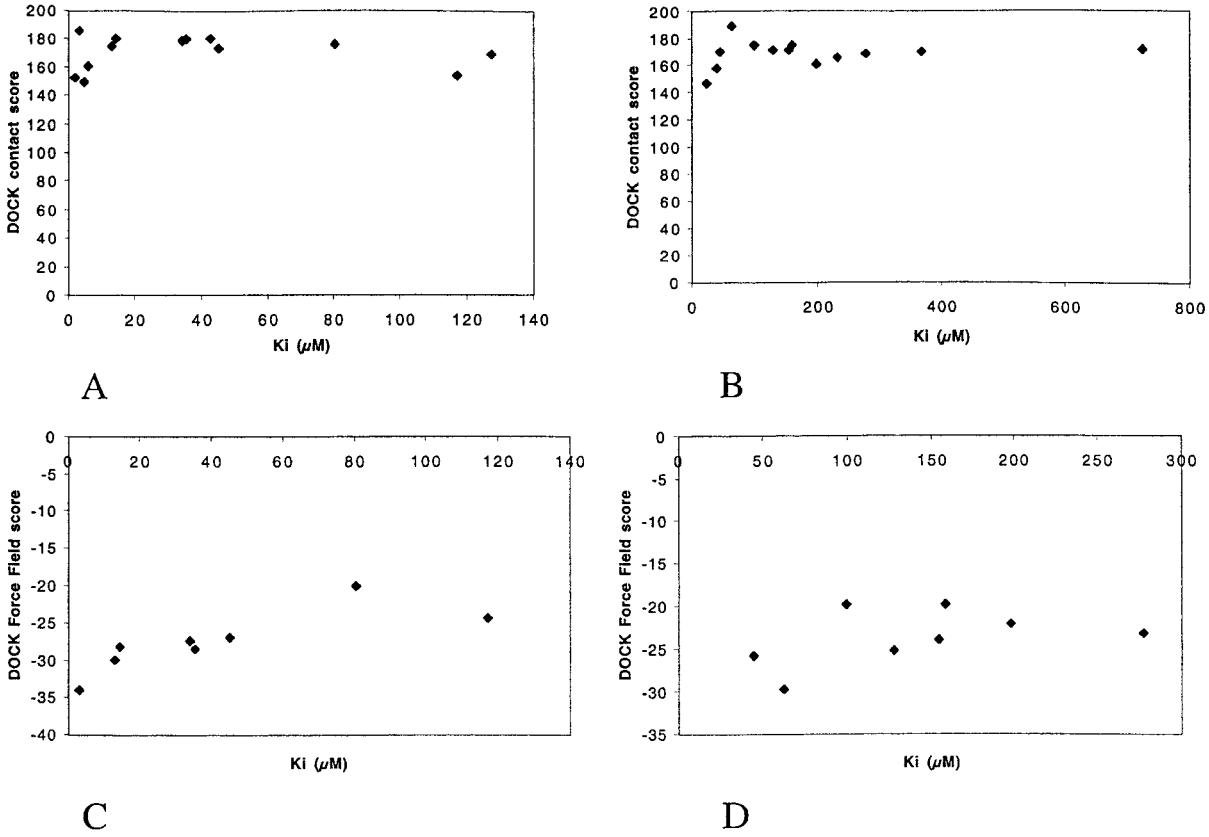

Figure 2. Correlation between experimentally determined inhibition constant *Ki* and DOCK scores for (A) contact score with *T. brucei* 6-phosphogluconate dehydrogenase; (B) contact score with sheep 6-phosphogluconate dehydrogenase; (C) force field score with *T. brucei* 6-phosphogluconate dehydrogenase; (D) force field score with sheep 6-phosphogluconate dehydrogenase.

The difference in binding affinity between bromocresol purple (**1**) and bromocresol purple sodium salt (**1a**) is intriguing, since the ring-opened sodium salt appears to be more active than the closed ring compound. However, under the assay conditions employed here, it would be expected that compound **1** would be converted to the sodium salt (**1a**). It appears this has not occurred or has only occurred partially with the more negatively charged sodium salt having superior activity. The predicted binding conformation for the closed-ring compound (**1**) is slightly different to that of the ring-opened compound (**1a**).

All of the triphenylmethane analogues assayed were selective for the parasite enzyme. The most selective compound was bromocresol purple, compound **1a**, which had a selectivity of about 40 fold; other notable compounds were **8, 9** and **16** (tetrabromophenolphthalein ethyl ester, tetraiodophenolsulfonephthalein, and brilliant green) which showed over 20-fold selectivity. The compounds were also shown to be competitive inhibitors with the substrate, 6-phosphogluconate, which implies that the compounds could be binding in the same binding site as the substrate.

Modelling

Structural data for both the *T. brucei* and sheep enzymes is available, hence it was decided to see if a model could be deduced to explain the binding of compounds in both a qualitative and if possible quantitative manner. To achieve this the structures of the compounds were firstly minimised using quantum mechanics calculations, and then docked into the active site.

Docking was achieved using the programme DOCK 3.5, which docks the compounds into the active site as a rigid body. The programme has two docking modes. In the contact mode, compounds are docked into the active site in optimum orientation according to steric parameters (Table 2). All of the

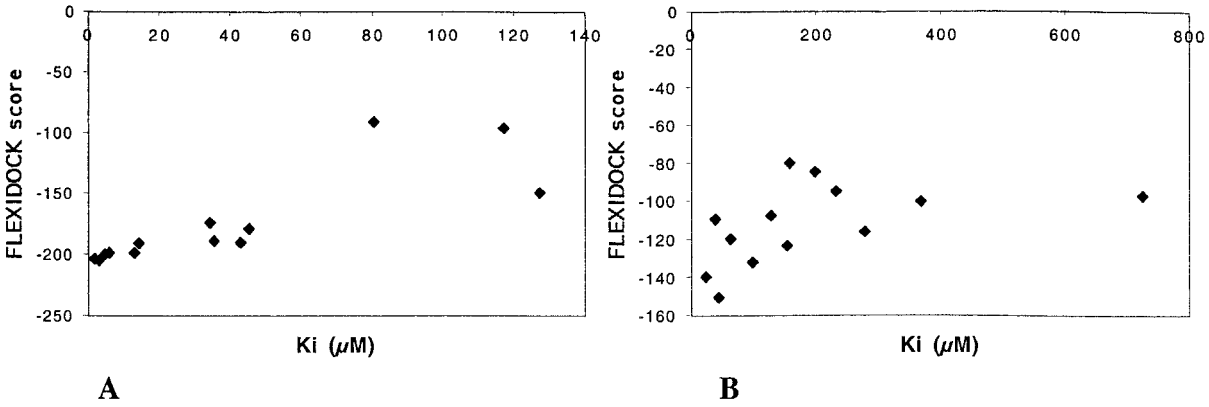

Figure 3. Correlation between experimentally determined inhibition constant K_i and FLEXIDOCK score for (A). *T. brucei* 6-phosphogluconate dehydrogenase and B. Sheep 6-phosphogluoncate dehydrogenase.

compounds (except compounds **13–17**) were docked into the active site of the *T. brucei* enzyme using the contact score (Table 2) (the higher the contact score the better the binding is predicted to be). There seems to be little correlation between activity against the *T. brucei* enzyme and contact score or between the sheep enzyme and contact score (Figures 2A and 2B). This is perhaps not surprising as the binding conformation of the molecule may not be the lowest energy conformation. In addition the contact score only takes account of steric factors (Van der Waals interactions) and not electrostatic interactions. The programme was not able to dock compounds **13–17** into the active site of the enzyme using our protocol. Compounds **13** and **17** are much bulkier than the other triphenylmethane derivatives and this may explain why they did not dock into the active site. It is unclear why compounds **14–16** did not dock into the active site as they are not likely to be significantly more bulky than compounds **1–13**.

The compounds were then docked into the active site using the programme DOCK 3.5 in force field score mode. Force field scoring takes into account electrostatic interactions between the inhibitor and the enzyme. However, fewer compounds were successfully docked (Table 2) (again the programme did not dock the compounds). Repeating the procedure, this time defining a larger volume for the active site gave no improvement in binding. Although fewer compounds were successfully docked, using force field scoring, there was some correlation between activity against the *T. brucei* enzyme and force field score, and between activity against the sheep enzyme and force field score (Figures 2C and 2D).

The orientations of molecules in the active site obtained by DOCK 3.5, in contact score mode, were then subject to searching using FLEXIDOCK, which is part of the SYBYL suite of software. In this approach, the compound undergoes optimisation of geometry in the active site, including conformational changes to the structure of the ligand, a feature not possible with DOCK 3.5. The 'interaction' energy is calculated using the Van der Waals, electrostatic and torsional energy terms of the TRIPOS forcefield. This gives a score which can be used to compare inhibitors. The results from these calculations are shown in Table 2. A better correlation was obtained between the FLEXIDOCK score and the K_i (*T. brucei* and sheep (Figures 3A and 3B)).

The putative binding of the natural substrate, 6-phosphogluconate, in the active site of the *T. brucei* enzyme is shown in Figure 4 and described in Table 3. Essentially the phosphate has strong electrostatic and hydrogen bonding interactions with the enzyme (binding with Tyr 193, Arg 289, and Arg 453). Then the 3- and 5-hydroxyl groups have hydrogen bonding interactions with the enzyme. In particular the 5-hydroxyl is very close to His 459 (1.7 Å). There also appears to be a hydrophobic contact between the carbon backbone of 6-phosphogluconate and Phe 456. The carboxylate group appears to have several weak interactions, with Lys 185 and Asn 189. Despite the low overall sequence identity between the *T. brucei* and sheep enzymes (33%), the active site residues are much more highly conserved, with all of the residues showing direct contact with the substrate being identical [13].

Table 3. Putative interactions between *T. brucei* 6-Phosphogluconate dehydrogenase and the substrate

C1 (COOH)	Weak electrostatic interaction with Lys 185 (5 Å) Weak interaction with the amide side chain NH of Asn 189 (4.0 Å).
C2 (OH)	Weak interaction with side chain NH of Lys 185 (4.0 Å). Weak H-bond with Gly 131 NH (3.5 Å)
C3 (OH)	Weak H-bond with side chain of Lys 185 (3.1 Å) Weak interaction with side chain NH of Asn 189 (3.4 Å)
C4 (OH)	No interactions
C5 (OH)	H-bond with His 459 (1.7 Å)
C6 (OPO_3^{2-})	Electrostatic interactions with Arg 289 (1.9 Å) and Arg 453. H-bond with Tyr 193 (2.0 Å)
Miscellaneous	Hydrophobic interaction of Phe 456 with the carbon chain

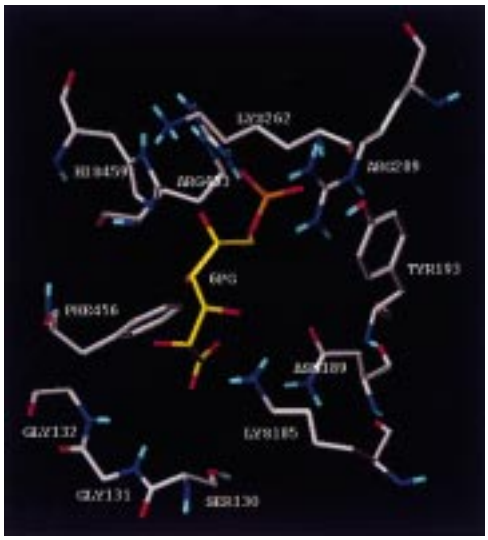

Figure 4. Key residues interacting with 6-phosphogluconate in the active site of *T. brucei* 6-Phosphogluconate dehydrogenase.

FLEXIDOCK predicts binding of the triphenylmethane derivatives in a slightly different orientation to 6-phosphogluconate, but utilising key residues from the active site. The predicted binding of compound **1a** is shown in Figure 5 for both the *T. brucei* and sheep enzymes. The fact that the natural substrate and the triphenylmethane derivatives occupy overlapping parts of the active site could explain the competitive nature of inhibition. The key interactions for the *T. brucei* enzyme are predicted to be: an electrostatic/hydrogen bonding interaction between the side chain of Lys 185 with the sulphonate or carboxylate functional group on the inhibitor. Another electrostatic/hydrogen bonding interaction between the phenolic group on a phenyl ring with Arg 289 (approximately 2.6 Å). The NH of Arg 289 also interacts with the halogen (∼2.9 Å), and there is a similar interaction between the halogen and the amide NH group of Gly 77 (∼2.2 Å). The side chain of Lys 262 interacts hydrophobically with the third phenyl ring (3.2–4.3 Å). In the case of tetrabromophenolphthalein ethyl ester (**8**) the ethyl group makes a hydrophobic interaction with the hydrophobic part of the side chain of Lys 185.

FLEXIDOCK predicts a similar interaction of the triphenylmethane derivatives and the sheep enzyme active site, with the major interactions occurring with Lys 183 and Lys 260. However in the case of the sheep enzyme, the distance between the two residues (as measured between the side chain amino groups) is further apart than the corresponding residues (Lys 185 and Lys 262) within the *T. brucei* enzyme (12.4 Å as opposed to 10.4 Å). The greater distance in the case of the sheep liver enzyme may mean that the interactions between the inhibitors and the active site are weaker, thus explaining the selectivity. It is of course possible that there is flexibility in the enzyme active site which would affect the Lys-Lys distances. However in a second sheep enzyme structure (pdb code: 2PGD) the Lys-Lys distance is 12.0 Å, indicating a similar orientation.

The differences in key interactions between the triphenylmethane derivative and the sheep enzyme predicted by this model are: the phenolic hydroxyl interacts with the guanidine group of Arg 287 (∼2.5 Å); but the halogen is further from the guanidine group (∼5.4 Å) weakening this interaction. Moreover the interaction of Gly 77 (*T. brucei*) is absent in the sheep enzyme as there is no corresponding residue at this location. Finally the distance between the third phenyl ring and the Lys 260 side-chain is longer (∼4.8–5.8 Å) in the sheep enzyme, suggesting a weaker hydrophobic interaction.

Multiple regression analysis

Multiple regression analysis was carried out on the data produced by FLEXIDOCK to probe the correlation between experimentally determined selectivities and those predicted from our model. The experimental

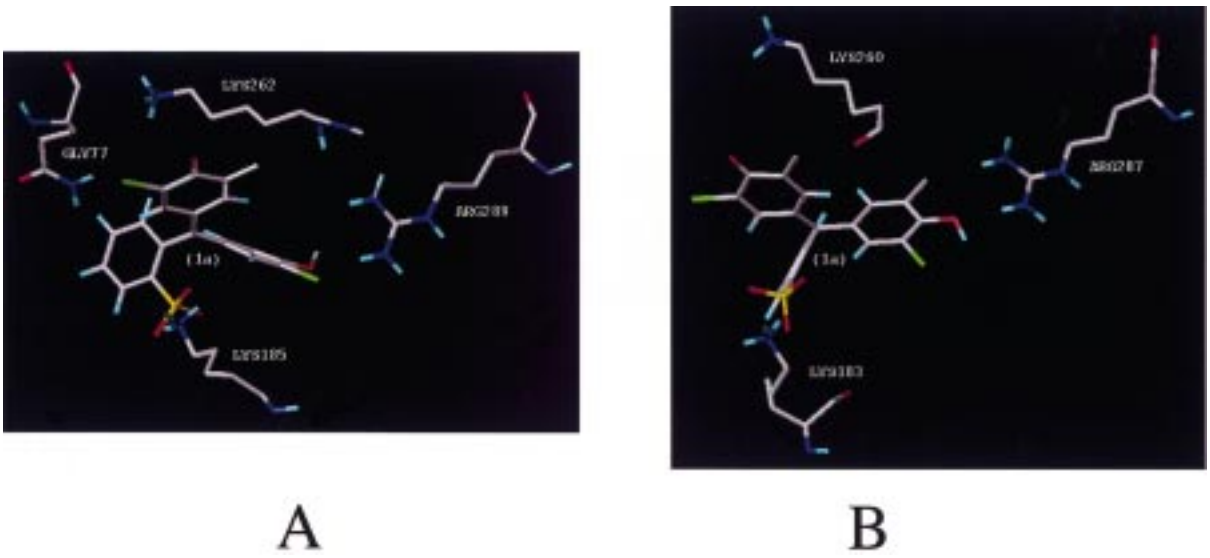

Figure 5. Key residues interacting with bromocresol purple, sodium salt (1a) in (A). The active site of *T. brucei* 6-phosphogluconate dehydrogenase and (B). The active site of sheep 6-phosphogluconate dehydrogenase.

selectivity (S_{exp}) was calculated:

$$S_{\text{exp}} = Ki(\text{sheep})/Ki(\textit{T. brucei})$$

There was no direct measure of predicted selectivity as the FLEXIDOCK scores are not the binding energy. Therefore a measure of predicted selectivity was defined as:

$$a = \{\text{FLEXIDOCK score } (\textit{T. brucei})\}/\{\text{FLEXIDOCKscore(sheep)}\}$$

TSAR [20] was used to define a relationship for all compounds for which a FLEXIDOCK score was obtained, with the exception of bromocresol purple (compound **1**), 12 compounds in total (Table 4). Compound **1** was not included as other compounds were assayed as the sodium salt. The relationship obtained for predicted selectivity (S_{pred}) was

$$S_{\text{pred}} = 1.0195a^5 - 1.4225 \quad \text{(equation)}$$

Regression coefficient, $r = 0.954$;
Cross validation, $r^2 = 0.858$; s value $= 3.67$;
F probability $= 6.807 \times 10^{-7}$

Most of the compounds showed very good correlation between predicted and experimental selectivity. The exceptions were compounds **7** and **8**; even in these compounds the prediction is reasonable which adds weight to the validity of the model.

Discussion

It is hoped that a structure-based approach to drug design will increase our ability to generate novel pharmacologically active molecules [2]. Combining the technologies of molecular biology, protein crystallography and computer-based algorithms capable of predicting the interaction between small molecules and the active sites of potential therapeutic targets is central to these expectations. This route to drug design may be particularly relevant in the development of novel reagents against tropical diseases where investment from the pharmaceutical industry is minimal [1]. For example DOCK has been used to discover novel inhibitors of proteases found in species of *Plasmodium* [21].

Here we report data from inhibitory studies on the activity of 6-phosphogluconate dehydrogenase from African trypanosomes using a series of substituted triphenylmethane derivatives. These compounds are important lead compounds for the development of potential drugs. Firstly the compounds show selectivity for *T. brucei* 6-PGDH over the corresponding mammalian enzyme. Secondly some of the inhibitors show some typical drug-like properties, in terms of lipophilicity and size. Simple analogues of the substrate, 6-phosphogluconate, are likely to have problems with membrane-permeability and delivery due to their highly charged nature and may show enzymatic instability, whilst some of the triphenylmethane

Table 4. Experimental and predicted selectivity from multiple regression analysis compared to experimental selectivity

No.	Name	Experimental selectivity	Predicted selectivity selectivity
1a	Bromocresol Purple, sodium salt	40.5	39.2
2	Bromochorophenol blue, sodium salt	4.3	6.9
3	Bromophenol blue, sodium salt	6.1	7.4
4	Phenol red, sodium salt	2.9	2.7
5	Rosolic acid	3.4	2.6
6	Tetrabromophenol blue, sodium salt	5.0	4.7
7	Tetrabromophenolphthalein	8.9	16.5
8	Tetrabromophenolphthalein ethyl ester	21.6	13.8
9	Tetraiodophenolsulfonephthalein, sodium salt	21	21.0
10	Triphenylmethane	1.9	0.6
11	Triphenolmethanol	1.7	0.5
12	Xylenol blue, sodium salt	5.7	7.1

derivatives investigated here (e.g., **5**, **7**, **8**) show better physicochemical properties, in terms of higher lipophilicity and lower number of H-bond donors and acceptors [22].

Initial structure activity relationships for activity have been established for the triphenylmethane derivatives. However further analogues need to be tested before a full understanding may be gained.

The compounds have been shown to be competitive inhibitors of the substrate 6-phosphogluconate. This suggests that the binding location of the inhibitors could overlap with the binding site of 6-phosphogluconate. (However it is also possible that inhibitors could be binding at a remote site from the active site, causing a change in conformation or accessibility of the active site [23], which could produce a pattern reminiscent of competitive inhibition.) This prompted us to try and model the binding of the inhibitors. Thus we were able to obtain a potential model of binding for compounds **1–12**, in which the inhibitors are predicted to bind at an overlapping site to the 6-phosphogluconate binding site. However DOCK did not successfully dock compounds **13–17** in the active site using our protocol, although these compounds also show competitive inhibition against the enzyme. We have no simple explanation of this, except perhaps for **13** and **17** which are larger compounds and possibly too large to be accommodated in the binding site.

FLEXIDOCK gives similar scores for the substrate, 6-phosphogluconate, in both enzymes (Table 2) which does not reflect their K_m values (3.5 μM for the *T. brucei* enzyme and 19 μM for the sheep enzyme). This may be due to the active site cavity being large compared to the substrate, giving rise to different modes of docking as compared to the triphenylmethane derivatives.

A further limitation of the model is that whilst the procedure views ligand as flexible, the active site is held static, whilst in reality it is dynamic. According to our model selectivity may depend to some extent on the distances between Lys 183–Lys 260 in the sheep enzyme and Lys 185–Lys 262 in the *T. brucei* enzyme. As mentioned before, this distance appears to be similar in a second structure of the sheep enzyme (2PGD) giving indication that this distance may indeed be longer in the sheep enzyme.

The lack of inclusion of all these compounds into the model points to a limitation. However, examination of the structure of the compounds reveals those that bind best (compounds **1–12**) are small and either negatively charged or neutral. The other compounds do not fit into this pattern; compounds **13** and **17** are significantly larger and zwitterionic at physiological pH; compounds **14–16** are small, but also positively charged. It would be surprising if compounds **14-16** did have a similar mode of action to compounds **1–12** as they are chemically very different. Examination of charge distribution around the active site (using the programme GRASP [24]), indicates that whilst the 6-phosphogluconate binding site is positively charged, there are some adjacent negatively charged areas on

the enzyme which could conceivably interact with positively charged inhibitors such as **14–16**.

Summary

In conclusion, we have discovered compounds that show selectivity for the *T. brucei* 6-phosphogluconate dehydrogenase over a counterpart from mammalian cells. Whilst these compounds require significant modification to become suitable as therapeutic agents, they represent an interesting starting point. We have also developed a model that can explain qualitatively how some of the compounds are selective for the parasite enzyme.

Triphenylmethanes were among the first compounds to be tested for trypanocidal activity [16], and there were early successes. These, however, never led to the derivation of practically useful trypanocides due to relatively low anti-parasite activity, plus substantial host toxicity. The products described in this study are unlikely to be directly useful as trypanocides, however, they do represent interesting leads for further development. Computer-based modelling algorithms have been shown to predict the nature of the binding of these compounds to 6-phosphogluconate dehydrogenase in a manner that explains the selectivity identified in comparative kinetic analysis. With refinement novel structures may also be identified which could have enhanced anti-parasite activity.

Acknowledgements

We would like to acknowledge the European Union (MB, SH & MPB) contract No. IC18CT98-0357, Government of Egypt (EE) and Cardiff Centre for Molecular Modelling (MHK) for funding; Dr Margaret Adams, University of Oxford for supplying coordinates.

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