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THE STRUCTURE-BASED DESIGN AND SYNTHESIS OF SELECTIVE INHIBITORS OF TRYPANOSOMA CRUZI DIHYDROFOLATE REDUCTASE

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Abstract: This paper describes the design and synthesis of potential inhibitors of *Trypanosoma cruzi* dihydrofolate reductase using a structure-based approach. A model of the structure of the *T. cruzi* enzyme was compared with the structure of the human enzyme. The differences were used to design modifications of methotrexate to produce compounds which should be selective for the parasite enzyme. The derivatives of methotrexate were synthesised and tested against the enzyme and intact parasites.

INTRODUCTION

Chagas' disease is a parasitic infection caused by the protozoan *Trypanosoma cruzi*. It is endemic in South America, where it is estimated that 16-18 million people are infected by the disease which accounts for many deaths and chronic ill-health.[1] The current drugs available to treat Chagas' disease have poor clinical efficacy and give rise to severe side effects. Therefore there is an urgent need to develop a new and more effective chemotherapeutic treatment.

The enzyme dihydrofolate reductase (DHFR) has been used successfully as a therapeutic target in a number of diseases. It is useful as a drug target as it is involved in the only biosynthetic pathway a cell has for biosynthesis of thymidine. In addition, the structure of the enzyme active site varies between species, allowing for selective drug design. Little has been done to investigate the utility of DHFR as a potential drug target in the case of *T. cruzi*.

As part of an ongoing project on the design and synthesis of inhibitors of protozoan DHFR we have created a homology model of the *T. cruzi* DHFR[2] using the published structure of the *Leishmania major* DHFR.[3] Extensive comparison between the human and *T. cruzi* DHFR active sites was then carried out.[2] This revealed differences at the active site in terms of residues present and shape of different binding sub-pockets. The main differences in terms of residues between the human and *T. cruzi* DHFR active site are shown in Figure 1. This shows the folate and the residues present in the human enzyme. The corresponding residues

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present in the *T. cruzi* enzyme are shown in parenthesis. One major difference concerns residues 20 and 21 (human numbering). In the human these are Gly-Asp which carry an overall negative charge, whilst in the parasite enzyme they are Arg-Ser which have an overall positive charge. These residues form part of the channel that occurs between the folate binding site and the NADPH binding site. It was reasoned that placing a negatively charged substituent on a known inhibitor at this location should decrease binding to the human enzyme due to charge repulsion with Asp21. This repulsion would not occur in the case of the *T. cruzi* enzyme, where ideally this modification should give rise to a favourable interaction with the Arg side chain. However in the model, the Arg side chain is oriented away from the tunnel between the folate and NADPH binding sites and is on the protein surface. But since the side chain is flexible it is not possible to exclude this interaction.

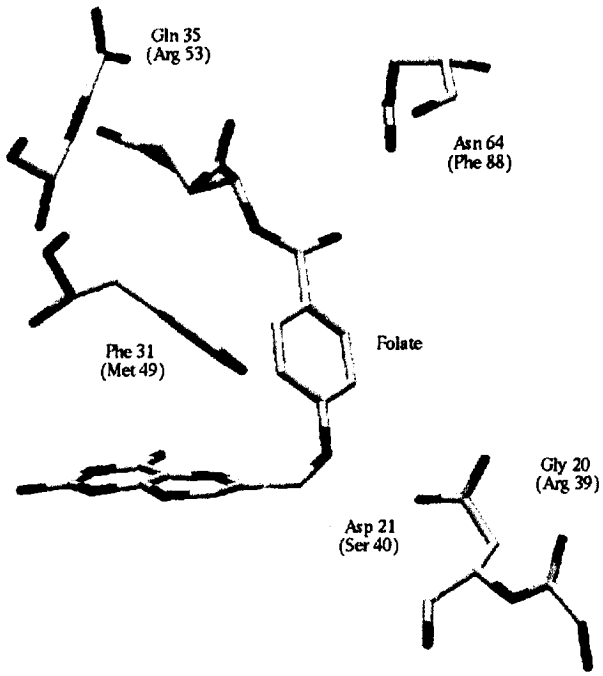
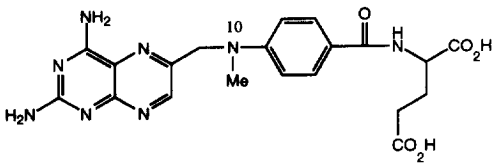


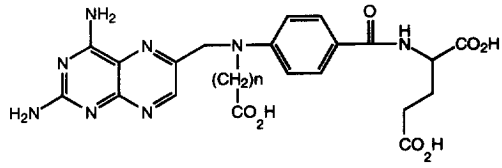
Figure 1: The active site of Human DHFR. Residues shown are those which show significant difference to the corresponding residues in T. cruzi (shown in parentheses)

It was decided to base modifications on the potent DHFR inhibitor methotrexate. This inhibitor interacts with an extensive part of the folate binding site. Methotrexate (Figure 2) was docked into the active site of the human enzyme using DOCK[4] and then Monte-Carlo conformational searching[5] carried out to predict how methotrexate binds in the active site. The bound conformation was similar to those observed in other methotrexate-DHFR complexes.[6] The distance from ^{10}N to the carboxylic acid side chain of Asp21 was 8.15Å. (The corresponding distance for the substrate folate is 7.65Å.) This suggested the negatively charged

group (carboxylic acid) could be appended to the ^{10}N group and would require a chain length of 4-6 carbons to interact with residues 20 and 21. This lead to the targets shown in Figure 2 being selected.



Methotrexate



Target Compounds: n= 3-5

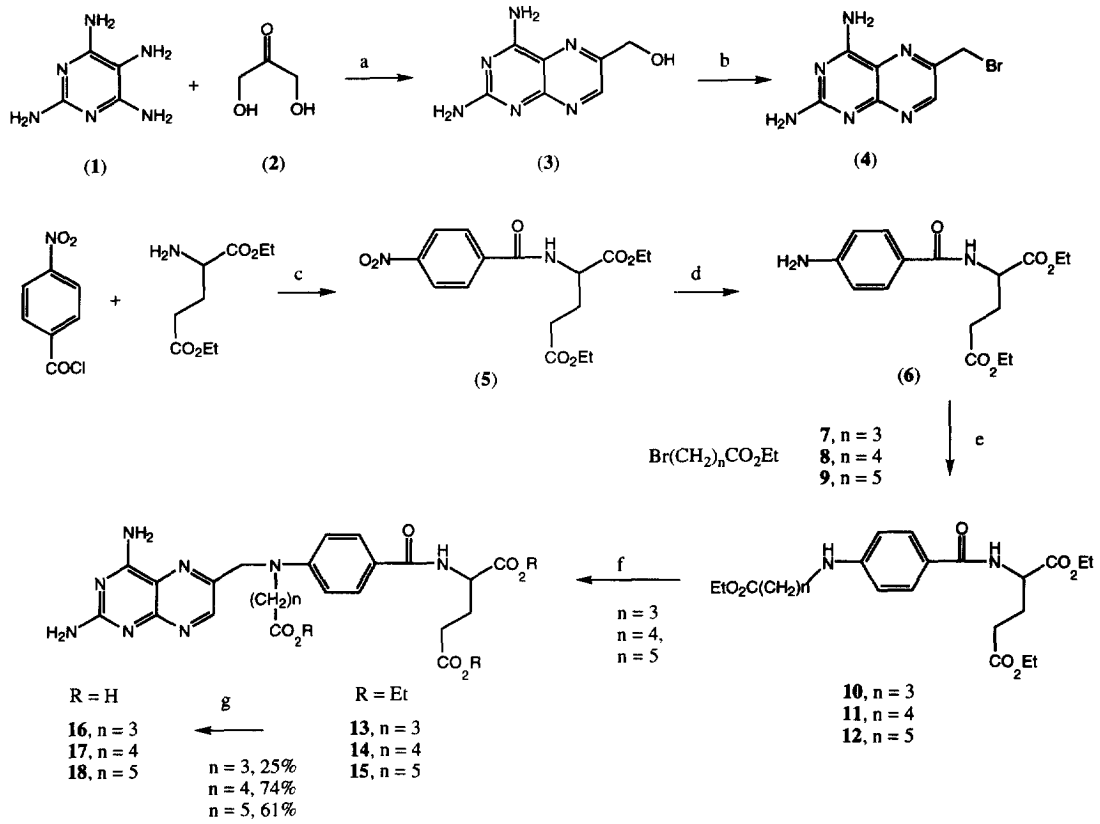
Figure 2

CHEMISTRY

The pteridine ring was synthesised by the methods reported by Baugh and Shaw[7] (Scheme). Thus 2,4,5,6-tetraaminopyrimidine (**1**) was condensed with dihydroxyacetone (**2**) under aerobic conditions. The major product was 2,4-diamino-6-hydroxymethylpteridine (**3**) which was purified by recrystallisation as the hydrobromide salt. Bromination[8] with bromine and triphenylphosphine yielded the bromopteridine (**4**), ready for condensation with the benzamide moieties.

The benzamide moieties were made by condensing 4-nitrobenzoyl chloride with the diethyl ester of glutamic acid to give the intermediate nitrobenzoyl amide (**5**). This was reduced using catalytic hydrogenation to give the amine (**6**) which was then alkylated with three different bromoesters (**7-9**). The products (**10-12**) were contaminated with dialkylated compound. It proved impossible to separate out the mono- and di-alkylated products by flash chromatography. However the dialkylated product is not reactive in the next step of the pathway and so it was carried on into the next step as an impurity.

Condensation between the amines (**10-12**) and the bromopteridine (**4**) gave the protected target molecules (**13-15**). These were then hydrolysed using sodium hydroxide solution to give the target molecules (**16-18**) which were purified by precipitation.



(a). i. NaOAc, cysteine, water, air. ii. HBr, EtOH; 35%. (b). Br_2, PPh_3, DMA. (c). pyridine; 97%. (d). H_2, EtOH, Pd/C; 94%. (e). i-Pr_2NEt, DMF, 100°C. (f). DMA, 65°C. (g). 5% NaOH, EtOH.

Scheme

BIOLOGICAL EVALUATION

Enzyme Assays

Compounds were then evaluated in enzyme assays against purified recombinant enzyme.[9,10] The Ki value was determined using Dixon plots (Table). A number of trends can be seen from the enzyme assays:

- The ethyl ester protected compounds (**13-15**) have much larger Ki s against the *T. cruzi* enzyme than the free carboxylic acids (**16-18**). This is not surprising as the carboxylic acids will be ionised at physiological pH and have ionic interactions with positively charged residues in the enzyme active site.[2]
- In the case of the *T. cruzi* enzyme the maximum inhibition is seen in the case of the shortest chain length and then the activity decreases as the chain length increases. This is the case for both series of compounds (**13-15** and **16-18**). In the case of the human enzyme, no such trend can be observed.
- All the compounds show a greater selectivity for the *T. cruzi* enzyme compared to methotrexate.

- For the free acid series (**16-18**), the compounds show a bigger drop in activity compared to methotrexate for the human enzyme (8.1-11.0 fold) than for the *T. cruzi* enzyme (2.8-4.8 fold) on introducing the negatively charged substituent.

These data support our hypothesis that introduction of a negatively charged substituent increases the selectivity for the parasite enzyme versus the human enzyme. However caution must be applied in interpretation of the data as the magnitude of the changes is not high and the compounds may adopt other binding modes in the active site than those we have predicted. Another finding is that the shorter 4-carbon chain length gives not only the best activity but also the best selectivity.

TABLE: Ki values for Inhibtion of Isolated Enzyme and *In Vitro* Against the Amastigote Stage of the Parasite in L-6 cells (mouse fibroblasts).

Compound	Enzyme Assays			Assay Against Amastigotes
	Ki (T. cruzi) nM	Ki (human) nM	Selectivity	IC_{50} μM
13	14.1	133	9.43	61.0
14	46.2	453	9.90	38.8
15	55.7	485	8.71	53.5
16	0.107	1.97	18.4	>171
17	0.131	1.45	11.1	>167
18	0.183	1.63	8.9	11.5
methotrexate	0.038	0.179	4.7	9.2

In Vitro Assays

The compounds were also evaluated *in vitro* against the amastigote (pathogenically important) stage of the parasite cultured in mouse fibroblasts (L-6 cells). [11] Unfortunately none of the compounds were very active, although the free acid **18** showed similar activity to methotrexate. There was no correlation between the *in vitro* activity of the compounds and their enzyme activity, suggesting factors other than enzyme inhibition are important for activity against the parasite. One possible factor is cellular uptake, as the most lipophilic compounds (**13-15, 18**) are the most active. Possibly the other carboxylic acids (**16, 17**) are too negatively charged for good uptake. The ester compounds (**13-15**) may be acting directly on the enzyme or being metabolised to the free acids *in vitro*.

CONCLUSION

In this approach we have used a comparison of the structure of a pathogen enzyme and the corresponding human enzyme to design compounds which should be selective for the pathogen. The compounds were then synthesised and evaluated. The data suggests that the structural modification carried out gives rise to some degree of selectivity. Whilst the compounds do not represent good therapeutic leads because of poor *in vitro* activity, the results validate the approach used.

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11. The *in vitro* activity against *T. cruzi* was determined in an assay using parasites expressing ß-galactosidase (Buckner et al., 1996). Briefly, L-6 cells were seeded into wells of a microtiter plate and infected the following day with trypomastigote forms of *T. cruzi*. Two days later the medium was removed and replaced by a serial drug dilution. After an incubation of four days the substrate chlorophenol red ß-D-galactopyranoside was added. The plate was read 2-6 hours later in an ELISA reader at 540 nm to quantify growth. An IC_{50}-value was then determined. [Buckner, F. S.; Verlinde, C.L.M.J.; La Flamme, A.C.; van Voorhis, W.C. *Antimicrob. Agents Chemother.*, **1996**, *40*, 2592-2597.]
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