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Novel Inhibitors of Leishmanial Dihydrofolate Reductase

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Abstract—The program DOCK3.5 was used to search the Cambridge Structural Database for novel inhibitors of *Leishmanial* dihydrofolate reductase. A number of compounds were obtained and screened against the enzyme and against the intact parasite *Leishmania donovani* and the related organisms *Trypanosoma brucei* and *Trypanosoma cruzi*. The compounds screened showed weak activity in both the enzyme assays and the in vitro assays.

There is an urgent need for the development of new drugs to treat the devastating disease leishmaniasis caused by organisms of the genus *Leishmania*. This disease causes huge suffering and economic hardship to people mainly in the developing world. The current drugs available to treat this disease suffer from difficulty in administration, toxicity, poor clinical efficacy and increasing resistance.[1]

Leishmania contain a bifunctional protein possessing the enzyme activity of both thymidylate synthetase and dihydrofolate reductase (DHFR) in contrast to mammalian cells, in which the enzyme activities reside on separate proteins.[2a] Pteridine metabolism is essential for growth of the parasite and DHFR is structurally distinct in *Leishmania* species,[2] suggesting that DHFR should be a target for chemotherapeutic development.[3] DHFR has been used successfully for the development of therapeutic agents: antimicrobial (trimethoprim); anticancer (methotrexate); antimalarial (pyrimethamine and cycloguanil); toxoplasmosis and *P. carinii* pneumonia (trimetrexate).[4] Unfortunately none of these classical antifolates are effective against leishmaniasis and many indeed are actually selective for the human enzyme over the parasite enzyme.[5]

We have been involved in the design, synthesis and biological evaluation of *Leishmanial* DHFR inhibitors,[6,7] all of which contain the 2,4-diaminopyrimidine motif. A new structural type might improve the selectivity and pharmacokinetic properties as well as reducing toxicity. The crystal structure of *Leishmanial* DHFR has been solved.[2a] Recent advances in the areas of three-dimensional database searching play an ever-increasing role in new lead generation,[8] and is especially useful when the structure of the molecular target (e.g., enzyme) is known.[9,10] Therefore we were interested to seek novel inhibitors of *Leishmanial* DHFR with a completely different basic chemical structure by Cambridge Structural Database (CSD) searching using the active site of *Leishmanial* DHFR enzyme. The approach that we adopted was to search the database by using the program DOCK, which was created by Kuntz and coworkers.[11] Several strategies were then adopted for a selection of compounds for testing. Compounds were assayed for activity against *L. major* and human DHFR and in vitro against the clinically relevant form of the parasite (amastigote form).

Modelling

Cambridge Structural Database

The CSD was selected as it contains the three-dimensional structures of a large number of compounds that have been obtained by X-ray crystallography. The advantage of this database is that the structures are well defined and usually in low energy regions of the

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conformational potential energy surface, while the disadvantage of this database is that some of the compounds are not readily available. Not all the CSD entries are drug candidates and it was reasoned that an inhibitor would be likely to contain an aromatic ring. Therefore a subsection of organic compounds were selected from the database containing a phenyl ring and/or heterocyclic structure prior to matching into the active site of the enzyme. This selection criterion detected a total of 25,684 compounds from the database for scoring (CSD release October 1996). Selection of compounds was made using QUEST.[12]

Screening of the database with DOCK3.5 in contact score mode

For the initial screening of the database, DOCK3.5 (the version available during the period of the project) was used in the contact scoring mode, which basically scores the compounds on steric fit within the active site of the enzyme (and does not take account of 'chemical' interactions). However this mode allowed rapid scoring of a large number of compounds. Contact scores were calculated for 20,568 compounds against the active site of leishmanial DHFR. The contact score varied between −10 and 290. Compounds with scores above ss170 were selected for further screening (1700 compounds).

Selectivity

Compounds need to show selectivity for the parasite enzyme over the human enzyme. An idea of selectivity was found by subtracting the contact score for the *L. major* enzyme from the contact score for the human enzyme. This is not a quantitative way to compare the score between the *L. major* and human DHFR, but may represent selectivity of these compounds for parasite DHFR in a qualitative way. Compounds were then selected for visual scoring based on a high contact score and high selectivity index. This was estimated by addition of the selectivity score (DOCK score of *L. major* less DOCK score of human) and activity score (DOCK score of *L. major*). This led to a working set of 174 compounds for further assessment.

Visual screening

Visual screening was carried out using MACROMODEL[13] along with the protein to visually assess the protein–ligand interactions. A number of parameters were considered. Firstly how well the compounds might interact with the active site in terms of electrostatic, hydrogen bonding and hydrophobic interactions. Secondly molecules which were very likely to show toxicity problems were eliminated. Finally synthetic accessibility was an important criterion for selection. For further scoring and analysis 86 compounds were listed.

Force field scoring

The list of 86 compounds was then subject to Force Field scoring within DOCK3.5, which takes into account chemical interactions between ligand and enzyme such as electrostatic interactions. Rigid-body minimisation of the enzyme–ligand structure was allowed before force field scoring for every structure. This has the effect of refining each ligand orientation generated prior to scoring it. The limitations of this minimisation parameter is that it only optimises the non-bonded interactions between the ligand and the enzyme, but is not able to modify the torsion angles and bond lengths within the ligand or enzyme. Taking account of chemical interactions in addition to steric interactions will lead to DOCK predicting a slightly different orientation of the ligands in the active site. In this scoring option the ligand–enzyme binding energy is estimated by a molecular mechanics calculation (the sum of the van der Waals attractive, dispersive interactions and coulombic electrostatic energies). The force field scores obtained varied from −256.29 to −26.32 (the lower the value the better the predicted interaction energy).

Compound selection

Following these selection criteria, five compounds were acquired for assay: VENGAW10 (**1**),[14] VENGEA10 (**2**),[14] DULHUN (**3**),[15] POKDUO (**4**)[16] and JUBVOR (**5**)[17] (Fig. 1 and Table 1).

Biological Screening

Enzyme assays

Compounds were then screened against recombinant *L. major* and human DHFR. The assay conditions were as reported.[6] The percentage inhibition for compounds

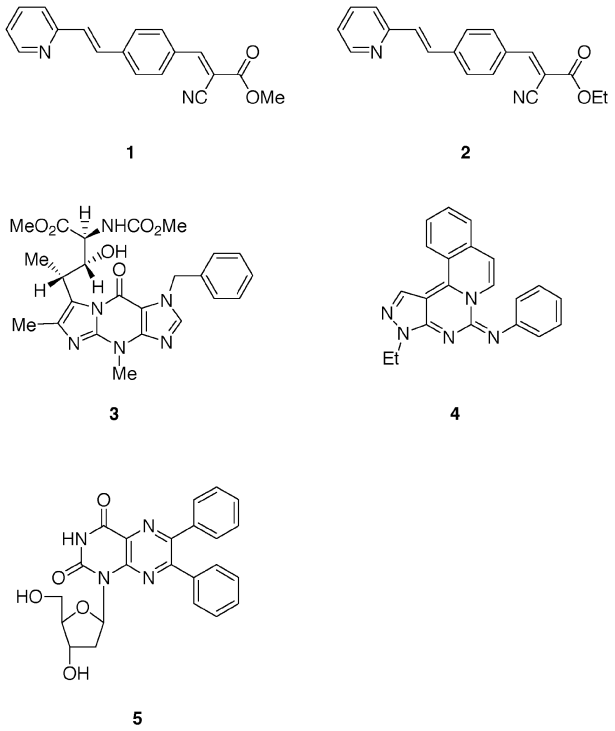

Figure 1. Compounds acquired for assay.

Table 1. DOCK3.5 search

Compound	CSD code	Contact score against *L. major* DHFR	Contact score against human DHFR	Force-field score against *L. major* DHFR	Force-field score against human DHFR
1	VENGAW10	219	165	−119.56	−85.3
2	VENGEA10	206	145	−73.85	−45.4
3	DULHUN	190	130	−100.96	−64.9
4	POKDUO	245	159	−158.64	−79.5
5	JUBVOR	239	185	−114.49	−75.4

Table 2. Results of enzyme assay

Compound	CSD code	*L. major* DHFR% inhibition/μM	Human DHFR% inhibition/μM
1	VENGAW10	32%/100	21%/15
2	VENGEA10	50%/100	43%/100
3	DULHUN	24%/100	16%/100
4	POKDUO	28%/100	10%/100
5	JUBVOR	50%/100	37%/50

Table 3. In vitro assay results against intact parasites

Compound	*L. donovani* IC_{50} μM	*T. brucei* IC_{50} μM	*T. cruzi* IC_{50} μM	L-6 cells MIC μM
1	56	16	30	>310
2	47	15	8.6	>296
3	<21	108	53	181
4	54	35	60	>265
5	~69	11	35	208

was determined in the presence of 30 μM concentration of substrate (Table 2). In most cases it was not possible to determine the IC_{50} values due to the lack of solubility of the compounds. Moderate inhibition was obtained in most cases although certain compounds were slightly selective for the *Leishmanial* enzyme.

In vitro assays

Compounds were also evaluated in vitro against the intact parasite (Table 3).[19] The form of the parasite found in the human was evaluated; in the case of *Leishmania*, this is the amastigote form which is an intracellular form. In this assay the parasites were cultured in mouse macrophages. *Leishmania donovani* was used for the assays. The species Leishmania is related to the trypanosomes *Trypanosoma brucei* and *Trypanosoma cruzi* which give rise to African trypanosomiasis and Chagas' disease, respectively. Evaluation was also carried out against the human stage of these organisms; in the case of *T. brucei* the free living trypomastigote stage (*T. brucei rhodesiense*) and in the case of *T. cruzi* the amastigote stage cultured in L-6 cells (rat skeletal myoblasts). The compounds showed weak antiparasitic activity. The greatest activity was seen in the case of *T. brucei rhodesiense*. This is probably due to the *T. brucei rhodesiense* being cultured axenically, whilst *L. donovani* and *T. cruzi* are cultured in rodent cells. However compound **2** is notable in its activity against *T. cruzi*. Cytotoxicity was assessed using L-6 cells.

Conclusion

Docking of compounds available in the database with the programe DOCK3.5 showed here to be a promising method for the identification of potential novel inhibitors of *Leishmanial* DHFR. Compounds were identified using a number of approaches: contact scoring, visual screening and finally force field scoring. Some compounds selected by this method showed moderate inhibition of DHFR and may be the basis for structure activity studies to identify new, more potent inhibitors. The compounds also showed weak antiparasite activity, although it is unknown whether the antiparasitic activity of these compounds is due to inhibition of DHFR

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19. In vitro assays were conducted as follows:

Leishmania donovani: Mouse peritoneal macrophages were seeded in RPMI 1640 medium with 10% heat-inactivated FBS into Lab-tek 16-chamber slides. After 24 h *L. donovani* amastigotes were added at a ratio of 3:1 (amastigotes to macrophages). The medium containing free amastigotes was replaced by fresh medium 4 h later. Next day the medium was replaced by fresh medium containing different drug concentrations. The slides were incubated at 37 °C under a 5% CO_2 atmosphere for 96 h. Then the medium was removed, the slides fixed with methanol and stained with Giemsa. The ratio of infected to non-infected macrophages was determined microscopically, expressed as percentage of the control and the IC_{50} value calculated by linear regression.

Trypanosoma cruzi: Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/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 -galactosidase (Lac Z) gene) were added in 100 μL per well with 2× of a serial drug dilution. The plates were incubated at 37 °C in 5% CO_2 for 4 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 4 days.

Trypanosoma b. rhodesiense: Minimum Essential Medium (50 μL) supplemented according to Baltz et al. (*EMBO J.* **1985**, *4*, 1273) 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.b. 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 (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 (*Acta Trop.* **1997**, *68*, 139). Fluorescence development was expressed as percentage of the control, and IC_{50} values determined.