Antimalarial drug discovery: development of inhibitors of dihydrofolate reductase active in drug resistance

David C. Warhurst

In view of widespread drug resistance in *Plasmodium falciparum*, new antimalarials are needed. Modelling techniques are being applied with some success to the design of drugs targeting dihydrofolate reductase (DHFR), and screening systems using recombinant enzyme are producing valuable data. The author reviews the interaction of inhibitors with probable active-site residues and examines new approaches. As the interaction of straight rigid drugs, like pyrimethamine, with the binding site of plasmodial DHFR is inhibited by the bulky Ser108→Asn resistance mutation, more flexible drugs, such as trimethoprim, may have a role in areas where this mutation is found.

The fight to control malaria is ongoing, success has eluded us. With 40% of the world's population exposed to the threat of malaria, there are an estimated 200 million clinical cases per annum and two million deaths. Moreover, with the spread of resistance to chloroquine (Avloclor®; Nivaquine®) in *Plasmodium falciparum* (malignant tertian malaria) since the 1960s, reaching Africa in 1978, more and more reliance is being placed on the antifolates, in particular the pyrimethamine/sulfadoxine potentiating combination (Fansidar®). This has been an invaluable second- or first-line treatment in areas of Africa where chloroquine is failing[1]. Although resistance to pyrimethamine (Daraprim®), which inhibits dihydrofolate reductase (DHFR), has long been recognized, resistance to Fansidar is now developing, posing a threat to control of morbidity and mortality[2].

In microorganisms, dihydrofolate (DHF) is sequentially synthesized from dihydropteridine pyrophosphate, *p*-amino benzoic acid and glutamate by dihydropteroate synthase (DHPS) and DHF synthase. Although vertebrates use exogenous folate, DHFR is vital because the product tetrahydrofolate, in both prokaryotes and eukaryotes, acts as a methyl-group donor in the universal thymidylate synthetase reaction during DNA synthesis. Antifolate agents effective on pathogens therefore need to be highly selective.

The currently used antimalarial antifolates, pyrimethamine and proguanil (Paludrine®), which were developed empirically in the 1940s and 1950s, are non-toxic and highly active. Unfortunately, resistance develops readily to both drugs.

Resistance to antimalarials

Resistance to pyrimethamine (PYR) *in vitro* is associated with point mutations in the parasite *DHFR* gene sequence (i.e. part of the sequence encoding the bifunctional enzyme dihydrofolate reductase/thymidylate synthase)[3]. Cycloguanil (CYC – the active metabolite of the prodrug proguanil) also selectively inhibits *P. falciparum* DHFR (PFDHFR), and identical or related point mutations also

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determine resistance. Serine in position 108 of PFDHFR is found in strains sensitive *in vitro* to both drugs, whereas a mutation to Asn108 is associated with resistance to PYR and a moderate loss of response to CYC. Thr108, together with an Ala16→Val mutation, confers resistance to CYC with only slight loss of response to PYR. Higher levels of PYR resistance are facilitated by mutations of Asp51→Ile and of Cys59→Arg, when associated with the Asn108 mutation. A mutation from Ile164→Leu in combination with Asp108 and one or both of the Ile51 or Arg59 mutations has been found in *P. falciparum* strains that are highly resistant to both drugs[4,5]. Other, rare mutations have also been described[6,7]. According to a field study, the PFDHFR-resistant genotype (Asn108 with Ile51 and Arg59) is also selected *in vivo* by chlorcycloguanil (CCY – the active metabolite of the antimalarial chlorproguanil, Lapudrine®)[8].

Only recently have biochemical studies on heterologously expressed PFDHFR become possible[9], and together with molecular modelling approaches[10,11], these are achieving some success in the development of new drugs that may circumvent resistance.

Structure of DHFR active site

The hydrophobic active site pocket of DHFR, which accommodates the heterocyclic ring system of substrate or drug, is terminated at one end by the methyl group of Thr in α-helix C (Thr45 in *Lactobacillus casei*, Thr57 in *Homo sapiens* and Thr/Ser/Asn108 in *P. falciparum*). At the opposite end, on α-helix B, are the carboxylic oxygens of Asp in microorganisms (Asp26 in *L. casei* or Asp54 in *P. falciparum*) or Glu in vertebrates. Antifolate drugs bind in the pocket by hydrophobic interactions with its lining residues, and by H-bonds between secondary and tertiary amino nitrogens and, in particular, the carboxylic oxygens of Asp or Glu.

In bacterial (e.g. *Escherichia coli* and *L. casei*) and vertebrate DHFR (*Gallus domesticus* and *Homo sapiens*), the *p*-amino benzoyl glutamate part of the substrate molecule is approximately perpendicular to the plane of the pteridine ring in the active site because of a bend at the -CH_2- group joining the two parts. The heterocyclic pteridine ring of DHF is H-bonded to the carboxyl group of Asp or Glu through its 2-amino group and N3. Methotrexate (MTX; Fig. 1a), one of the first antifolates to be developed, mimics DHF very closely and is known to fit the active site in DHFR in a similar position to DHF, with one important distinction, the pteridine ring is rotated so that N1 and 2-amino of MTX H-bond to carboxyl instead of N3 and 2-amino[12].

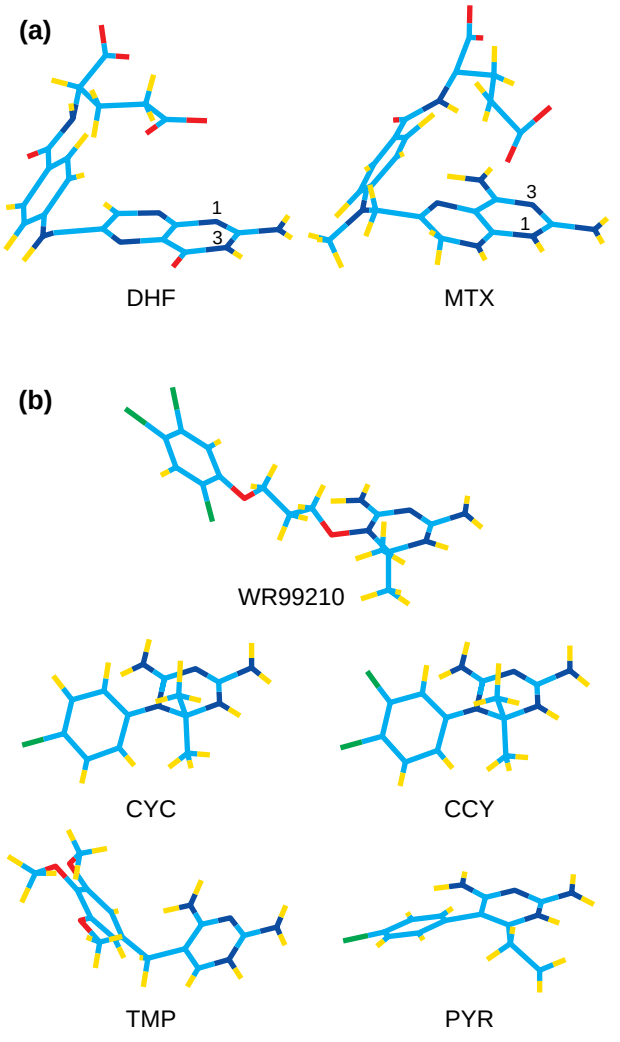

Figure 1. *(a) Active-site conformations of dihydrofolate (DHF) and of methotrexate (MTX). (b) Optimized structures of dihydrotriazines WR99210, cycloguanil (CYC), chlorcycloguanil (CCY) and of pyrimidines trimethoprim (TMP) and pyrimethamine (PYR). Colours show: carbon, light blue; nitrogen, dark blue; hydrogen, yellow; oxygen, red; chlorine, green.*

The bend at -CH_2- between the two rings is not possible in the highly active antimalarials PYR (a phenyl pyrimidine) or CYC and CCY (phenyl dihydrotriazines), but it is found in the active-site conformation[13,14] of the antibacterial benzyl pyrimidine antifolate trimethoprim (TMP), which is used in combination with sulfonamide as sulfamethoxazole/TMP (cotrimoxazole; Septrin®) in malaria treatment[15,16]. Although both PYR and TMP are 5-substituted 2,4-diamino-pyrimidines, examination of chemical formulae and molecular

modelling of CYC and CCY confirms the close conformational similarity between PYR, CYC and CCY (Fig. 1b). They consist of a diamino-substituted pyrimidine or dihydro-1,3,5-triazine ring, mimicking the pteridine ring of DHF, joined directly and rigidly to a *p*-chlorobenzene ring. The N1 of TMP, like that of MTX (Ref. 17), is protonated and H-bonds with the active-site carboxylate side chain of Asp in *E. coli*, *Staphylococcus aureus* and *L. casei*; other H-bonding and van der Waals interactions are also conserved at the binding site.

Flexible antifolates

A recent unpublished study (T. Jelinek, pers. commun.) and earlier work suggest that antifolates like TMP that have a bend between the rings may be effective in pyrimethamine-resistant *P. falciparum*. TMP/sulfonamide sensitivity in *P. falciparum* does not always parallel PYR/sulfonamide sensitivity. An early clinical study[18] comparing the quinine-, chloroquine-, PYR- and proguanil-resistant 'Camp' strain from South East Asia with a sensitive 'Uganda' strain, in non-immune volunteers, found that TMP/sulfalene treatment was effective in both strains. In their discussion, the authors suggested that 'the action of PYR and of TMP in inhibiting [dihydrofolate] reductase by binding to the enzyme has a different molecular basis'. More recently it was concluded[19] that *in vitro* cross-resistance between PYR and TMP and between TMP/sulfamethoxazole and PYR/sulfadoxine appeared not to be absolute. There was a marked reduction in sensitivity to PYR alone and sulfadoxine alone in the chloroquine, PYR and sulfadoxine-resistant Thailand K1 strain (which has the Ser108→Asn mutation) compared with the sensitive F32 Tanzanian strain, whereas sensitivities to TMP and sulfamethoxazole in the two strains were similar.

Even more flexibility between the two rings (limited in MTX and TMP) is seen in the structure of a new antimalarial under development – WR99210 (Ref. 25, Fig. 1b), which also has activity in PYR resistance. These observations suggest that the relationship between the 108 residue, the drug and the carboxylic oxygens of Asp may be important for the activity of antimalarial antifolates in resistant strains. It has provided the stimulus for the present study.

Active site mutations associated with resistance

All the bacterial residues equivalent[20] to mutation sites in PFDHFR that are known to have an effect on pyrimethamine/cycloguanil sensitivity *in vitro* are also found around the active site in the *L. casei* or *E. coli* DHFR crystal structures (see alignment, Fig. 2). The most widely studied drug associated with DHFR is MTX, and coordinates for a good crystal structure of the ternary complex with MTX and NADPH of DHFR from *L. casei* are available in the Brookhaven Structural Database (ID: 3dfr)[21]. The probable effects of some of the mutations in PFDHFR are discussed below, following examination of the crystal structure using the HyperChem (release 3) molecular modelling package.

β-sheet A

In *L. casei* DHFR, the backbone oxygen of Leu4 H-bonds with the 4-amino group of MTX or TMP. The equivalent residue in PFDHFR is Ile14. Two residues further along β-sheet A is Ala6, conserved in *P. falciparum* as Ala16 and identical in DHFR sequences from a wide range of other organisms. The mutation to the bulkier Val16 occurs in *P. falciparum* in one form of CYC resistance. The methyl carbon of the Ala6 side chain protrudes into the binding site in the 3dfr structure, and is only 3.9 Å from the 2-amino nitrogen of the drug, which is crucial for H-bonding with Asp. Van der Waals interactions between these two atoms are likely, and will stabilize the position of the drug in its interaction with the Asp26 carboxylic acid oxygens. The side chain of Ala is 1.54 Å in length, and that of Val is branched with two methyl groups each 2.49 Å from the backbone. It is probable that a change from Ala16→Val (which has been found only in *P. falciparum*) would significantly alter the orientation of the drug in its interaction with Asp54 in PFDHFR (or Asp26 in *L. casei*), and, especially for rigid drug structures, with the crucial residue 108 at the opposite end of the binding pocket.

α-helix B

In α-helix B of the *L. casei* active site is found Asp26; its carboxylate side chain H-bonds to the N1 and 2-amino groups of MTX and TMP, and two common mutation sites in *P. falciparum* occur around the equivalent Asp54 residue, Asn51→Ile and Cys59→Arg (Leu23 and Arg31 in *L. casei* respectively).

In the 3dfr structure the methyl carbon of Leu23 side chain is only 3.62 Å from the -CH_2- carbon of the carboxylate side chain of Asp26, strongly indicative of van der Waals interactions. The orientation and degree of protrusion of the carboxylic oxygens into the hydrophobic cavity will be stabilized by Leu23. In wildtype *P. falciparum* the equivalent residue is Asn51, and if this has a similar function, then the resistant mutation to Ile51, with a short but

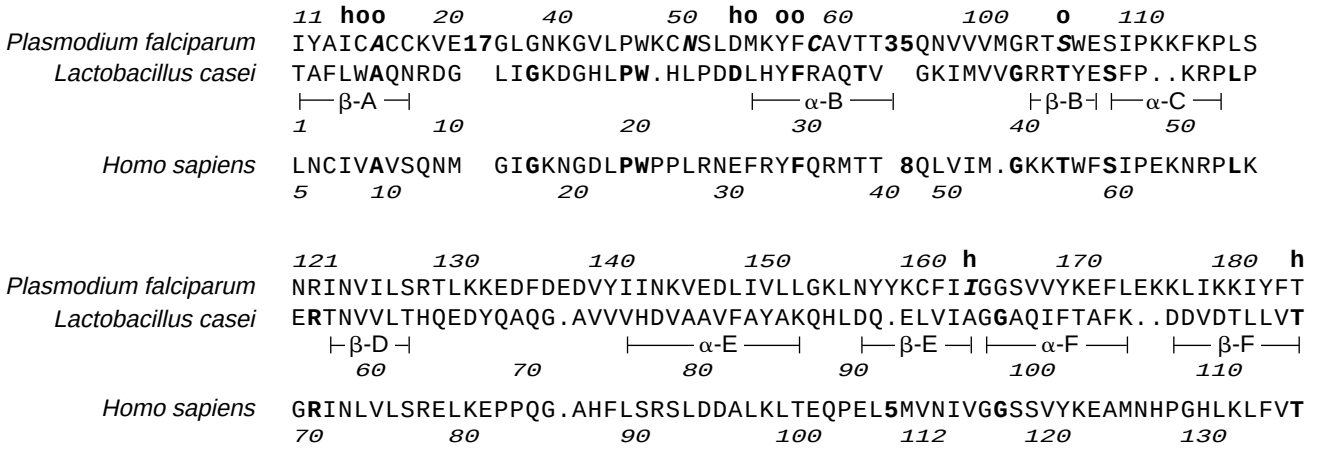

Figure 2. *Alignment of active site-related portions of* P. falciparum, L. casei *and human dihydrofolate reductase sequences. In the* P. falciparum *sequence there are two loops of 17 and 35 residues (shown as numbers in bold face) inserted between active site residues. The letter* **h** *marks residues interacting by H-bonding with methotrexate (MTX) and trimethoprim (TMP) in* L. casei*, and* **o** *marks residues showing van der Waals interactions with MTX and TMP. Location of* α*-helices and* β*-sheets is indicated. Residues in bold in the* L. casei *sequence are widely conserved.* P. falciparum *residues where mutation is associated with resistance to PYR or CYC are in bold italics.*

bulky side chain, will have the effect of altering the location of the Asp54 carboxylic oxygens and the orientation of their H-bonds with the drug.

Bacterial Phe30 is conserved as Phe58 and its ring is involved in interaction with both rings of the drugs and of the substrate DHF.

In the same helix, the side chain of Arg31 in *L. casei* protrudes into the solvent outside the enzyme globular protein. In *P. falciparum* the wildtype residue is Cys59, which has lower hydrophilicity and which may have an impact on the orientation of the α-helix. The Cys59→Arg mutation, as suggested by Hyde[3], is likely to influence the position of the helix, as the equivalent residue in the chicken enzyme (Gln36 in human) was shown to be in van der Waals contact with phenyl dihydrotriazine inhibitors, closely similar to cycloguanil and pyrimethamine. Among other things, this may affect orientation of H-bonds between drug and side chain of Asp26.

α-helix C

In α-helix C of *L. casei* DHFR, Thr45 corresponds to Thr/Ser/Asn108 in PFDHFR, located at the opposite end of the binding site to Asp54. Threonine is conserved in this position in a large range of other organisms and its side chain methyl carbon, located 4.3 Å from the closest carbon (-CH_2-) of MTX in *L. casei*, is clearly an important component of the hydrophobic pocket enclosing the pteridine ring. Together with Ala16→Val (see above), Thr108 gives CYC resistance in *P. falciparum*, whereas Asn108 alone gives some resistance to PYR and CYC, and enhances resistance to CYC given by Ile164→Leu (see below). The side chain methyl carbon of the equivalent Thr in the chicken enzyme complexed with phenyl dihydrotriazine, is close enough to the drug to be involved in van der Waals interactions[22]. Importantly, the hydroxyl oxygen of the Thr side chain and backbone amino both H-bond with the adenine nucleotide 5′ phosphate group in the NADPH cofactor[23]. This means that the position and the nature of this residue can influence both enzyme activity and drug binding.

Alternative 108 residues (Ser, Thr and Asn) seen in natural isolates of *P. falciparum* interestingly have side chain hydroxyl or carboxyl groups capable of H-bonding, and the expressed enzymes[9] have similar K_{cat}/K_m values (6.8, 2.6 and 3.7, respectively). However, for Cys and Gln the values are 0.5 and 0.7, respectively, despite the existence of H-bonding capacity. DHFR with Gly108 or Ala108 (each lacking a side chain H-bonding group) have values of 1.1 and 1.5, respectively. When side chain length or bulk are plotted against drug (PYR, CYC) K_i, the last Gly/Ala108 variants are less affected by drug than expected, whereas those with Cys/Gln108 fit the negative correlation seen for Thr/Ser/Asn108 (see Box 1).

Box 1. Analysis of residue 108

The hypothesis that changes at residue 108 would affect activity of both PYR and CYC in the same direction was investigated as follows, using as the activity parameter the mean of PYR and CYC nanomolar K_i values taken from the study of Sirawaraporn and colleagues[9].

Measurements on models of α-helices (HyperChem) and calculations of structural and physicochemical parameters (MOLGEN) were made to investigate the probable effect of the charge, bulk and lipophilicity of residue 108 on the interaction of PYR and CYC with the *P. falciparum* enzyme (Table 1).

Table 1. Physicochemical characteristics of residue 108 compared with antifolate K_i for expressed *P. falciparum* DHFR

Residue 108	Calculated charge on side chain non-H atom distal to backbone[a]	Distance of the atom from backbone[a] (L, Å)	Molecular weight of side chain (MW)	Side chain[b] (log π)	Molar refractivity[b] (MR)	Surface area[b] (A)	Volume[b] (V)	Mean K_i of PYR and CYC (nM)
Thr	-**CHOHCH$_3$** (−0.26)	2.5	45	0.08	13.01	29.6	72.8	1.5
Ser	-CH$_2$**O**H (−0.35)	2.4	31	−0.27	8.26	21.2	51.8	2.1
Ala	-**C**H$_3$ (−0.25)	1.5	15	1.09	6.35	16.9	42.3	3.0
Cys	-CH$_2$**S**H (−0.08)	2.8	47	0.32	14.39	29.9	64.8	4.5
Gly	no side chain							12.0
Asn	-CH$_2$CO**N**H$_2$ (−0.42)	3.7	58	−1.03	14.47	33.6	79.2	14.0
Gln	-CH$_2$CH$_2$CO**N**H$_2$ (−0.43)	4.9	72	−0.40	19.09	42.2	100.7	126.5

[a]Optimization of drug structures: in HyperChem Release 3 (Autodesk) the drug was built with the relevant ring nitrogen (N1 except for inhibitors **1** and **2**) protonated, and geometry was optimized *in vacuo* using the MM+ molecular mechanics force field and the Polak-Ribiere conjugate gradient method [default termination condition of 0.1 Kcal/(Åmol)]. Then the charges were recalculated by a semi-empirical calculation using the AMI programme and the single-point method (programme defaults, with an overall charge of +1). The procedure of geometry optimization and semi-empirical charge calculation was repeated once. The torsion angles between the rings for the two phenyl dihydrotriazines were found to be around 70°, as reported for active site conformations of these agents by Matthews *et al.*[22], while that for the phenyl pyrimidine pyrimethamine was 42°.

[b]Calculations using MOLGEN: to carry out calculations in the MOLGEN suite, the isolated amino acid side chains were made into complete structures by adding hydrogen to the initial carbon atom. Thus the side chain of alanine became CH_4 and that of serine CH_3OH. These structures were optimized (as above) in HyperChem and the files read into MOLGEN. It was necessary to recalculate charges at this stage before proceeding further. Conformationally-independent estimates of log π and molar refractivity were calculated using Viswanadhan's fragmentation method.

Table 2. Correlation of log K_i versus physicochemical characteristics of residue 108

log K_i vs	r	*P*
L	0.991	<0.01
L^2	0.989	<0.01
MW^2	0.954	<0.01
MR^2	0.895	<0.05
V^2	0.926	<0.05
A^2	−0.902	<0.05

For residues at 108 with side chains 2.4–4.9 Å in length, (five out of seven of the residues available for analysis) side chain length and bulk [i.e. molecular weight (MW), molar refractivity (MR) volume (V) and surface area (A)] are significantly correlated to reduced sensitivity (Table 2). The charge on the distal non-H atom and the calculated log π (lipophilicity) of the side chain apparently had no impact.

In the case of the remaining two residues, where a side chain was not present (Gly108) or short (1.5 Å; Ala108), the K_i values were anomalous – the trend, if any, being in the opposite direction to that shown by the rest of the group. It may be that the extra space is too large for adequate hydrophobic interaction, but the impossibility of the side chain H-bond to NADPH (see above) may mean that the position of the residue is less rigidly constrained, and this may affect the efficiency of drug binding.

β-sheet E

In *L. casei*, Ala97 in β-sheet E is involved, via its backbone oxygen, in H-bonding with the 4-amino nitrogen of MTX and of TMP. The side chain methyl carbon is located 3.85 Å from the same atom. It would be of interest if such a close approach is also seen in *P. falciparum*, as mutation of the equivalent residue (Ile164→Leu) gives resistance to CYC (Ref. 9), and is enhanced by the Ser108→Asn mutation. Although Ile is more bulky than Ala (side chain 3.05 Å vs 1.56 Å, respectively), Leu is also bulky and longer (side chain 4.034 Å) than Ile. This change could affect H-bonding between Asp54 and the drug.

β-sheet F

Finally, bacterial Thr116 in β-sheet F H-bonds through its side chain with a fixed water molecule, that is H-bonded to the MTX or TMP 2-amino group, and, with a further H-bond, it stabilizes the position of the Asp26 (Asp54 in PFDHFR) carboxylic acid group. Thr116 is conserved as Thr185 in PFDHFR.

Prediction and design of new drugs

We have as yet no crystalline structure of the active site of PFDHFR, but there is much information available on the bacterial enzyme, and the degree of conservation of the active site between DHFR sequences of *P. falciparum* and other bacteria encourages modelling studies. Toyoda *et al.*[10] prepared a model structure for PFDHFR (details not yet published). They selected from a database of available chemicals, compounds with bifunctional basic groups that could form stable H-bonds in plane with a carboxyl group. Optimized molecular models were docked with the enzyme model on computer, selecting 32 with suitable characteristics for experimental test against recombinant wildtype PFDHFR. Two compounds (**1**, a triazinobenzimidazole, and **2**, a pyridoindole, also known as Trp-P-2; Fig. 3) were found to be active with (high) K_i values against recombinant wildtype enzyme of 0.54 μM and 8.7 μM, respectively[10]. This study serves as a demonstration of the feasibility of the use of a model to detect new lead compounds for further modification and improvement.

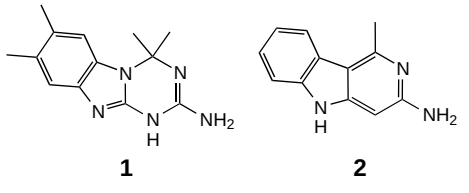

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Figure 3. *Inhibitors* ***1****, a triazinobenzimidazole, and* ***2****, a pyridoindole, were found to be active with* K_i *values of 0.54 and 8.7 μM, respectively, against recombinant* P. falciparum *dihydrofolate reductase*[20].

In more recent work, a model of PFDHFR based on the crystal structure of that from *Leishmania major* was constructed (details not yet published). The authors confirm earlier predictions[3,24] that there is potential for steric interference between the *p*-chlorine substituent of PYR and the resistant residue Asn108, reporting the synthesis and testing of two successful modifications of the drug[11] (with shorter rigid length) designed to avoid this problem. Compounds CC83 (*des*-chloro, *m*-methoxy pyrimethamine), and SO3 (*m*-chloro pyrimethamine) showed K_i values of 1.07 nM and 0.30 nM, respectively (cf. 1.5 nM for PYR), against recombinant wildtype DHFR. Against doubly mutated enzyme (Cys59→Arg and Ser108→Asn), the relative resistance factors were 13 and 8 compared with 48 for pyrimethamine. Activity was retained *in vivo* in preliminary experiments in mice.

Insights from modelling studies

The above studies show the potential of modelling for the improvement of old and the discovery of new antifolate antimalarials.

Using the HyperChem suite it is possible to replace MTX in the active site of 3dfr by other antifolate structures, such as PYR, CYC, CCY, WR99210 and TMP (Fig. 4). Protonated N1 in each case replaces N1 of MTX in favourable H-bonding orientation with a carboxyl oxygen of Asp26.

On examination of the drug structures fixed in the active site (PYR is shown in Fig. 4b), it is clear that the *p*-chlorine of PYR, CYC and CCY – a phenyl pyrimidine and two phenyl dihydrotriazines – are likely to approach much more closely to residue 45 (108 in PFDHFR) than do the equivalent substituents of MTX, TMP or WR99210, apparently because of the longer rigid length of the molecule in the binding plane (Table 3). Matthews *et al.*[22] point out that unlike a benzyl pyrimidine such as TMP, phenyl dihydrotriazines (and phenyl pyrimidines) have no side-chain torsional freedom in relation to the heterocycle except for simple rotation about the single bond connecting the two rings. Their discussion implies that longer inhibitors of this type have to fit in the bacterial enzyme in a way that reduces the binding affinity to the binding pocket.

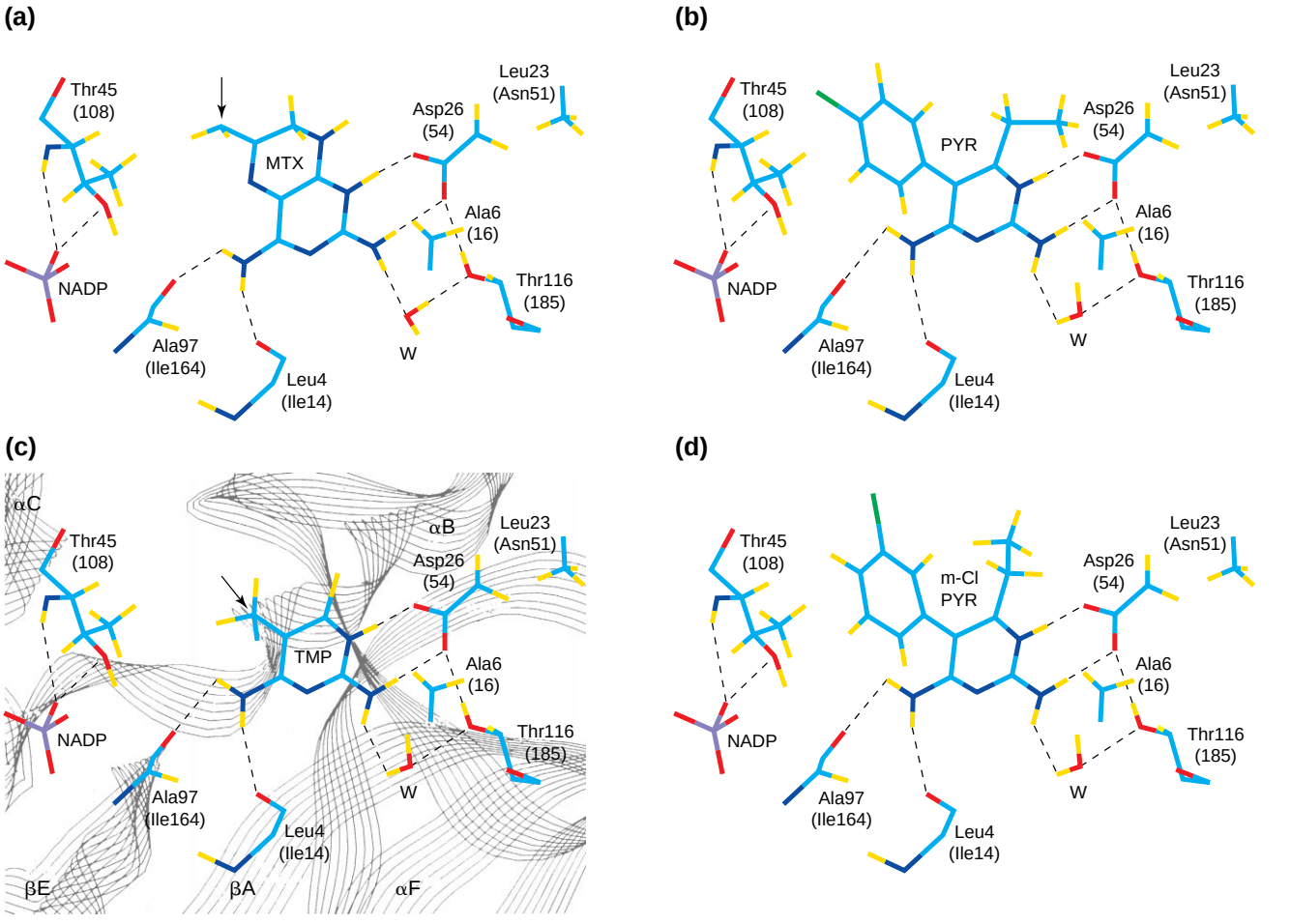

Figure 4. *Antifolate drugs in active site of* L. casei *dihydrofolate reductase (DHFR) to illustrate H-bonding relationships with surrounding residues, and of Thr45 with NADP (dotted lines). Equivalent residues for* P. falciparum *DHFR are shown in parenthesis. The methyl groups of Ala6 and Leu23 are indicated. For clarity, some parts of the residues are erased. (a) Shows methotrexate (MTX) and (b) pyrimethamine (PYR) in the active site, the* p*-chlorine of PYR is in close proximity to the side chain methyl group of Thr45 (108). The position of the* p*-amino benzoyl glutamate part of MTX (omitted) is indicated by an arrow. (c) Shows trimethoprim (TMP), the position of the trimethoxy phenyl ring (omitted) is indicated by an arrow. Positions of important α-helices and β-sheets are shown. (d) SO3 (*m*-Cl pyrimethamine; Ref. 11) in active site shows the increased distance between the methyl side chain of Thr45 (108) and the drug. Colours show: carbon, light blue; nitrogen, dark blue; hydrogen, yellow; oxygen, red; phosphorus, violet; chlorine, green.*

PYR, CYC and CCY have not been particularly useful against bacteria. The K_i for bacterial DHFR of four phenyl dihydrotriazines[22] similar to CYC had a minimum value of 245 nM, whereas that of TMP was 7.9 nM. On the basis of the measurements here, it is likely that the relative insensitivity of bacteria to phenyl dihydrotriazines (and phenyl pyrimidines) may be related to the short distance between the relevant part of the drug molecule (the large chlorine atom in the case of PYR and CYC) and the nearest active-site residue, Thr45 (Table 3). The van der Waals distance for contact and interaction between the *p*-chlorine of PYR or CYC and the methyl carbon of the Thr45 side chain should be no less than 3.6 Å and is likely to approach 4.0 Å. Even the general inhibitor MTX has the -CH_2- carbon 4.3 Å from the methyl carbon of Thr45 in the ternary complex (3dfr).

Residue 108

There is now sufficient evidence that the length and bulk of the side chain of residue 108 is an important plasmodial resistance determinant for PYR and CYC. It appears that

Table 3. Drug and binding-site parameters

Drug	Drug atom 'X' closest to Thr45[a]	Distance from X to CH_3 of Thr45[a] (Å)	Estimated extra space[b] (Å)	Torsion angle between the two rings	Rigid length[c] (Å)
Methotrexate	>C-**C**H_2	4.3	−0.7	NA	7.66
Trimethoprim	>C-**C**H_2-	5.4	−1.8	NA	5.51
Pyrimethamine	>C-**Cl**	2.1	1.5	42.4	9.96
CC83	>**C**H	3.0	0.6	42.3	8.23
SO3	>**C**H	3.1	0.5	43.0	8.24
Cycloguanil	>C–**Cl**	1.8	1.8	71.6	9.85
Chlorcycloguanil	>C–**Cl**	1.9	1.7	72.0	9.86
WR99210	>C-**O**-	5.4	−1.8	NA	5.26
Inhibitor **1**	>C-**C**H_3	1.7	1.9	NA	9.30
Inhibitor **2**	>**C**H	2.8	0.8	NA	7.83

[a]Distance in Å from X to CH_3 of Thr45 in *L. casei* DHFR. Optimization of amino acid structures: in HyperChem the selected amino acids were included in an H-bonded α-helix, and geometry and charge were calculated as in Table 1.

[b]Estimated extra space Å needed to allow binding of drug to *L. casei* DHFR assuming 3.6 is optimal.

[c]Rigid length (Å) of the heterocyclic ring system of the drug, and non-H substituent, X, measured from 2-amino N or equivalent. NA, not applicable.

the rigid length (RL) from the 2-amino group to *p*-phenyl substituent, in the case of phenyl substituted pyrimidines (PYR) or dihydrotriazines (CYC), or as far as the bend (often -CH_2-) in the more flexible agents (MTX and TMP), is a very important parameter. This is probably because of H-bond-related restriction of the movement of residue 108, and the sterically restricted requirement for H-bonding of the Asp54 to the heterocyclic ring system of the drug. The space for binding is finite, and, should the 108 side chain be too large (such as Asn108), will result in a poor fit (resistance) for drugs with an RL >8.7 Å.

The measured distance between the Thr45 methyl carbon and Asn26 carboxyl carbon in 3dfr is 12.4 Å. By looking at the estimated mean extra space needed by PYR, CYC and CCY at the *L. casei* DHFR active site (and assuming a minimal distance of 3.6 Å for van der Waals interaction), it is possible to arrive at an estimate of length for the same region of the malarial active site (from the carboxyl carbon of Asn54 to the methyl carbon of Thr108) of at least 14 Å, a difference of 1.6 Å. This is 1 Å longer than the human DHFR site (complex with deazafolate, 2dhf) measured in the same way[25].

Residue 108 mutations have a greater impact on antimalarial activity of PYR than on CYC. When modelled into the bacterial active site, the -CH_2- carbon of the ethyl side chain of the pyrimidine ring of PYR is only 3.2 Å distant from a carboxyl oxygen of the Asp26 side chain. Although the position of the oxygen is capable of variation by rotation around the carboxyl carbon bond, the ideal H-bonding with this atom appears to be planar with the ring, so the presence of an ethyl (or methyl) group in this position on PYR is likely to constrain the orientation of the H-bonds and, hence, affect the activity of the drug should the length of the site be reduced. Examination of the dihydrotriazine drugs (CYC and CCY) in the site, indicates that the two methyl groups attached to the ring tetrahedral carbon will be on either side of the ring, more distant from the Asp carboxyl oxygen, and may even serve to stabilize the position of the H-bond in the plane of the ring.

Precursor of greater resistance

It has been proposed that the single mutation at residue 108 is an essential precursor to further mutations in DHFR that give higher levels of resistance to antifolates. On the basis of recent studies by Sirawaraporn *et al.*[9] on resistance to PYR and CYC using a synthetic *PFDHFR* gene expressed in *E. coli*, it has been suggested that if agent(s) could be developed that inhibit both the wildtype and the Asn108 single mutant, then emergence of that precursor would be prevented and development of resistance significantly delayed. Perhaps then, the resistance produced by changes in residue 108 is only found for drugs with long, rigid structure, like PYR, CYC and CCY, but not for more flexible drugs.

These observations support the concept that variation of residue 108 may exert its important influence on the activities of PYR, CYC and CCY through steric interference with the chlorine *p*-substituent. This effect is likely to be appreciably less important for TMP and other flexible drugs.

It follows that in the development of new drugs effective against resistant malarial DHFR, the RL from the 2-amino group to the *p*-phenyl substituent, in the case of phenyl-substituted pyrimidines or dihydrotriazines, or as far as the bend (often -CH_2-) in the more flexible agents, is a very important parameter. This is probably because of constraints on the position of residue 108 (Thr45 in *L. casei*), which needs to H-bond to NADP, and upon the position of the heterocyclic ring system of the drug, which has H-bond contacts with three residues and a water molecule in the binding site.

Although the anticancer agent MTX, with an RL of 7.66 Å, is active on resistant and sensitive strains of *P. falciparum in vitro*, it is too toxic to use *in vivo* because it fits the mammalian DHFR well. Nevertheless, other antifolate drugs that also have an RL <8.7 Å, such as TMP, are likely to be effective against the most common mutant form of PFDHFR, Ser108→Asn. This brings up the possibility that in areas where antifolate resistance is widespread, the sequential or even combined use of TMP and PYR, probably combined with sulfonamide, may be advantageous.

Modelling novel antimalarials

The five newly discovered compounds mentioned earlier may provide a test of the model described here that was originally developed for MTX, TMP, PYR and CYC (Table 3).

WR99210 is a metabolite of the proguanil-related prodrug PS15, and has a dihydrotriazine ring like CYC (Ref. 26). It is bent at a ring-bridging oxygen, in a similar position to the -CH_2- bridge of TMP. It is known to be active in the presence of the Asn108 mutation[27,28] and would be predicted to have such activity on the basis of this modelling study.

For **1**, it appears that the overall clearance between Thr108 and drug methyl carbon is similar to that seen for CYC, and activity in PYR/CYC-sensitive PFDHFR should be expected. It seems unlikely, however, that similar activity will be seen against the PYR/CYC-resistant parasite. In **2**, there is more space between Thr108 and drug, leading to the expectation that activity may be seen in resistance. However, the distance between amino N4 of the drug and Ala97 (Ile164 in PFDHFR) is apparently not optimal for H-bond formation. If relationships were the same in PFDHFR this would predict the observed lower activity of **2** compared with **1** against the wildtype enzyme.

Both compounds CC83 and SO3 were designed to have extra clearance near residue 108, which agrees with activity against PYR-sensitive/resistant parasites. The lower intrinsic activity of CC83 may be related to the loss of the chlorine substituent, which will significantly reduce the lipophilicity. However, the calculated log π values are 2.28 for CC83 and 3.05 for SO3, compared with 3.05 for PYR, thus, it does not appear that the difference in lipophilicity is a major factor. It is highly encouraging that activity is seen in the mouse system in both these analogues.

As the relationship between the length of the binding site and the rigid length of an antimalarial drug appears important in determining selective toxicity, there may have to be a trade-off between effectiveness against resistant parasites and effects on the host. It is known that toxicity of WR99210 is higher than that of PYR or CYC (Ref. 29). It must be recognized that the risk of toxicity is enhanced in drugs with shorter rigid length, and modification of other parts of the molecule (as seen in the trimethoxy benzyl ring of TMP) may be necessary to achieve suitable therapeutic indices.

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