Dihydropteroate synthase: an old drug target revisited

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Folic acid is an important cofactor for the biosynthesis of a wide range of metabolites and is an essential component of the diet for higher eukaryotic organisms, including man. Prokaryotes and some lower eukaryotes can synthesize folic acid de novo from para-aminobenzoic acid (pABA) and GTP via a multi-step series of reactions, some of which are shown in Figure 1 [1–3]. 7,8-Dihydropterin is formed from pABA by the enzyme dihydropteroate synthase (DHPS). DHPS therefore sits at the junction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by lysised by GTP cyclohydrolase (not shown in Figure 1). An aldolase step is carried out on 7,8-dihydropterober by the enzyme dihydropteroate aldolase (DHNA), followed by a pyrophosphokinase step catalysed by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), using ATP. The product of this reaction, 6-pyrophosphomethyl-7,8-dihydropterin (DHPPP), is then used as a substrate for dihydropteroate synthase (DHPS). DHPS (E.C. 2.5.1.15) catalyses the formation of a carbon–nitrogen bond, joining the pterin moiety within DHPPP to pABA to form dihydropteroate and pyrophosphate. DHPS therefore sits at the conjunction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by GTP cyclohydrolase (not shown in Figure 1). An aldolase step is carried out on 7,8-dihydropterober by the enzyme dihydropteroate aldolase (DHNA), followed by a pyrophosphokinase step catalysed by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), using ATP. The product of this reaction, 6-pyrophosphomethyl-7,8-dihydropterin (DHPPP), is then used as a substrate for dihydropteroate synthase (DHPS). DHPS (E.C. 2.5.1.15) catalyses the formation of a carbon–nitrogen bond, joining the pterin moiety within DHPPP to pABA to form dihydropteroate and pyrophosphate. DHPS therefore sits at the conjunction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by GTP cyclohydrolase (not shown in Figure 1). An aldolase step is carried out on 7,8-dihydropterober by the enzyme dihydropteroate aldolase (DHNA), followed by a pyrophosphokinase step catalysed by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), using ATP. The product of this reaction, 6-pyrophosphomethyl-7,8-dihydropterin (DHPPP), is then used as a substrate for dihydropteroate synthase (DHPS). DHPS (E.C. 2.5.1.15) catalyses the formation of a carbon–nitrogen bond, joining the pterin moiety within DHPPP to pABA to form dihydropteroate and pyrophosphate. DHPS therefore sits at the conjunction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by GTP cyclohydrolase (not shown in Figure 1). An aldolase step is carried out on 7,8-dihydropterober by the enzyme dihydropteroate aldolase (DHNA), followed by a pyrophosphokinase step catalysed by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), using ATP. The product of this reaction, 6-pyrophosphomethyl-7,8-dihydropterin (DHPPP), is then used as a substrate for dihydropteroate synthase (DHPS). DHPS (E.C. 2.5.1.15) catalyses the formation of a carbon–nitrogen bond, joining the pterin moiety within DHPPP to pABA to form dihydropteroate and pyrophosphate. DHPS therefore sits at the conjunction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by GTP cyclohydrolase (not shown in Figure 1). An aldolase step is carried out on 7,8-dihydropterober by the enzyme dihydropteroate aldolase (DHNA), followed by a pyrophosphokinase step catalysed by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), using ATP. The product of this reaction, 6-pyrophosphomethyl-7,8-dihydropterin (DHPPP), is then used as a substrate for dihydropteroate synthase (DHPS). DHPS (E.C. 2.5.1.15) catalyses the formation of a carbon–nitrogen bond, joining the pterin moiety within DHPPP to pABA to form dihydropteroate and pyrophosphate. DHPS therefore sits at the conjunction of two biosynthetic pathways, one from GTP to folic acid and the other from chorismate, which ultimately forms pABA. In the final section of the pathway, dihydrofolate is formed by
The addition of glutamate to dihydropteroate, via dihydrofolate synthase, and is then used as the substrate for dihydrofolate reductase.

The fact that the folate biosynthesis enzymes are absent from man has made them an attractive target for the design of antimicrobial drugs. A good illustration of this rationale is the sulphonamides, which have been widely used in clinical practice for 60 years as antimicrobial and antiparasitic agents [4]. Sulphonamides act as analogues of pABA and are known to be specific inhibitors of folate biosynthesis [5–7]. (The structure of a simple sulphonamide, sulphanilamide, is shown in Figure 1.) The introduction of sulphonamides in the late 1930s had a dramatic effect on the treatment of bacterial infections. For example, fatality rates in cases of meningococcal meningitis fell from over 80% to 20% largely as a result of their introduction [8]. The use of sulphonamides to treat bacterial infections declined rapidly after the introduction of penicillin, but they are still in clinical use for the treatment of non-bacterial infections. Sulphasalazine is used as a first-line treatment for infection by Pneumocystis carinii, a common infection in patients with AIDS [9]. In these cases sulphamethoxazole is administered in conjunction with the dihydrofolate reductase inhibitor trimethoprim, illustrating how two specific inhibitors of folate metabolism can act in concert. This approach is also used in treating the malaria parasite Plasmodium falciparum, through the use of Fansidar®*, which is a combination of the dihydrofolate reductase inhibitor pyrimethamine and the sulphonamide sulfadoxine [10]. The value of antifolate chemotherapy in the treatment of a wide range of human pathogens is therefore beyond dispute.

Studies of the inhibition of Escherichia coli DHPS by sulphonamides have found that they act as alternative substrates for DHPS both in vitro and in vivo, forming the corresponding 7,8-dihydropterin-sulphonamide as product [11,12]. Diffusion studies showed that the 7,8-dihydropterin-sulphonamide derivatives diffused readily from the microbial cell and no

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**Figure 1**

Reactions catalysed by dihydropteroate synthase (DHPS), 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) and dihydroneopterin aldolase (DHNA)

![Reactions catalysed by dihydropteroate synthase (DHPS), 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) and dihydroneopterin aldolase (DHNA)](image-url)
evidence was found for their accumulation. In vitro the dihydropterin-sulphonamides were shown to be relatively poor inhibitors of the folate biosynthesis enzymes, including DHPS and HPPK [11]. The evidence suggests, for E. coli at least, that sulphonamides inhibit folic acid biosynthesis mainly by competition with pABA, thus reducing the net flux of the pathway to folic acid, rather than through any indirect effects of the 7,8-dihydropterin-sulphonamide derivatives on other enzymes.

The sequences of several DHPS genes have now been reported from a wide range of pathogenic organisms, including Streptococcus pneumoniae, E. coli, P. carinii, Staphylococcus aureus, Neisseria meningitidis, P. falciparum and Toxoplasma gondii [13-19]. It is interesting to note that DHPS from some eukaryotic sources occurs as a bifunctional complex with HPPK; this is the case for P. falciparum, T. gondii and Pisum sativum [15,19,20]. In P. carinii, DHNA, HPPK and DHPS all occur on one polypeptide chain [16,21]. Widespread resistance to sulphonamides has been documented in many organisms and several studies have been published that relate a sulphonamide resistance phenotype to specific mutations within the dhps gene. Lopez et al. [22] identified a 6-bp insertion in the SulA dhps gene that appeared to correlate with sulphonamide resistance in S. pneumoniae. More recently, this work has been extended by Maskell et al. [23], who compared the SulA gene sequences from 12 clinical isolates of S. pneumoniae and found that 3- or 6-bp insertions between Arg-58 and Tyr-63 accounted for the resistance phenotype. In other cases resistance seems to occur by substitution of amino acids at certain positions. For example, the increasing resistance to sulphonamides in the malaria parasite has been shown to correlate with amino acid substitutions in at least one of five different positions within the Plasmodium dhps gene [10,15]. Sulphonamide resistance phenotypes in the meningitis pathogen N. meningitidis appear to have arisen through horizontal gene transfer from other Neisseria species, forming mosaic gene structures [18,24]. Some meningococcal strains appear to acquire resistance by insertion of a dipeptide into the dhps gene sequence although, interestingly, this does not occur at the same position as in the streptococcal enzyme. dhps genes from other sulphonamide-resistant meningococcal strains contain amino acid substitutions at positions 31, 84 and 194. The relative importance of each of these mutations has been evaluated by site-directed mutagenesis and study of the kinetic properties of the mutant enzymes [25]. It was found that mutation from a sensitive to a resistant form of the enzyme increases the $K_m$ for pABA to some extent, but appears to have a greater effect on the $K_i$ for sulphonamide.

The study of the structure and mechanism of DHPS has been accelerated by the recent description of the crystal structures of DHPS from S. aureus and E. coli [17,26]. The structure of the S. aureus DHPS apoprotein was determined to a resolution of 2.2 Å, and the structure of a complex with a substrate analogue, 6-pyrophosphomethyl-pterin, was also determined to 2.4 Å. The E. coli DHPS structure was determined to a slightly higher resolution, 2.0 Å, and the structures of a binary complex with DHPPP and a pseudo-ternary complex with 6-hydroxymethyl-7,8-dihydropterin (DHP), pyrophosphate and sulphanilamide were also determined. Both enzyme structures adopt a TIM-barrel type fold, with the active site located at the C-terminal end of the β-barrel (Figure 2). A comparison of the alignments of DHPS amino acid sequences from a wide range of sources with both crystal structures reveals some interesting minor differences. For example, DHPS from S. pneumoniae contains an insertion of approximately 25 amino acids that does not occur in other sequences and appears to fall between the fifth β-strand and fifth α-helix of the TIM barrel. Similarly, the P. falciparum gene sequence predicts an insertion of about 30 amino acids between the seventh β-strand and α-helix. The functional significance of these insertions, if any, is unclear at present.

The substrates bind in a cleft that is lined with residues that are highly conserved in all DHPS amino acid sequences. The plane of the pterin ring lies parallel to the axis of the β-barrel, with the pyrophosphate group pointing outwards. A network of hydrogen bonds from the side chains of residues in the protein is responsible for recognition of the pterin ring. These comprise (in the E. coli numbering scheme) Asp-96, Asn-115, Asp-185 and Lys-221, and are all residues that are absolutely conserved in sequence alignments. Met-139 and Phe-190 may also play an important part in recognition of the pterin ring by forming hydrophobic contacts, and these residues are also highly conserved. Thr-62 plays an important part in the recognition of the α-phosphate group, with two hydrogen bonds from main chain and side chain atoms. Interest-
ingly, this position is occupied by a serine in the *Plasmodium dhps* amino acid sequence and this portion of the structure is subject to mutation in *dhps* sequences from sulphonamide-resistant strains (see below). The β-phosphate group is effectively sandwiched between the side chains of Arg-63 and Arg-255, and additional hydrogen bonds are contributed by Asn-22 and His-257. This elaborate network of hydrogen bonds suggests how pyrophosphate, a product of the reaction, could be recognized independently by the enzyme.

The sulphanilamide lies across the outside of the pterin binding cleft and its recognition involves fewer residues than DHPPP. The ligand is surrounded by Arg-63, Pro-64, Arg-220, Lys-221 and His-257, all of which are conserved except Arg-63, which is Asn and Gly in the *Toxoplasma* and *Plasmodium* sequences respectively. One side of the sulphanilamide appears to be exposed to bulk solvent, which could explain why such a variety of sulphonamide derivatives are effective inhibitors of the enzyme. The aniline nitrogen atom on sulphanilamide is located 3.65 Å from C11 within DHP, the atom that is the target of attack by the nitrogen atom in catalysis. This appears to be too far for a reaction to occur efficiently, although the DHP and DHPPP ligands appear to occupy identical positions in space. As the authors point out, it seems likely that a subtle rearrangement of the relative positions of DHPPP and sulphanilamide is required for catalysis [26]. Indeed, it is noteworthy that the positions of the main chain atoms in the *E. coli* structure are essentially unchanged in the apoprotein, binary and pseudo-ternary complexes. In this respect the situation is similar for the *S. aureus* crystal structure. The structure was determined alone and in complex with the substrate analogue 6-pyrophosphomethyl-pterin: again, there were no significant structural changes on binding of the ligand. The *S. aureus* crystal structure does differ from the *E. coli* DHPS in one important respect: electron density arising from the 6-pyrophosphomethyl-pterin ligand was found in only one monomer of the non-crystallographic dimer, suggesting a half-of-sites reactivity [17]. Additional evidence for this hypothesis was obtained from steady-state kinetic measurements, which showed substrate inhibition at high concentrations of pABA. The authors proposed a second, non-catalytic, binding site for pABA in *S. aureus* DHPS which would affect the rate of reaction when occupied. The crystals of the *E. coli* enzyme contain a monomer in the asymmetric unit and so monomers within the dimer are related by crystallographic symmetry. Occupancy of only one binding site per dimer cannot be observed in this crystal form. Detailed studies of the kinetics of *E. coli* DHPS have not shown any evidence for substrate inhibition at high concentrations of pABA and DHPPP, although product inhibition studies suggested an ordered binding mechanism, with DHPPP binding first [27]. However, the authors found this scheme difficult to reconcile with the variation of velocity with substrate concentration, which was characteristic of a ping-pong reaction scheme. Furthermore, it would appear that the lack of substantial changes in structure on binding of DHPPP, as observed in the *E. coli* crystal structure, do not agree well with the results of the kinetic studies.
Figure 3
Location of \textit{P. falciparum} sulphonamide resistance mutations

The sites of the five sulphonamide resistance mutations in \textit{P. falciparum} identified by Wang et al. \cite{10} are shown, superimposed on a chain trace of \textit{E. coli} DHPS (PDB accession code IAJO). The residues are numbered according to their equivalent positions in the \textit{E. coli} DHPS amino acid sequence. The diagram was constructed using Molscript \cite{28}.

It is interesting to examine the distribution of the sulphonamide resistance mutations across the DHPS structure. Figure 3 shows the location of the five sites of point mutation identified by Wang et al. \cite{10} that are involved in mediating resistance to sulphonamides in \textit{Plasmodium}. The mutations fall towards one end of the protein and are generally located close to the sulphamamide binding site. Thr-62 and Arg-63 form direct contacts with sulphamamide and DHPPP and it is plausible that mutations in these positions in the \textit{S. aureus} enzyme is less clear: sequencing of the \textit{dhps} genes from several sulphonamide-resistant clinical isolates identified multiple mutations in each isolate that were distributed across the structure and not localized close to the ligand binding sites. It is unclear at present whether these are all required to confer sulphonamide resistance.

In conclusion, it is clear that the study of DHPS as a drug target has now entered an exciting new phase. Although the enzyme has been known for many years, it is now being revisited with modern molecular biological and biophysical techniques. This has opened up new avenues of possibilities for the rational design of antifolates which were clearly not open to medicinal chemists 60 years ago.

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1 Shiota, T., Disraely, M. N. and McCann, M. P. (1964) \textit{J. Biol. Chem.} \textbf{239}, 2259–2266
Mechanistic diversity of β-lactamases

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The ‘β-lactamase cycle’

In a recent issue, the American magazine Time cited the discovery of penicillin as one of the most important achievements of the 20th century. This choice, which is particularly striking because this finding was not due to American scientists, underlines the impact of the development of antimicrobial chemotherapy on the life of the ordinary citizen. Over 50 years the penicillin family has grown to a large number of compounds which represent the vast majority of the currently available antibiotics and share a common chemical feature, the four-membered β-lactam ring. They kill bacteria by inactivating the membrane-bound ß-turn pep- tidases [1], essential enzymes in the synthesis of the bacterial cell wall (Figure 1). However, bacteria have reacted by developing various resistance mechanisms whose spreading was favoured by the strong selective pressure created by the intensive, and sometimes abusive, utilization of antimicrobial agents. Four such mechanisms have presently been identified: the production of enzymes which efficiently hydrolyse the amide bound in the β-lactam ring (the β-lactamases), the synthesis of modified ß-turn pep- tidases, whose affinity for β-lactams is drastically reduced, a decrease of the outer membrane permeability in Gram-negative species and, mainly in the genus Pseudomonas, the appearance of active efflux systems which pump the antibiotic out of the periplasm. Although the resistant ß-turn-peptidases are responsible for an increasing number of clinical problems, exemplified by the emergence of the