Published in final edited form as:
Curr Drug Targets. 2007 January ; 8(1): 31–47.

Targeting Purine and Pyrimidine Metabolism in Human Apicomplexan Parasites

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Abstract

Synthesis *de novo*, acquisition by salvage and interconversion of purines and pyrimidines represent the fundamental requirements for their eventual assembly into nucleic acids as nucleotides and the deployment of their derivatives in other biochemical pathways. A small number of drugs targeted to nucleotide metabolism, by virtue of their effect on folate biosynthesis and recycling, have been successfully used against apicomplexan parasites such as *Plasmodium* and *Toxoplasma* for many years, although resistance is now a major problem in the prevention and treatment of malaria. Many targets not involving folate metabolism have also been explored at the experimental level. However, the unravelling of the genome sequences of these eukaryotic unicellular organisms, together with increasingly sophisticated molecular analyses, opens up possibilities of introducing new drugs that could interfere with these processes. This review examines the status of established drugs of this type and the potential for further exploiting the vulnerability of apicomplexan human pathogens to inhibition of this key area of metabolism.

Keywords

Plasmodium; *Toxoplasma*; *Cryptosporidium*; nucleotide biosynthesis; salvage pathways; drug resistance; drug targets; folate metabolism

INTRODUCTION

Replication of DNA and its transcription to the various RNA species necessitate a constant and substantial supply of the constituent nucleotides, a particularly demanding requirement in the case of rapidly dividing cells or pathogens, for example cancer cells or malaria parasites. DNA synthesis utilises the pyrimidine deoxyribonucleotide 5′-triphosphates dCTP and dTTP, and the purine deoxyribonucleotide 5′-triphosphates dATP and dGTP; RNA synthesis requires the ribose counterparts of C, A and G, together with uridine 5′-triphosphate, UTP. In addition, certain of these molecules and related derivatives are particularly important as sources of chemical energy (e.g. ATP, GTP), nucleotide-based enzyme cofactors (e.g. NAD^+, FAD, FMN), precursors of more complex molecules (e.g. the conversion of GTP to pterins and folates), intracellular second messengers (e.g. cAMP) and switch signals (e.g. GDP/GTP) involved in metabolic and gene regulation. Their critical participation in many aspects of cell function and replication makes their biochemical pathways attractive targets for drug intervention. If such intervention depletes the dNTP

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pool sufficiently, DNA replication is arrested, but even the induction of nucleotide imbalances can lead to misincorporation of base types during replication, leading to fatal genetic damage [1].

In principle, the above nucleotides and/or their nucleoside and nucleobase precursors can be obtained in several ways: synthesis *de novo*, direct acquisition by salvage or by subsequent interconversions to yield the appropriate repertoire of purines and pyrimidine derivatives found in the nucleic acids and other important molecular species. Drugs aimed at disrupting such processes have been successfully used against protozoan parasites of the phylum Apicomplexa, such as *Plasmodium* and *Toxoplasma*, for many years, although selection of resistant strains is a major and ever-increasing challenge, particularly in the case of malaria parasites [2]. However, the recent completion of genome sequences, together with ongoing biochemical, genetic and bioinformatic investigations, now pave the way for the possibility of introducing new drugs targeted at these processes, to help circumvent the problems of resistance and widen the choices for prophylactic or therapeutic intervention. This review will examine the major pathways involved, the status of established drugs disrupting these pathways, and the potential for further exploiting the vulnerability of apicomplexan human pathogens to inhibition of these key areas of metabolism.

Several apicomplexan parasites affect human health to a major degree. Principal among these are the highly host-specific malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. Two other species lead to malaria in humans, *Plasmodium malariae* and *Plasmodium ovale*, but these are less widespread and cause far fewer infections [3]. Of the four, *P. falciparum* is normally the only species that can cause the death of the host, the fate of at least one million people a year so infected (mainly young children) [4], although this figure is likely to be a significant underestimate [5]. Even if not fatal, attacks of malaria cause considerable levels of morbidity that add substantially to the economic and public health burden of many developing countries in tropical and sub-tropical areas [6]. The *P. falciparum* genome sequence was essentially completed in 2002 [7], and at the time of writing, 10x shotgun coverage of the whole genome of *P. vivax* is also available, with annotation [8] (PlasmoDB: http://www.plasmodb.org; TIGR: http://msc.tigr.org/p-vivax). *Toxoplasma gondii* is a highly promiscuous parasite found worldwide that is capable of infecting a wide range of cell types in most warm-blooded animals (mammals and birds). Although infections in humans are normally asymptomatic, they can cause severe morbidity and death in immunocompromised people (especially organ transplant and AIDS patients), as well as in congenitally infected foetuses or newborns. This opportunistic parasite is the most frequent cause of secondary central nervous system infections in the immunocompromised [9]. Another opportunist of the apicomplexan phylum, also with a global distribution and an ability to infect a wide range of animals, is *Cryptosporidium*. This organism infects epithelial cells of the small intestine and is responsible for large waterborne outbreaks of cryptosporidiosis, which is normally manifest as acute but self-limiting enteritis in healthy adults, but as severe chronic disease with substantial morbidity and mortality among AIDS patients and young children [10]. Unlike for *Plasmodium* and *T. gondii*, there is no fully effective clinical treatment for *Cryptosporidium*. Partly because of major technical difficulties in culturing this organism in the laboratory or passaging it through animals, again in contrast to *T. gondii* and species of *Plasmodium*, aspects of the basic biology of *Cryptosporidium* remain poorly understood and direct drug screening is difficult. However, knowledge of its key metabolic pathways, genome organization and organellar complement has increased considerably since the completion of the genome sequences of *C. parvum* [11] and its close relative *C. hominis* [12] (see also CryptoDB; http://www.cryptodb.org), two important species that infect humans. *T. gondii* as an experimental organism is greatly facilitated by the ease with which transfection and genetic manipulation can be carried out [13, 14], and its genome sequence is also currently at the level of an

annotated 10x shotgun coverage (ToxoDB: http://www.toxodb.org; TIGR: http://msc.tigr.org/t_gondii). Transfection is also possible in certain *Plasmodium* species [15-17], but is technically considerably more demanding and less versatile. However, this technology has been of major utility in investigating, for example, the molecular basis of drug resistance in these organisms, particularly for *P. falciparum* [18].

Among the other apicomplexans, parasites of the genus *Isospora* cause intestinal disease in several mammalian species. Human intestinal isosporiasis is caused by *Isospora belli* and pathology in immunocompetent patients can include diarrhoea and steatorrhoea, with a range of other unpleasant symptoms. Infections by *Isospora* in humans are less frequent and generally less serious than for the other parasites described above. Similarly, *Sarcocystis*, while quite common in man, is rarely diagnosed, as the pathology is slight. Moreover, no genome sequencing projects for these two genera are being pursued in the major sequencing centres as yet. The remainder of this article will thus be concerned with *Plasmodium, Toxoplasma* and *Cryptosporidium*, as the key members of the Apicomplexa that have by far the greatest impact on human health. All three of these parasites occupy nutrition-rich, intracellular environments at certain stages of their life cycles, and exhibit a variety of adaptations that include metabolic deficiencies relative to free-living eukaryotes. The resulting simplified metabolic pathways often contain a rate-limiting enzyme indispensable to the parasite but not essential in the host. Moreover host and parasite homologues may be sufficiently dissimilar to allow the discovery or design of specific inhibitors of the parasite protein [19]. Examples of both of these phenomena are common in the nucleotide-processing pathways.

ACQUISITION OF PYRIMIDINES AND THEIR DERIVATIVES

The Apicomplexa are considered generally, but not universally, to be capable of synthesising pyrimidines from the simple amino acid precursors glutamine and aspartic acid by essentially the same mechanisms as found in other eukaryotes, including the human host. This is a six-step process whereby the pyrimidine derivative orotic acid is first assembled, then added to a ribose-5-phosphate moiety to form orotidine 5′-monophosphate. This is subsequently converted *via* decarboxylation to the parent mononucleotide uridine 5′-monophosphate (UMP), which is the precursor of all other pyrimidine nucleotides, Fig. (**1**). After a double phosphorylation of UMP to UTP, CTP synthase (CTPS; EC 6.3.4.2) converts the latter to CTP in a rate-limiting step, which is the only known route for *de novo* synthesis of cytosine derivatives. Although early studies were unable to detect significant levels of CTPS in *P. falciparum* [20], despite its apparent status as an indispensable enzyme, the gene encoding this enzyme is present in malaria parasites [21], as it is in *T. gondii* and *Cryptosporidium* [12]. The potential of a CTP synthase inhibitor, 3-deazauridine, has been investigated as an anti-cancer [22] or anti-HIV agent [23], but not yet as a possible measure against apicomplexan parasites. As well as the above ribonucleotides, deoxyribonucleotides must be made, and in most organisms the conversion to the deoxyribose forms takes place at the level of nucleoside diphosphates, catalysed by ribonucleotide reductase (RNR; EC 1.17.4.1) (discussed later). Formation of thymine nucleotides requires methylation of dUMP to produce dTMP by thymidylate synthase (TS; EC 2.1.1.45) in the folate pathway, an area of metabolism dealt with in some detail below, as it has long been a prime target for antimicrobial agents, including those directed at *P. falciparum* and *T. gondii*.

In the human host, both biosynthesis and salvage of pyrimidines occur at significant levels, and if the former is blocked, as in hereditary orotic aciduria, the condition can be relieved by administration of uridine. By contrast, salvage of pyrimidines is thought to be of little, if any, importance in the malaria parasites, which may relate to the fact that mature mammalian erythrocytes (the host cells in which the parasite resides for most of the period

of infection) lose their ability to synthesise pyrimidines *de novo*. The dependence of malaria parasites on pyrimidine biosynthesis and the ability of the host to use salvage pathways makes the *de novo* pathway in the parasite a potentially effective drug target. By contrast, analysis of the genomic sequence of *Cryptosporidium* indicates that it lacks genes for all six of the enzymes involved in *de novo* synthesis, and is thus completely dependent on salvage from the host for its pyrimidines, as it is for its purines (see later). Indeed, *Cryptosporidium* is deficient in many biosynthetic capabilities and its genome encodes numerous transporters to facilitate salvage of essential precursors from the host [12]. Thus, the loss of the *de novo* synthesis capability for pyrimidines in this organism is compensated for by three salvage enzymes. These are a monofunctional uracil phosphoribosyltransferase (UPRT; EC 2.4.2.9), a bifunctional protein with UPRT and uridine kinase (UK; EC 2.7.1.48) activities, and thymidine kinase (TK; EC 2.7.1.21), Fig. (**1**). The UK-UPRT and TK enzymes appear so far to be unique to *Cryptosporidium* within the Apicomplexa. UK enzymes are capable of processing cytidine as well as uridine, and *C. parvum*, unlike *T. gondii*, is susceptible to cytosine-arabinoside (Ara-C), a pro-drug that is activated by UK [24]. Interestingly, phylogenetic analysis suggests that a number of the genes involved in nucleotide metabolism in *Cryptosporidium* ultimately derive from protozoal, algal or bacterial sources, and that in particular, the gene encoding TK in this organism was acquired by horizontal transfer from a proteobacterium, while that for UK-UPRT may have originated from an algal symbiont [25].

The importance of the balance between salvage mechanisms and *de novo* biosynthesis is well illustrated in the case of *T. gondii*, which falls between *Plasmodium* and *Cryptosporidium* in its abilities to salvage pyrimidines. As discussed below, this parasite cannot salvage thymidine or cytidine, nor can it utilise the thymine and cytosine nucleobases themselves [26]. However, it has retained the ability to salvage uracil, from which the T and C nucleotides can be produced, and parasites disabled in the pyrimidine biosynthetic pathway by gene knockout are able to survive if exogenous concentrations of uracil are high enough. Interestingly, though, such parasites are severely compromised to the extent that, while still able to infect mice, they are unable to kill them, even if the mice are not immunocompetent [27]. Not only does this suggest that pyrimidines are at insufficient concentrations in a mammalian host, at least for the limited salvaging abilities of *Toxoplasma* (although clearly not the case for *Cryptosporidium*), but that targeting the enzymes of pyrimidine synthesis in this organism is a potentially powerful approach. Uracil is efficiently imported into the parasite, and uptake of radiolabelled uracil is traditionally used to measure growth rates and the effects of inhibitors. The key enzyme for uracil salvage in *T. gondii* is UPRT, which adds a ribose 5-phosphate moiety to convert the base to UMP. Importantly, mammalian cells lack this activity, opening up the possibility of using uracil analogues that could be selectively processed by the parasite UPRT to toxic metabolites, a consideration that also applies to *Cryptosporidium*. It was recently shown that substituted uridines can be successfully incorporated into the RNA of *T. gondii* itself or of human cells expressing *T. gondii* UPRT heterologously [28]. Moreover, the properties of this enzyme have been characterised [29, 30] and it has been crystallised in the presence of several ligands, including its substrate uracil and the important pro-drug 5-fluorouracil, with resolution to *ca*. 0.2 nm [31]. Although 5-fluorouracil itself is a toxic anticancer drug, these studies should facilitate the design of pyrimidine-based pro-drugs that are more suitable as agents to combat toxoplasmosis.

The key regulatory enzyme in pyrimidine biosynthesis in those apicomplexan parasites that have retained this pathway is the first member, carbamoyl phosphate synthetase II (CPSII; EC 6.3.5.5), which is activated by 5-phosphoribosyl-1-diphosphate (5-phosphoribosyl pyrophosphate, PRPP) and inhibited by the end-product UTP [32]. CPSII molecules from the apicomplexan parasites are monofunctional, as in bacteria, but differ from those found in

other eukaryotes, which are either bi- or trifunctional with respect to activities further down the pathway. These enzymes thus differ significantly in their architecture and properties from those of the human host [33, 34]. This includes peptide insertions in the glutamine amidotransferase and carbamoyl phosphate synthetase domains of these proteins that are absent from bacterial, plant, fungal and mammalian counterparts [35]. Growth of *T. gondii* can be strongly inhibited by the glutamine antagonist acivicin, which targets the glutamine amidotransferase activity associated CPSII. Interestingly, this activity has been targeted in *P. falciparum* in a quite different way, utilising inhibition by a ribozyme [36]. This takes advantage of the fact that the CPSII mRNA transcript contains two large insertions absent in other homologues, including that from humans. Such insertions are not uncommon in malarial transcripts and represent highly specific targets of RNA sequence that could in principle be exploited for nucleic acid-based therapies.

The other important enzymes in *de novo* UMP synthesis comprise aspartate carbamoyltransferase (ATCase; EC 2.1.3.2), which catalyses the second step, and whose gene and product have been characterised in *T. gondii* [37], dihydroorotase (DHOase; EC 3.5.2.3), which catalyses the third step, whose gene has been cloned from *P. falciparum* [38] and *T. gondii* [39], dihydroorotate dehydrogenase (DHODH; EC 1.3.3.1) the fourth (see later), orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10) the fifth, and orotidine 5′-monophosphate decarboxylase (OMPDC; EC 4.1.1.23), the sixth, Fig. (**1**). In *P. falciparum* and *T. gondii*, separate genes encode OPRT and OMPDC [38], which have been shown in the malaria parasite to associate into a complex comprising two subunits of each enzyme [40, 41], with different kinetic properties from the host UMP synthase, where these activities are combined on a single bifunctional protein. Analogues of orotate (the substrate of OPRT) substituted at the 5′ position have been shown to inhibit pyrimidine synthesis in *P. falciparum* and in the rodent parasites *P. berghei* [42, 43] and *P. yoelii* [44]. Such treatment was able to cure mice of infection with the latter, as long as uridine was co-administered to permit host salvage. This type of compound is attractive as it may act against the parasite, not only by directly inhibiting OPRT, but also by undergoing processing to a UTP derivative that may inhibit CPSII [45] or a dUMP derivative that can inhibit TS (see later). A large number of orotate analogues have also been systematically tested against *Toxoplasma* OPRT and structural features necessary for strong binding defined [46]. Four derivatives in particular were found in this study that had been observed in earlier work [47] to be much less active against the mammalian enzyme. Inhibitors of malarial DHOase and OMPDC have also been investigated and their effectiveness monitored by the accumulation of blocked intermediates [45].

THE FOLATE PATHWAY IN THYMIDINE SYNTHESIS

Drug intervention to block pyrimidine synthesis has traditionally taken the form of targeting the folate pathway, as the major reaction in which folate cofactors are involved is in the conversion of deoxyuridine 5′-monophosphate (dUMP) to deoxythymidine 5′-monophosphate (dTMP). For every molecule of the latter required for incorporation into DNA *via* dTTP, a molecule of tetrahydrofolate (THF), in the form of the 5,10-methylene derivative, is oxidised to dihydrofolate (DHF) as its one-carbon unit is transferred to dUMP, Fig. (**2**). If the supply of THF is cut off or severely reduced, either by blocking *de novo* synthesis of folate molecules, blocking their salvage from the host, blocking the recycling of DHF to THF, or a combination of these, then the parasite will die from thymidine starvation. In the case of *Plasmodium* and *Toxoplasma*, this cannot be ameliorated by salvage of thymidine from the host, as neither of these parasites has this capability [27], consistent with the lack of TK-encoding gene candidates in their genome sequences. In order to meet their dTMP synthesis needs for replication, these parasites are thus completely dependent upon sufficient dihydrofolate reductase activity (DHFR; EC 1.5.1.3) for DHF to THF reduction,

as well as on serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) to produce 5,10-methylene-THF from serine, and on TS to transfer the one-carbon unit from this intermediate to dUMP, Fig. (**2**). The DHFR and TS activities in these organisms, as in other protozoa and plants, are found together on a bifunctional protein encoded by a single *dhfr-ts* gene. *Cryptosporidium* also produces the DHFR-TS and SHMT required for folate recycling, but is exceptional in harbouring a *tk* gene that again appears to be of bacterial origin, and which therefore might offer a good target for novel inhibitors to be used in combination with antifolates.

The folate cofactors required for the thymidylate cycle may in principle be acquired by a parasite either by *de novo* synthesis and/or by salvage from the host [48]. *P. falciparum* is capable of using both routes, as shown by several studies using radioactive precursors and monitoring the products by HPLC [49-51]. Thus, label in guanine or guanosine, incorporated *via* GTP, is found in the major product 5-methyltetrahydrofolate, as is label in folic acid or folinic acid (leucovorin; 5-formyltetrahydrofolate) provided exogenously. Import of folates has also recently been demonstrated in *T. gondii* [52], but like *P. falciparum*, the genes for *de novo* folate synthesis are present as well, with several activities confirmed in experiments utilising recombinant gene products ([53]; J. Smith, F. Flett, J E Hyde and P F G Sims, unpublished data). By contrast, *Cryptosporidium* lacks any identifiable genes encoding folate biosynthesis enzymes and thus appears to be wholly dependent upon salvage of folates from the host, although no experimental studies investigating this phenomenon have been reported to date.

As well as the primary precursor GTP, whose purine ring is rearranged into that of a pterin by GTP cyclohydrolase I (GTPC; EC 3.5.4.16), the biosynthetic pathway for folate requires a source of *para*-aminobenzoic acid (pABA). This is linked to the pterin moiety by dihydropteroate synthase (DHPS; EC 2.5.1.15) to produce a pteroate derivative, which in turn is monoglutamated by dihydrofolate synthase (DHFS; EC 6.3.2.12) to give DHF, Fig. (**2**). Again, in principle, the pABA can be obtained exogenously from the host or as a product of the shikimate pathway *via* chorismate, which in other organisms also provides a route to the aromatic amino acids. Genes encoding some of the enzymes of this pathway have been identified in *P. falciparum* [7, 54] and *T. gondii* [55, 56]. These include a putative bifunctional PabAB protein from *P. falciparum* that would contribute two of the three activities that make up the pABA synthetase responsible for conversion of chorismate to pABA [57]. Thus, the two activities of PabAB constitute the aminodeoxychorismate synthase (EC 6.3.5.8) that transfers the amido group from glutamine to chorismate. A separate protein, PabC, with aminodeoxychorismate lyase activity (EC 4.1.3.38), then converts the resulting intermediate to pABA. However, there is no evidence that the aromatic amino acids can also be derived from chorismate in these organisms. The shikimate pathway is absent in its entirety from *Cryptosporidium*, as deduced from the complete genome sequences [11, 12], in keeping with its status as the most metabolically streamlined of the apicomplexans considered here. Thus the molecular basis of this organism's apparent sensitivity to the shikimate pathway inhibitor glyphosate [55] is unclear, although the high levels used in these experiments (>5 mM) suggest that other, less specific, targets may be involved, particularly as the inhibition could not be relieved by providing exogenous pABA.

ANTIFOLATES AND DRUG RESISTANCE

For many decades, the only drugs in clinical use deployed against malaria and *Toxoplasma* that directly affect pyrimidine metabolism were the antifolates, which are still heavily used today, despite losing their efficacy against malaria in many areas of the world [58]. The principal antimalarial antifolate drugs are pyrimethamine (PYR), proguanil (PG;

metabolised *in vivo* to the active form cycloguanil, CG), and the sulfa drugs, which include sulfonamides such as sulfadoxine (SDX), and sulfones such as dapsone. PYR and CG target DHFR, and thus folate recycling, achieving their pharmacological utility by binding to the parasite enzyme several hundred-fold more tightly than to its human counterpart. The sulfa class of drugs target DHPS, and thus *de novo* folate biosynthesis, which is absent in the human host. Although PYR and PG were initially administered as single agents, resistance arose rapidly and it was only in combination with sulfa drugs that effective clinical formulations were developed that are still widely used today to combat chloroquine-resistant malaria, particularly in Africa [59]. This arises from the potent synergy observed between the components of these combinations [60] and retardation of the onset of resistance that is characteristic of carefully chosen drug formulations [2]. A similar combination of PYR and sulfadiazine (SDZ) is used extensively as the major treatment for life-threatening toxoplasmic encephalitis (TE). These drugs are effective against the rapidly dividing tachyzoite form of *T. gondii*, but do not affect the bradyzoite (cyst) stages, which act as a permanent reservoir of infection in the host. Acute therapy for TE has to be followed by lifelong maintenance therapy with PYR/SDZ at lower dosages. Unfortunately, such a regime, which is much more severe than that used to treat malaria, can induce dangerous side effects, including allergic reactions and haematotoxicity. At present, however, alternative treatments using PYR combined with the antibiotic clindamycin or other antiparasitic drugs are less effective, although in studies of treatments for ocular toxoplasmosis, a combination of pyrimethamine with azithromycin appears to be of comparable efficacy as pyrimethamine-sulfadiazine, but results in less severe and less frequent adverse reactions [61].

Mutations in the *dhfr* Gene Conferring PYR and CG Resistance

The established antifolates are very cheap drugs and parasite resistance to this class of inhibitors is of major importance, as it compromises one of the very few effective treatments affordable in many African countries. Therefore, although known not to be completely predictive of clinical failure in malaria treatment, the genetic changes in *P. falciparum* involved in resistance to the individual components of antifolate combinations have been investigated in considerable depth. The discovery of variant *P. falciparum* DHFR sequences in strains with differing degrees of resistance [62-64] led rapidly to the demonstration that high-level PYR resistance results from the accumulation of mutations in the *dhfr* domain of the bifunctional *dhfr-ts* gene, principally at codons 108, 59 and 51, where allelic variation gives rise to the amino acid changes S108N, N51I and C59R. Alteration of the 108 codon appears to be the essential first step, after which the additional mutations can accrue to increase further the level of PYR resistance [65]. In South-East Asia, a fourth alteration, I164L, is now commonly seen, which in combination with the changes at codons 51, 59 and 108, bestows complete resistance of *P. falciparum* to achievable physiological concentrations of PYR/SDX [66-69]. As yet, such quadruple mutant parasites have not been detected in African parasite populations, but they have been reported recently from the Indian island of Car Nicobar [70]. Residues 16 (A16V) and 164, as well as an alternative alteration in position 108 (S108T), are also involved in resistance to CG, whose structure is very similar to that of PYR [71, 72]. The causal relationship between resistance and a series of structurally modified enzymes has been conclusively proven in several ways [65, 73-75], including the powerful but technically challenging technique of *in vitro* transformation of drug-sensitive parasites with vector constructs bearing mutant forms of *dhfr*, which then acquire the predicted levels of PYR resistance [76].

In the case of *T. gondii*, PYR-resistant parasites have been reported [13], but the *dhfr* genes from the strains monitored did not show variation from the wild-type sequence. Other mechanisms can be envisaged, such as gene amplification or promoter mutations increasing

levels of mRNA and hence of the DHFR target, but at present there are no data that shed light on the molecular basis of PYR resistance in this parasite, other than the demonstration that mutations deliberately engineered into sensitive parasites that mimic those seen in *P. falciparum dhfr* similarly confer PYR-resistance [77], and that artificial truncations within the linker region between the DHFR and TS domains also reduce sensitivity to PYR [78]. The former observation has been used as a basis to explore the individual roles and structural implications of the various mutations [77, 79]. Moreover, the engineered strains of *T. gondii* have also been used in competitive growth studies, both *in vitro* and *in vivo*, to explore the degree to which given patterns of mutations affect the fitness of the organism and thus gain insight into the trade-off between the expression of a highly drug-resistant enzyme and the reduction in the efficacy of that enzyme in catalysing turnover of its normal substrate [80]. Related to this point, there is some recent evidence of amplification in *P. falciparum* of the first gene in the folate biosynthetic pathway, in strains that are carrying mutations in the downstream *dhps* and *dhfr* genes. The hypothesis here is that up-regulated expression of GTPC might increase flux through the pathway to compensate for a presumed reduction in turnover efficiency of these drug-resistant forms of the DHPS and DHFR enzymes [81].

Cryptosporidium is naturally resistant to the clinical antifolate drugs, and it was originally proposed that amino-acid residues different from those found at the critical sites of DHFR in *Plasmodium* species might bestow resistance to the drugs effective on those organisms [82]. On this basis, novel inhibitors of the *Cryptosporidium* enzyme have been investigated [83, 84], as have its crystal structure [85, 86] and detailed kinetic properties [87]. However, given the recent discovery of a *tk* gene and the demonstration of its encoded activity in *C. parvum*, this organism appears to have a direct route to dTMP by phosphorylation of thymidine salvaged from the host, and it has been proposed that this bypass mechanism is likely to be a major contributor to the observed resistance to current antifolate drugs [25].

Mutations in the *dhps* Gene Conferring Resistance to Sulfa Drugs

The sulfa drugs target DHPS, which, like DHFR, forms one domain of a bifunctional protein, in this case with hydroxymethylpterin pyrophosphokinase (HPPK-DHPS) in both *P. falciparum* [88, 89] and *T. gondii* [90]. DHPS links pABA to 6-hydroxymethyl-7,8-dihydropterin, forming dihydropteroate in the step preceding dihydrofolate synthesis, while HPPK (EC 2.7.6.3) catalyses the step preceding that of DHPS, Fig. (**2**). Progress along similar lines to DHFR has also been made in exploring the basis of resistance to the sulfa drugs. In the case of *P. falciparum*, where numerous analyses of both laboratory and field samples of diverse geographical origins have been conducted, polymorphisms in codons 436, 437, 540, 581 and 613 in the *dhps* domain in general correlate with varying levels of SDX resistance in the laboratory or Fansidar (PYR plus SDX) resistance in the field [88, 91-95], although exceptions have been reported, and other factors may well be involved [96, 97]. The most common resistant DHPS variant in Africa combines A437G and K540E [92-94, 98], while a different double mutant, A437G plus A581G, appears to be frequent in South-East Asia [92]. In South America, samples with all three of these mutations are often observed [72, 93, 99]. Mutations in the 436 codon (which give rise to Ala, Phe or Cys, from the wild-type Ser) and the 613 codon (Ala to Ser or Thr) are rarer, but in a triple combination with the A437G change, confer upon the enzyme the highest levels of sulfa-drug resistance measured so far [88, 100]. Overall, the key alteration appears to be A437G, which is seen in the great majority of mutant DHPS sequences observed to date, although only rarely by itself [92].

As for *dhfr*, later studies have explored in detail the relationship of the *dhps* mutations to drug resistance, including inhibition assays of variant recombinant enzymes [91, 101] and

measurements of resistance levels in sensitive parasites stably transformed *in vitro* with the different mutant forms of the *dhps* gene [100]. More recently, studies of microsatellite markers flanking the coding regions of both the *dhfr-ts* and *hppk-dhps* genes in *P. falciparum* from numerous geographical locations show convincingly that the highly drug-resistant forms do not occur by step-wise accumulation of mutations in frequent, independent events, but rather arise apparently very rarely. They then spread quite rapidly across malarial regions, as drug challenge gives them a strong selective advantage [72, 94, 102], a discovery that has important implications for the management of malaria prevention and treatment [103].

Although surveys of the *dhps* gene in *T. gondii* have been much less extensive than for *P. falciparum*, a range of clinical specimens from human cases of toxoplasmosis [53] and meat samples in the human food chain [104, 105] have been investigated. Non-synonymous mutations have been seen in 4 codons to date, i.e. N407D, E474D, R560K and A597E. However, of these, only the change in codon 407 appears to be associated with sulfa-drug resistance [53], conferring upon the recombinant HPPK-DHPS enzyme an IC_{50} value for SDZ about 30-fold higher than that for wild type, and comparable levels of cross-resistance to other clinically useful sulfa drugs. Interestingly, this codon is equivalent to the key codon 437 of *P. falciparum dhps* described above, although the amino-acid changes are different. As yet, reports of SDZ-resistant strains of *T. gondii* identified genotypically are still rare, although PYR-SDZ therapy fails in a significant proportion of patients for reasons that in many cases cannot be explained by poor compliance [106].

Novel Antifolate Drugs and Targets

Expression of *P. falciparum* DHFR-TS or the DHFR domain alone from allelic variants of the gene in heterologous systems was used extensively to provide material for kinetic and inhibitor studies. However, for many years it proved difficult to obtain protein in sufficient quantity and purity for crystallographic studies, despite complete resynthesis of the gene with *E. coli* codon usage to avoid the problems associated with the highly A+T-rich DNA of *P. falciparum* [107]. In the meantime, several 3D models of the enzyme were published, based on homology modelling to known DHFR crystal structures from other organisms [108-110]. Such models led to the design and synthesis of novel PYR derivatives able to bind effectively to PYR-resistant DHFR [109, 111, 112], and the screening of a wide range of commercially available compounds *in silico* for potential tight binding. From the latter, 21 promising candidates were tested *in vitro*, among which two with low- or sub-micromolar inhibition constants were identified as potentially attractive leads, particularly as they differed structurally from other families of DHFR inhibitors [108]. Recently, some 17 years after the *P. falciparum dhfr-ts* gene was first cloned, crystal structures were eventually obtained for the protein from both PYR-sensitive and PYR-resistant parasites, in association with anti-DHFR drugs [113, 114], giving considerable impetus to the search for new compounds inhibitory to this activity [115-118] and providing a firmer basis for computer predictions of specificities for different types of inhibitor [119]. The crystal structures also help to rationalise how the resistance mutations described above alter the enzyme conformation such that drug binding is greatly reduced, while permitting sufficient processing of normal substrate [120]. Modelling studies have also been extended to the DHFR-TS molecule of *P. vivax*, where mutations similar to those seen in *P. falciparum* lead to antifolate resistance [121], and more recently, crystal structures of the enzyme from wild-type and resistant parasites complexed with PYR and one of its derivatives have been reported [122].

As yet, attempts to obtain structures for HPPK-DHPS or the DHPS domain alone from either *P. falciparum* or *T. gondii* have not succeeded, and again, researchers investigating sulfa-drug binding have been dependent upon models based on X-ray diffraction patterns of

single-domain DHPS molecules from bacteria such as *E. coli* [123], *Staphylococcus aureus* [124], *Mycobacterium tuberculosis* [125] and *Bacillus anthracis* [126]. Such models suggest that all five of the polymorphic amino acid residues observed in *P. falciparum* DHPS are likely to form part of the only solvent-accessible channel connecting the external milieu to the catalytic centre [125, 127]. However, the recent publication of X-ray data for the HPPK-DHPS unit from the trifunctional yeast DHNA-HPPK-DHPS analogue, the first such study from a eukaryotic organism, should further facilitate analysis and understanding of the bifunctional parasite enzymes [128].

Determination of 3D structures by X-ray crystallography or NMR studies enhances enormously the ability to tailor existing drugs and promising lead compounds in the search for novel inhibitors. However, empirical screening of compound libraries is also an important route to the same goal. One such antimalarial compound that arose in this way, and has been the subject of much subsequent research, is the diaminotriazine WR99210. This is a potent anti-DHFR agent that inhibits parasites at sub-nanomolar levels, binding into the folate-binding pocket in a manner distinct from that seen with PYR and CG [113, 129]. Crucially, this drug is capable of killing the PYR-refractory parasites that carry the quadruple DHFR mutations at codons 51, 59, 108 and 164 [130-132]. Although considered for a long time to be too toxic for use in humans, and with problems of bioavailability, fresh interest in this compound has been aroused by the suggestion that it be administered as its phenoxypropoxybiguanide precursor, PS-15 [133], or an analogue thereof [134], which would be metabolised *in vivo*, similar to the way that PG is converted to the active form of CG in the liver. Such a regime is considered more likely to be tolerated by the host and to circumvent limited solubility, although problems with PS-15 itself have led to further attempts to find leads amongst this family of biguanides [135]. Importantly, it has been shown that, at least in the case of *P. vivax*, the greater the resistance to PYR, the greater the sensitivity of the parasites to WR99210. It has thus been proposed that if PYR and WR99210 were used in combination, selection of parasites resistant to both drugs would be strongly retarded [136]. More recent studies have explored the detailed mode of action of WR99210 and related derivatives, to identify compounds capable of killing parasites of both species carrying the DHFR alleles that confer the highest levels of resistance to PYR [137].

Although compounds such as PYR and WR99210 are known to target the active site of DHFR, inhibition by binding elsewhere on the molecule has also been reported, as in the case of the fluorescein derivative eosin B [138]. This compound inhibits the *T. gondii* enzyme and growth of the parasite at sub-millimolar levels, but is much more potent against strains of *P. falciparum*, where it appears to act on a variety of sites additional to DHFR. One novel approach to screening new compounds cheaply and rapidly for anti-DHFR activity involves inserting the gene into a DHFR-deficient yeast strain to restore prototrophy with respect to thymidine synthesis (as well as the folate-dependent production of adenine, methionine and histidine), and observing the effects of these compounds on the growth of the organism on agar plates [74]. This has been carried out with the *dhfr* genes from *C. parvum, P. falciparum, T. gondii* and man, as well as for the fungal-like parasite *Pneumocystis carinii*. A large number of novel substituted pyrimidine or quinazoline compounds were investigated, 22 of which were lipophilic and intended to bypass potentially problematic folate transport mechanisms. This led to the identification of several compounds that were effective against the enzyme from PYR-resistant *P. falciparum*, as well as six that powerfully inhibited the *C. parvum* enzyme in this context, but were nonselective in that they also strongly affected human DHFR [83]. Additionally, derivatives of the established antibacterial antifolate trimethoprim were investigated and found to be highly selective against *Cryptosporidium* DHFR relative to the host enzyme, but not especially potent. Conversely, another large screen of 93 lipophilic di- and tricyclic diaminopyrimidine derivatives against recombinant DHFR from human and bovine isolates

of *C. parvum* [84] revealed several compounds that were more potent than trimethoprim, but were less selective than the latter for *C. parvum* relative to human DHFR. Nevertheless, sub-micromolar concentrations of certain of these compounds inhibited *in vitro* growth of *C. parvum* in host cells in the presence of thymidine and hypoxanthine, suggesting that such inhibitors, in combination with leucovorin (folinic acid), might be efficacious against infections of *Cryptosporidium*, despite the presence of TK activity in this organism, as described earlier.

The success and widespread use of anti-DHFR inhibitors over several decades and the continuing potential of such inhibitors for refinement [139, 140] begs the question as to whether inhibition of the other enzymes in the thymidylate cycle part of the folate pathway might also prove to be effective targets. As TS is essential for conversion of dUMP to dTMP, this enzyme has also been investigated in some detail. Indeed, this enzyme is targeted by many anti-cancer drugs, which fall into the two main categories of folate and nucleotide analogues [141]. In this case, however, the human and parasite enzymes are much less divergent than is seen for DHFR, raising the prospect that adequate selectivity might be too difficult to achieve. At least two strategies to circumvent this problem have been proposed. The pyrimidine analogue 5-fluorouracil (5FU) was one of the first TS inhibitors to be developed as an anticancer drug, but is not transported efficiently into *P. falciparum* [20]. Unlike uracil and uridine derivatives, however, orotate analogues are readily taken up by the parasite and incorporated in the pyrimidine synthesis pathway, resulting in a metabolite toxic to TS [142-144]. This phenomenon affects the parasite more severely, since mammalian cells salvage orotate derivatives relatively poorly. Thus, 5-fluoroorotic acid (5FO) is processed to 5-fluoro-2′-deoxyuridylate, which inactivates TS by covalent binding to the active site and inhibits cultured parasites in the nanomolar range [142, 144, 145]. *In vivo* animal studies were also promising [42, 44], and the efficacy of 5FO could be increased by the addition of uridine, which mammalian cells but not parasites can salvage, thus bypassing the toxicity of 5FO to the host. However, the poor pharmacokinetic properties of this compound have probably limited its further development. A similar strategy takes advantage of the inability of the parasite to salvage sufficient thymidine, of which again the host cells are capable. Thus, one compound that is of the folate analogue type, 1843U89, was effective against drug-sensitive as well as drug-resistant *P. falciparum*, an inhibition that could not be reversed with thymidine, whereas mammalian cells were unaffected by this compound at concentrations up to 1,000-fold higher when thymidine was present [146].

The third enzyme of the thymidylate cycle, SHMT, shows a significant increase of its activity in cancerous cells during S-phase, when DNA is being replicated [147] and, given its essential role in this cycle, its inhibition is predicted to strongly affect cell growth. The *shmt* gene and its product have been characterised functionally in both *P. falciparum* [148-150] and *T. gondii* (J Smith, J E Hyde and P F G Sims, unpublished data). The enzyme is also present in *Cryptosporidium*, where, as in yeast and other organisms, there are two clearly recognisable *shmt* genes, which are predicted to encode respectively a cytosolic and a mitochondrial form of the protein. In the different *Plasmodium* species there is a less well conserved gene that might possibly encode a second, mitochondrial SHMT [151], but such a candidate has not yet been identified in *T. gondii*. In general, however, SHMT activity has been studied as a potential drug target much less than have the DHFR and TS components of the cycle, either for cancer treatment [152] or to inhibit parasites [153]. Analogues of its two substrates, serine and THF, have been investigated [154], and the powerful DNA replication inhibitor mimosine, a plant amino acid, has been shown to primarily target SHMT [155], but no drugs have as yet progressed to clinical trials. Although the parasite enzymes are not as highly conserved with respect to their human counterparts as TS, they do show sufficient similarity to suggest that identifying differential inhibitors of sufficient specificity may again

be problematic. However, a recent homology modelling study of *P. falciparum* SHMT indicates that its active site is somewhat larger than that of the human enzyme, and ways of how this and other subtle differences in geometry might be exploited in the design of folic acid analogues are suggested [156].

While similarities between human and parasite proteins are an important consideration for the folate recycling enzymes, all of those involved in *de novo* folate production, Fig. (**2**), except for the first (GTPC), are absent from host cells and thus in principle could represent a highly attractive family of drug targets [157]. In bacteria, where the folate biosynthetic pathway is near-ubiquitous, mutations or deletions of the relevant genes generally result in non-viability. Other than DHPS, however, none of these enzymes has as yet been targeted clinically in either *P. falciparum* or *T. gondii*. Crystallographic data now exist for bacterial versions of all of these enzymes (Protein Data Bank; http://www.rcsb.org/pdb), providing a structural basis for homology modelling of the parasite proteins. GTPC is regarded as a potentially good target for rational drug design [158], and the catalytic domain of the *P. falciparum* enzyme is only 37% identical (62% similar) to the human enzyme, suggesting that it might be sufficiently dissimilar from its host counterpart to permit effective discrimination [149]. Moreover, in other microorganisms, conversion of the purine ring of GTP to a pterin ring by GTPC is the rate-limiting step in folate biosynthesis, and the enzyme can play a key role in cell-cycle regulation [159]. Inhibitors of *E. coli* dihydroneopterin aldolase (DHNA; EC 4.1.2.25) have been investigated [160, 161] and compounds effective at sub-micromolar concentrations described [161]. Unexpectedly however, neither this enzyme nor its gene has yet been identified in malaria parasites or *Toxoplasma*, despite completion or near-completion of the genome sequences of several *Plasmodium* species and that of *T. gondii*, as well as biochemical evidence that radiolabelled guanine-based precursors are converted to folate and thus are presumed to pass through a DHNA-catalysed step, as in other organisms that effect *de novo* folate biosynthesis. At this point therefore, the possibility cannot be excluded that there is a novel route to the HPPK reaction that somehow bypasses the conventional DHNA step. HPPK is also seen as a particularly attractive target [158] and inhibitors based on binding to its two substrate pockets (for 6-hydroxymethyl-7,8-dihydropterin and ATP) have been described [162]. The unusual bifunctional nature of the HPPK-DHPS protein in *P. falciparum* and *T. gondii* might also permit development of novel inhibitors that are able to block both activities. As well as the clinically validated sulfa-drugs, high potency pterin analogues have been developed against bacterial DHPS [163], and inhibitors of DHFS have also been investigated in *E. coli* [164] and *Neisseria* [165]. *P. falciparum* and *T. gondii* are again unusual in that they are the only eukaryotes known to date that express a protein from a single gene that has both DHFS and folylpolyglutamate synthase (FPGS; EC 6.3.2.17) activity ([166]; J. Smith, J E Hyde and P F G Sims, unpublished data). Although not particularly well conserved with respect to the monofunctional human FPGS, it was again thought that careful inhibitor design might be required to achieve sufficient selectivity. Interestingly though, the recently determined crystal structure of the bifunctional *E. coli* enzyme has revealed a binding site for dihydropteroate (the substrate of the DHFS activity) that is quite different from the folate binding site used by FPGS enzymes [167], indicating that there may well be scope for differential inhibition of parasite DHFS-FPGS enzymes. After the first reaction catalysed by GTPC, all enzymes in the *de novo* pathway described above use pterin derivatives as substrates, but reports of testing or developing pterin analogues as potential inhibitors of the various steps in folate synthesis are relatively rare [126, 163, 168, 169], possibly due to the generally poor solubility of these compounds.

Inhibition of Folate Salvage

An important factor in considering this area of metabolism as a drug target is that, unlike most bacteria, *P. falciparum* can salvage folate from external sources, as well as synthesise it *de novo*. This is known to strongly antagonize DHPS inhibitors when tested *in vitro* [170, 171], but the extent to which salvage from the host can meet the needs of this parasite *in vivo* is still uncertain, and may vary among different strains [48, 172], as well as in individual hosts. Similarly, despite a recently proven capability [52], it is not yet clear how important folate salvage is to *T. gondii*, although *Cryptosporidium* must depend on this route exclusively (see above). In general, the higher the concentration of folate derivatives in the intracellular pool, the less effective will be the action of the antifolate drugs. Individually, this is especially marked for the sulfa-based drugs targeted at DHPS [172], although susceptibility levels to PYR are also affected [67, 170]. Interestingly, transfection studies on *P. falciparum* indicate that complete blockage of biosynthesis by knockout of the *dhps* gene cannot be compensated for by salvage of exogenous folate, but if a low level of DHPS activity is retained by targeted mutagenesis of the gene, parasites are viable in folate-containing medium. This indicates that an element of *de novo* synthesis, possibly located in a separate cellular compartment, is apparently essential for parasite growth [51]. As far as drug treatment is concerned, it follows that if uptake of folate can be selectively impeded, then the efficacy of the antifolate drugs should be enhanced. The first attempts in this direction were taken using the anti-gout drug probenecid [173, 174], which acts as a transport inhibitor, where it was observed that concentrations of probenecid at physiologically acceptable levels were apparently able to potentiate the activities of both PYR and SDX. Enhancing the effectiveness of the traditional, cheap antifolates in this way represents a promising area for further exploration, Moreover, recent bioinformatic analyses of *P. falciparum* [175], *Cryptosporidium* and *Toxoplasma* [52] have yielded candidate proteins with characteristics of the folate/biopterin transporter family known as FBT or BT1, which includes functionally characterised members in the trypanosomatid parasite *Leishmania* [176], cyanobacteria and plants [177]. If the apicomplexan FBT homologues are indeed shown to be involved in the import of folates into the cell, this would represent an important new development in antifolate inhibitor research.

INHIBITION OF PYRIMIDINE SYNTHESIS OUTWITH THE FOLATE PATHWAY

Atovaquone is a more recently introduced inhibitor, licensed as a clinical antimalarial in combination with PG (as Malarone), and as a treatment for acute toxoplasmosis in humans. This highly substituted 2-hydroxynaphthoquinone derivative affects pyrimidine synthesis in a quite different way from the antifolate drugs, in that it acts as a structural analogue of coenzyme Q (CoQ; ubiquinone) in the mitochondrial electron transport chain. The electron flow is involved in maintaining the membrane potential of the mitochondrion, essential for the transport of proteins and small molecules in and out of the organelle [178]. Atovaquone was shown to collapse the membrane potential of the rodent parasite *P. yoelii*, which remains unaffected when exposed to unrelated antimalarials such as chloroquine and tetracycline [179]. The rapid effect on respiration results from inhibition by the drug of cytochrome *b* in the bc_1 complex (complex III) of the electron transport chain of the parasite [180]. Atovaquone has a selective action on parasite electron transport as the latter employs a CoQ_8 complex (i.e. one carrying 8 isoprene units on the aromatic ring), a homologue that differs from the CoQ_{10} type found in humans. Interference with the transfer of electrons in turn inhibits dependent enzymes, including DHODH, which resides on the outer membrane of the parasite and which must pass electrons to CoQ when it oxidises dihydroorotate to orotate during the fourth step of pyrimidine synthesis, Fig. (**1**). Native dihydroorotate dehydrogenase activity has been characterised in *P. falciparum* [181] and recombinant protein expressed heterologously from the genes cloned from *P. falciparum* [182] and *T.*

gondii [183], leading to a crystal structure for the malarial enzyme [184]. Existing strong inhibitors of human DHODH were found to bind the *P. falciparum* enzyme very weakly, suggesting that species-specific compounds could ultimately be found where the situation is reversed [182]. This conclusion was vindicated in a recent high-throughput study of over 200,000 candidate molecules, the most effective of which inhibited the malarial enzyme at nanomolar levels, and was four orders of magnitude less effective against the human enzyme [185].

When used in monotherapy, atovaquone was found to rapidly select for resistant parasites [186]. To investigate the genetic basis of this phenomenon, resistant lines of *P. yoelii* [180], *P. berghei* [187] or of *P. falciparum* [188] were derived in the laboratory and analysed for DNA polymorphisms. This revealed either single or double point mutations in the cytochrome *b* gene of different lines that correlated with atovaquone resistance *in vitro*. In *P. yoelii* the mutations affected one or more of the codons 258-272; in *P. falciparum* the same region was involved (codons 272-284), together with a mutation in codon 133, and in *P. berghei* the affected codons were 133, 144 and 284. A further mutation at codon 268 of *P. falciparum* was also found in parasites isolated from a patient suffering parasite recrudescence following atovaquone treatment [188]. In several of the lines analysed, the mutations were associated with up to a several thousand-fold reduction in parasite susceptibility to the drug. The cytochrome *b* gene of *T. gondii* has also been characterized from wild type and atovaquone-resistant strains, where mutations encoding M129L (equivalent to the M133I mutation in *P. falciparum*), and I254L were found in the latter [189]. The binding of atovaquone to the yeast cytochrome bc_1 complex has also been studied as a model system [190]. Molecular modelling from these several studies suggests that the altered residues are all clustered within a highly conserved region of the molecule that represents the ubiquinol oxidation pocket of the bc_1 complex, where the drug can interact with the Rieske iron-sulfur protein component that transfers electrons. When used in combination with PG, the emergence of parasite resistance is considerably retarded, although recently, reports of clinical failures using Malarone as treatment (rather than prophylaxis) have appeared [191-194]. Mutations of codon 268 (Y268S or Y268N) of the cytochrome *b* gene have been associated with some, but not all of these failures. As described earlier, PG is metabolised *in vivo* to CG, an anti-DHFR inhibitor that would not be expected to retain activity against the multidrug-resistant parasites found in South-East Asia, yet the Malarone combination is clearly highly efficacious in this area. The biochemical basis of the synergy between these two components is thus not yet clear and may not involve the DHFR activity. *Cryptosporidium* lacks a mitochondrial genome but possesses an organelle that appears to retain certain mitochondrial functions, including an atypical electron transport system [12]. However, the sparse evidence available suggests that atovaquone is unable to significantly retard the growth of this parasite [195].

ACQUISITION OF PURINES

Almost all parasitic protozoa studied to date have been found to be incapable of *de novo* synthesis of the purine nucleotides, and genome sequencing of such organisms confirms that the genes involved in purine synthesis in mammals and other eukaryotes are indeed absent [196]. The parasites must therefore rely on salvage of these molecules or their precursors from the host to provide the necessary building-blocks of nucleic acids and other nucleotide derivatives. As the range of purines found in these parasites appears to represent a normal complement, clearly mechanisms must exist, not only for their uptake from the host, but also for the necessary interconversions among the various base types.

Import of Purines or their Derivatives

The first step in the salvage of purines is transport of the relevant molecules into the parasite. The parasite cytoplasm is separated from that of the host by the parasitophorous vacuolar membrane, which is thought to be freely permeable to small molecules [197], and the parasite plasma membrane, which is not, and which must therefore employ transporters, also known as permeases, to regulate import and export [198]. Such permeases in general facilitate the passage of nucleobases, nucleosides, or both, and any potential antiparasite drugs that are analogues of these molecules must also be processed through a permease. The properties of these molecules are therefore an important consideration in developing new therapies [199].

The first high affinity nucleobase transporter to be identified in an apicomplex an parasite w as a *T. gondii* molecule named TgNBT1, able to transport hypoxanthine, and probably guanine and xanthine, but not adenine [200]. Two nucleoside transporters have also been characterized in this organism and others are predicted from the genome sequence. TgAT1 transports adenosine, guanosine and inosine, but with too low an affinity for it to be the principal route for adenosine entry [201, 202]. However, a second transporter, TgAT2, shows a much higher affinity for a range of nucleosides, including adenosine, as well as for the bases hypoxanthine and guanine. TgAT2 is also bound by several purine nucleoside analogues, such as tubericidin and adenine arabinoside (Ara-A), and is considered to be a strong candidate for a possible therapeutic target [200].

Adenosine is known to be rapidly imported into the malaria parasite [203] and the genes for two transporters predicted to process nucleosides or nucleobases were identified in the original analysis of the *P. falciparum* genome [7], one of which had been previously characterized as encoding a product named PfNT1 [204] or PfENT1 (for ‘equilibrative nucleoside transporter’) [205]. This protein, now thought to be a major player in both nucleobase and nucleoside import [206, 207], has been localized by immunoelectron microscopy to its expected position on the parasite plasma membrane [208]. It is expressed throughout the asexual life cycle, but is significantly elevated during the early trophozoite stage, just prior to the onset of DNA synthesis. Its properties differ in important ways from mammalian nucleoside transporters, including an unexpected ability to transport the L-stereoisomer of adenosine, as well as the normal D-form, and an insensitivity to established inhibitors of mammalian ENTs, phenomena that could potentially be exploited in the design of antiparasite inhibitors [204, 205]. Although PfENT1 mediates rapid transport of adenosine into cultured parasites, it appears to have quite a broad substrate specificity for both purine and pyrimidine nucleosides [204-206], despite the fact that little use appears to be made of the latter, as described earlier. *Plasmodium* is also able to import the bases adenine, hypoxanthine, xanthine and guanine with the participation of this, and possibly other related transporters, two further putative candidates for which have been more recently proposed on bioinformatics grounds, making a total of four thus far [175]. However, parasites in which the gene encoding PfENT1 has been knocked out become auxotrophic under normal physiological conditions for hypoxanthine, inosine (the ribonucleoside form of hypoxanthine) and adenosine, with the reduction in hypoxanthine uptake being especially pronounced [207]. This further emphasizes the key role of this particular permease in parasite viability. In principle, GMP can be acquired by salvage of guanine using a phosphoribosyltransferase activity (see below), or by synthesis from other bases or nucleosides *via* a series of reactions completed by GMP synthetase (EC 6.3.5.2). However, the erythrocyte is poor in guanine, xanthine and guanosine, and lacks a guanosine kinase [20], so utilisation of hypoxanthine is of major importance. GMP synthetase has been characterized in *P. falciparum* and its gene shown to express maximally around S-phase. The antibacterial GMP synthetase inhibitor psicofuranine was shown to retard growth of the parasite at comparable concentrations to that required to inhibit *E. coli*, and to be

antagonized by exogenously provided guanine, supporting the notion that GMP is indeed predominantly synthesised *via* this enzyme [209].

In common with the other apicomplexans parasites, no gene encoding adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) has been identified in *Plasmodium* [196], an enzyme that is highly conserved in other organisms. Thus, despite earlier reports of APRT activity in all three of the parasites considered here, it is now thought most likely that where adenine is used, it is first deaminated by an adenine deaminase activity (AD; EC 3.5.4.2) to hypoxanthine, which can then be phosphoribosylated (see below). In *Cryptosporidium*, salvage in fact appears to depend solely upon the import of adenosine, and a requisite adenosine transporter gene has been identified [11], representing the only obvious gene of this type in the genome [196].

Interconversion of Salvaged Purine Derivatives

The mechanisms by which *Plasmodium, Toxoplasma* and *Cryptosporidium* undertake purine salvage are diverse, both with regard to primary sources and to routes of interconversion, Fig. (**3**). As described above, hypoxanthine is regarded as the key precursor of the other purines in *Plasmodium*. Degradation of exogenous hypoxanthine with xanthine oxidase strongly inhibits parasite growth [210], and hypoxanthine is commonly used as a nutritional supplement in malarial cultures, or for radioactive labelling as a monitor of growth. A key source of hypoxanthine is thought to be the deteriorating human erythrocyte infected by the parasite, in which ATP is catabolised to hypoxanthine *via* ADP, AMP, IMP and inosine [211, 212]. The adenosine nucleosides, though available within the erythrocyte, cannot be efficiently salvaged directly as the required adenosine kinase (AdoK; EC 2.7.1.20) activity of the parasite is found to be very low [20], and indeed no gene encoding this activity has yet been identified in *Plasmodium*. Moreover, purine nucleoside phosphorylase (PNP; EC 2.4.2.1), while able to cleave other purine nucleosides by phosphorolysis to the free base, is unable to do this efficiently with adenosine. However, there is a high level of adenosine deaminase (AdoD; EC 3.5.4.4) activity from both host and especially the parasite, which instead converts the adenosine to inosine [20]. Given the ability of the parasite to selectively import the L-stereoisomer of adenosine, described above, L-adenosine analogues have been investigated and shown to inhibit parasite AdoD at picomolar levels but to have no effect on the mammalian counterpart [213]. The inosine that is produced by AdoD in normal metabolism is then converted by PNP to hypoxanthine, and both the human and parasite PNP enzymes are also highly expressed in infected erythrocytes [20]. Based on different conserved sequence motifs, human PNP is classified as a family 2-type enzyme, members of which have a trimeric quaternary structure and act preferentially on 6-oxopurines such as guanosine and inosine, while many bacterial PNPs fall into family 1-type, hexameric enzymes acting on both 6-oxopurines and 6-aminopurines, such as adenosine. The product of the *P. falciparum pnp* gene, while more closely resembling the family 1-type, does not fit well into either category [214] and exhibits the ability to process alternative substrates, such as 5′-methylthioinosine, which is a by-product of the polyamine biosynthetic pathway [215]. Human PNP activity is powerfully inhibited by immucillins, developed originally as anticancer and immunosuppressive drugs that mimic the transition state conformation of mammalian PNP. However, owing to a close similarity in transition-state configurations, certain of these inhibitors are also found to bind to the parasite enzyme with sub-nanomolar inhibition constants [214, 216, 217]. The binding constants to the human enzyme are in general even lower, so that PNP activity from both sources can be very effectively blocked. Therapy using these compounds would depend on the fact that humans have alternative routes to the purine nucleotides, but the parasite does not. However, crystal structures of both enzymes [218-221] reveal important structural differences between them, which can explain the unusual ability of the *P. falciparum* enzyme to process 5′-methylthioinosine.

These data have been exploited in a later generation of immucillins to produce at least one derivative that binds the parasite PNP some 100-fold more tightly than the human enzyme [215, 220].

Interestingly, *T. gondii* PNP, while very similar at the sequence level to *P. falciparum* PNP (41% identity), exhibits somewhat different properties. Although inosine and guanosine are major substrates for *T. gondii* PNP, as they are for the malarial enzyme, the *Toxoplasma* enzyme is effectively unable to process 5′-methylthioinosine as a substrate, consistent with an unexpected absence of polyamine biosynthetic capability in this organism [222]. Moreover, although strong immucillin inhibitors of *T. gondii* PNP have been identified, parasite growth is barely affected by high concentrations of these compounds, as efficient purine salvage *via* AdoK is sufficient to maintain viability (see below).

Given the central role of hypoxanthine in the metabolism of *P. falciparum*, hypoxanthine/guanine (EC 2.4.2.8), and xanthine phosphoribosyltransferase (EC 2.4.2.22) activities are critical for purine salvage and are found to localise in this parasite to a single HXGPRT enzyme [223], whose 3D structure in association with transition-state analogues has been studied by X-ray crystallography and NMR [224]. The ribonucleosides adenosine and inosine are converted to hypoxanthine, while guanosine is converted to guanine. This is followed by HXGPRT-catalysed ribophosphorylation of these two bases, as well as of xanthine. Both GMP and AMP can be formed from the IMP that results from ribophosphorylation of hypoxanthine, and GMP can also be obtained from XMP, Fig. (**3**). Sequence alignments, site-directed mutagenesis and 3D modelling of malarial HXGPRT with its homologues from human, *T. gondii* and other species indicate that the His-196 residue neighbouring the active site plays an important role in determining the specificity of the enzyme. If His-196 is converted to Lys, as found in the human enzyme, xanthine and guanine are no longer substrates for the enzyme, whereas conversion of hypoxanthine to IMP is unaffected [225]. This is a good example of how subtle variations in sequence can give rise to important differences in properties among otherwise closely related orthologues, and indicates that effective differential inhibition may be achievable for many such targets with careful drug design. Immucillin-based compounds related to the PNP inhibitors described above, but phosphorylated at the 5′ position, have been modelled on the transition state of malarial HXGPRT. These compounds are extremely powerful inhibitors with binding constants >1,000-fold tighter than that of the native substrate, but do not as yet discriminate between host and parasite enzymes [226]. To screen for other inhibitors of malarial HXGPRT, an *E. coli*-based system expressing the human and *P. falciparum* genes in parallel has been developed [227], along similar lines to the yeast-based assay for DHFR-TS inhibitors described earlier [74].

Toxoplasma gondii tachyzoites rapidly take up hypoxanthine and guanine [228]. These can be processed by two active isozymes of HXGPRT encoded by a single gene whose transcript is differentially spliced at one exon to yield the isozymes I and II, which differ by a 49 amino acid insertion present in isoform II near to its N-terminus [229, 230]. Localization studies have demonstrated that palmitoylation of Cys residues within this insertion directs isoform II to the inner membrane complex of the parasite, whereas isoform I remains in the cytosol. However, both forms exhibit closely similar activities *in vitro* and are expressed in parallel throughout the life-cycle stages, so the *in vivo* significance of these findings is as yet unclear. *T. gondii* HXGPRT has long been considered as an attractive target for drug intervention [231, 232] and crystal structures for this enzyme have been reported [233, 234], together with detailed studies of the catalytic mechanism [234]. Although HGPRT and XPRT activities were also claimed to be present in crude extracts of *Cryptosporidium* sporulated oocysts [235], no evidence of a gene or genes capable of encoding the corresponding enzymes has been found in the completed genome sequence of

either *C. parvum* [11] or *C. hominis* [12]. As the incidence and average number of introns in these genomes are low (5% and 5-20% of ORFs in these two species, respectively), such a well conserved gene would be expected to be readily identifiable, if it were present. Furthermore, direct biochemical assay indicates a complete lack of HXGPRT activity in *C. parvum* and there is strong evidence that salvage of adenosine provides the only source of purines in *Cryptosporidium*, which is converted by AdoK to AMP [25]. This is in turn deaminated to IMP by AMP deaminase (EC 3.5.4.6), an activity found in all three parasites. Inosine 5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) and GMP synthetase then provide the route to the guanine nucleotides, Fig. (**3**). IMPDH is the rate-limiting enzyme in the multistep conversion of AMP to GMP and is highly susceptible to inhibition by compounds such as ribavirin and mycophenolic acid, which are already in clinical use as immunosuppressive and antiviral agents, and show significant anticryptosporidial activity in culture [25]. The key role of IMPDH and the availability of numerous lead compounds make this a very attractive target. Interestingly, the IMPDH from *Cryptosporidium* also appears to have a bacterial origin, unlike the orthologues from the other Apicomplexa and the human host [11, 236]. As well as phylogenetic data supporting the view that it derives from an epsilon-proteobacterium, experimental evidence has been obtained by functionally expressing the *C. parvum* gene in *E. coli* and showing that, like bacterial IMPDH enzymes, the protein product is much more resistant to mycophenolic acid than the mammalian enzymes, as well as possessing kinetic properties quite different from human IMPDH [237]. These characteristics add strongly to its attraction as a target for the development of selective inhibitors of the parasite. *T. gondii* expresses a mycophenolic acid-sensitive IMPDH, but, similar to the situation with *T. gondii* HXGPRT outlined above, two alternatively spliced transcripts of the single gene can be detected, with the smaller protein product lacking the so-called active-site flap [238], although again, the significance of such a product *in vivo* is not yet clear. Although the gene encoding IMPDH has also been identified in the *P. falciparum* genome, a coupled assay designed to simulate the situation *in vivo* utilised purified recombinant human IMPDH together with parasite HXGPRT in a drug screen for novel inhibitors, as these activities operate in tandem in the salvage pathway [239]. As well as the GMP synthetase activity mentioned earlier, genes encoding adenoylsuccinate synthase (EC 6.3.4.4) and adenoylsuccinate lyase (EC 4.3.2.2) have been characterized in *P. falciparum* [240, 241], completing the set required for the interconversion of AMP, IMP, XMP and GMP, Fig. (**3**).

In the case of *T. gondii*, it has been possible to utilise parasite transfection to explore the major pathways of purine acquisition by gene knockout. Adenosine is incorporated into nucleotides in this organism at a much higher rate than any of the other purine bases or nucleosides [228], and the two key enzymes that are involved in this process are HXGPRT, described above, and AdoK. If either is disabled, the parasite is still viable, but deletion of both of the encoding genes is not possible. From measurements of the relative fitness of the two types of knockout parasite, the route using adenosine *via* AdoK was shown to be more important than that using hypoxanthine or the other purines [196], as predicted from earlier biochemical studies [242]. This makes AdoK an even more attractive target for chemotherapeutic intervention than HXGPRT for this parasite, although humans of course also express AdoK. However, adenosine analogues in the form of 6-benzylthioinosine derivatives have been discovered that, when phosphorylated by AdoK, become selectively toxic to the parasite enzyme [243, 244]. The search for new inhibitors of AdoK will be greatly facilitated by X-ray crystallography studies of the *T. gondii* enzyme, [245, 246], which have highlighted significant differences between human and *T. gondii* AdoK, including a 5 amino-acid deletion in one of the segments connecting the two domains in the latter. This leads to a large difference in the relative orientation of those domains between the human and parasite enzymes, which in turn is reflected in a novel mechanism of catalysis by the latter.

OTHER TARGETS AND CONCLUDING REMARKS

Other essential aspects of metabolism directed towards the production or processing of nucleotides or nucleic acids have been considered as potential drug targets, particularly where there are clear differences in primary structure or biochemical properties between the parasite and host analogues. *P. falciparum* expresses separate RNA guanylyltransferase (EC 2.7.7.50) and RNA triphosphatase (polynucleotide 5′-phosphatase, E.C.3.1.3.33) enzymes, two of the three activities that are required to form the 5′-cap structure on all mRNA molecules, and the triphosphatase has been shown to resemble fungal and viral homologues that are structurally and mechanistically unrelated to those found in mammals, which comprise a bifunctional molecule with the guanylyltransferase activity [247]. The effectiveness of anticancer and antibacterial agents that target the topoisomerases required to relieve torsional stress in DNA molecules undergoing replication or transcription have led to a considerable body of work on the protozoal enzymes, including type I and type II activities from *P. falciparum* [248]. Similarly, the malarial adenylyl cyclase and guanylyl cyclase activities that produce the cyclic nucleotides cAMP and cGMP differ considerably from their mammalian counterparts. These nucleotides, which act as second messengers in other organisms, are involved in the regulation of differentiation into gametocytes, a sexual stage that is essential for the onward transmission of the parasite from the human host to a new mosquito vector [249]. Several candidates are relevant to both the purines and pyrimidines, for example the repair mechanisms used by *P. falciparum* to correct apurinic or apyrimidinic sites on DNA appear to differ from those of the human host [250]. Another attractive target is RNR [251], an enzyme required for the conversion of ribonucleotides to their deoxy-equivalents, which is rate-limiting in DNA replication and essential for cell viability. Given the absence of many of the *de novo* synthesis enzymes from *Cryptosporidium*, this activity could provide an attractive alternative target within the area of nucleotide metabolism, and may prove to be of utility in the case of the other apicomplexan parasites. To provide deoxyribonucleotides in the right ratio for DNA synthesis, RNR is under complex regulatory control. Eukaryotic RNRs are heterotetramers with two large (alpha or R1) and two small (beta or R2) subunits. Genes encoding both types of subunit have been cloned and characterized from *C. parvum* [252], and from *P. falciparum* [253, 254] where the RNR activity is reported to show characteristics of allosteric control [255]. Such control would be expected to differ between *P. falciparum* and the human host, given the highly skewed (81%) A+T base composition of this parasite's genome. Interestingly, *P. falciparum* has recently been shown to utilise two different small beta-type subunits, R2 and R4, that differ significantly in their primary sequence, but appear to co-localize in native complexes. Although other organisms also express more than one small subunit type, the physiological significance of this phenomenon in *P. falciparum* is as yet unclear [256]. Inhibition of RNR has been much studied in the field of cancer research and a variety of compound types could in principle act as lead compounds for antiparasite inhibitors [251]. These include iron chelating and radical scavenging agents, as well as nucleoside derivatives [257] and others that act either by inhibiting translation, inactivating the R1 or R2 subunits, or preventing the critical association between them [258]. The last type includes peptidomimetic inhibitors designed to exploit the marked sequence divergence between the C-termini of the human and malarial R2 subunits to selectively block in the latter case their interaction with the large subunits of the RNR tetramer [251].

The enzymes of purine and pyrimidine production, involved in both *de novo* biosynthesis and interconversions of salvaged precursors, provide a rich source of potential targets for combating the important apicomplexans parasites that infect humans, but as yet, only a limited number of drugs that mainly target folate synthesis or recycling, and thus thymidylate production, have achieved the status of licensed clinical agents. The range of nucleotide-linked reactions considered here, while extensive, is not exhaustive. The

biochemical reactions of the parasitic protozoa, and in particular the above enzymes, show many differences with respect to their human hosts. However, as has been pointed out in a previous review, despite the large and expanding number of potential antiparasite targets, assessments of the most promising should be carefully carried out to validate them as *bona fide* candidates before 'commitment to long-term studies on development of specific inhibitors' [19]. There are of course considerable hurdles to overcome in the many steps between identification of a seemingly attractive enzyme target and the eventual formulation of a safe and effective medicine, where problems of solubility, effective delivery, toxicity to the host and other potential pitfalls must be tackled [259]. Realistically, novel antiparasitic drugs targeting this area of metabolism are perhaps most likely to evolve from leads that have been well studied in the search for new agents that inhibit the replication of cancer cells, which share a common need with protozoal parasites for the rapid acquisition, conversion and deployment of nucleotides and their derivatives.

Acknowledgments

Relevant work carried out in the author's laboratory was funded principally by grants 056845 and 073896 from the Wellcome Trust, UK.

ABBREVIATIONS

AD	Adenine deaminase
AdoD	Adenosine deaminase
AdoK	Adenosine kinase
AIDS	Acquired immunodeficiency syndrome
APRT	Adenine phosphoribosyltransferase
ATCase	Aspartate carbamoyltransferase
cAMP	Cyclic AMP
CG	Cycloguanil
CoQ	Coenzyme Q
CPSII	Carbamoyl phosphate synthetase II
CTPS	CTP synthase
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate synthase
DHNA	Dihydroneopterin aldolase
DHOase	Dihydroorotase
DHODH	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
5FO	5-Fluoroorotic acid
FPGS	Folylpolyglutamate synthase

5FU	5-Fluorouracil
GTPC	GTP cyclohydrolase I
HPPK	Hydroxymethyldihydropterin pyrophosphokinase
HXGPRT	Hypoxanthine/xanthine /guanine phosphoribosyltransferase
IMPDH	Inosine 5′-monophosphate dehydrogenase
NAD^+	Nicotinam ide adenine dinucleotide
OMPDC	Orotidine 5′-monophosphate decarboxylase
OPRT	Orotate phosphoribosyltransferase
pABA	*Para*-aminobenzoic acid
PabAB	Bifunctional protein constituting aminodeoxychorismate synthase activity
PabC	Protein constituting aminodeoxychorismate lyase activity
PG	Proguanil
PNP	Purine nucleoside phosphorylase
PRPP	5-Phosphoribosyl pyrophosphate (5-phosphoribosyl-1-diphosphate)
PYR	Pyrimethamine
RNR	Ribonucleotide reductase
SDX	Sulfadoxine
SDZ	Sulfadiazine
SHMT	Serine hydroxymethyltransferase
TE	Toxoplasmic encephalitis
THF	Tetrahydrofolate
TK	Thymidine kinase
TS	Thymidylate synthase
UK	Uridine kinase
UPRT	Uracil phosphoribosyltransferase

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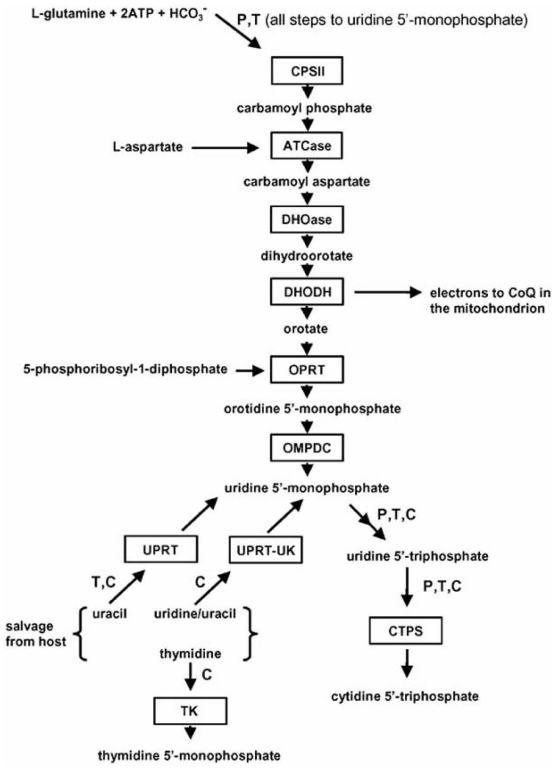

Fig. (1).

De novo and salvage pathways to uridine 5′-monophosphate, the precursor of other pyrimidine nucleotides. P,T,C indicate that the pathway or step is utilised in *Plasmodium, Toxoplasma* and *Cryptosporidium* respectively. *Cryptosporidium* is also able to salvage thymidine *via* a thymidine kinase activity to form dTMP (see also Fig. (**2**)) and to salvage cytidine *via* the bifunctional UPRT-UK enzyme to form CMP (not shown). For abbreviations of enzymes (boxed), see text.

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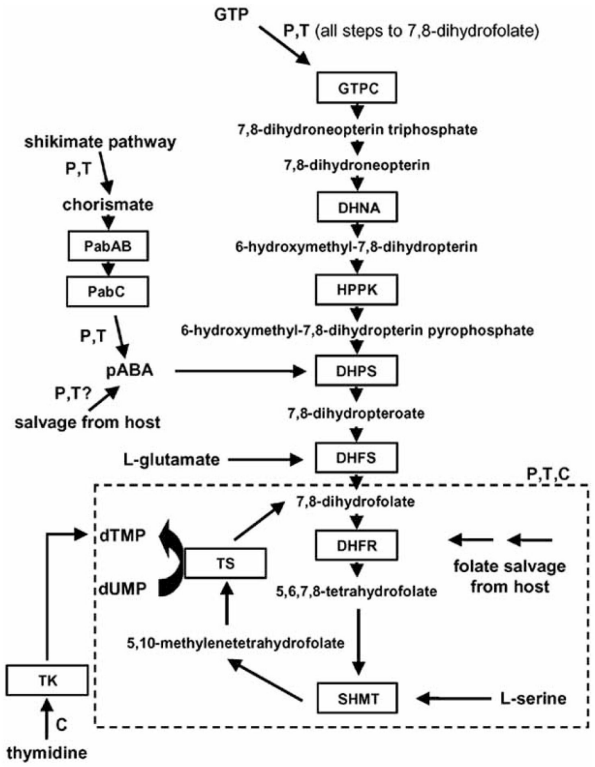

Fig. (2).

De novo and salvage pathways to reduced folate, and (dashed boxed) the thymidylate cycle. Polyglutamated forms of folate (known to be the preferred substrates in other organisms, and experimentally demonstrated in *P. falciparum* [49, 50]) are omitted for clarity. The triphosphate group is removed from 7,8-dihydroneopterin before the DHNA step, but it is not yet known whether this is by a non-enzymatic loss of pyrophosphate and subsequent removal of the final phosphate by a non-specific phosphatase activity, or whether the entire step is enzymatically catalysed. Genes encoding a DHNA activity are as yet unidentified in *Plasmodium* and *Toxoplasma*. The oxidation state of salvaged folate is unspecified, although the major form in the human host is 5-methyltetrahydrofolate. Experimental evidence for folate salvage in *P. falciparum* and *Toxoplasma* has been established, but is lacking for *Cryptosporidium*, despite its evident necessity (see text). The targets of current antifolate drugs in clinical use against malaria and toxoplasmosis are DHPS and DHFR. P,T,C indicate that the pathway or step is utilised in *Plasmodium, Toxoplasma* and *Cryptosporidium* respectively. For abbreviations of enzymes (boxed), see text.

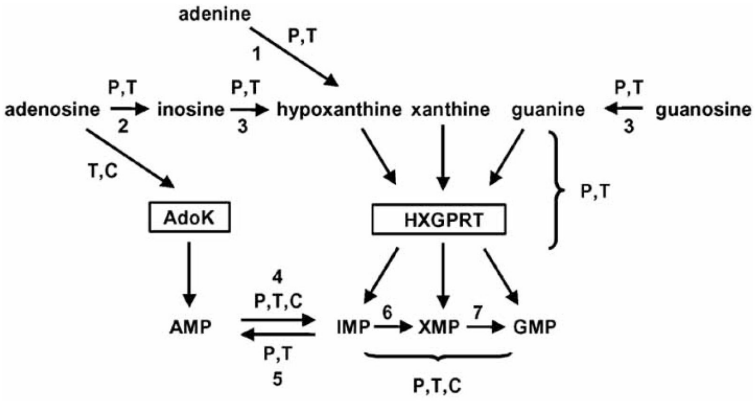

Fig. (3).

Primary purine salvage pathways. P,T,C indicate that the pathway or step is utilised in *Plasmodium, Toxoplasma* and *Cryptosporidium* respectively. In *Toxoplasma*, the only parasite of the three capable of all of the reactions depicted, the predominant route to the purine nucleoside monophosphates is *via* AdoK. Numbered enzymes are as follows: 1, AD; 2, AdoD; 3, PNP; 4, AMP deaminase; 5, adenoylsuccinate synthase + adenoylsuccinate lyase; 6, IMPDH; 7, GMP synthetase. Note also the ability of *P. falciparum* PNP to process 5′-methylthioinosine arising from the polyamine biosynthetic pathway (see text). Figure adapted from [196]. For abbreviations of enzymes, see text.