Targeting DHFR in parasitic protozoa

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Parasitic apicomplexans are responsible for some of the most severe worldwide health problems, including malaria, toxoplasmosis and cryptosporidiosis. These parasites are characterized by a bifunctional enzyme, dihydrofolate reductase-thymidylate synthase (DHFR-TS), which has a crucial role in pyrimidine biosynthesis. Inhibitors of DHFR have been successful in the treatment of toxoplasmosis and malaria. However, there is currently no effective therapy for cryptosporidiosis, and despite early successes against malaria, resistance to DHFR inhibitors in malaria parasites has now become a global problem. Novel DHFR inhibitors, designed using the recently revealed crystal structures of the enzymes from two parasitic protozoa, are in development.

The phylum apicomplexa contains some of the world's most dangerous protozoan parasites. The most important disease caused by Apicomplexan protozoa is malaria, caused by four species of *Plasmodium*: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*. Of the four species, *P. falciparum* is responsible for the most severe form of malaria, cerebral malaria, and causes the majority of morbidity cases. *Plasmodium falciparum* are inoculated into the bloodstream of a vertebrate host by an infected female *Anopheles* mosquito during bloodfeeding [1]. The symptoms of malaria include fever, chills, headache, delirium, severe anemia, heart attack, cerebral hemorrhages and kidney failure. Each year, 500 million individuals are affected by malaria and around three million individuals die from the disease; children under five years of age living in sub-Saharan Africa comprise the majority.

Toxoplasmosis is caused by *Toxoplasma gondii*, a parasite that can infect all warm-blooded animals [2]. Congenital toxoplasmosis, caused by transmission between a pregnant woman and fetus, can result in abortion, neonatal death or fetal abnormalities [3]. The most common route of transmission to humans is through ingestion of tissue cysts in infected meat or oocysts in food or water contaminated with feline feces [2]. The main symptoms of toxoplasmosis include fever and sore throat, lymphadenopathy, splenomegaly and hepatomegaly. Immunocompromized patients can develop more severe forms of the disease because previously acquired, latent *T. gondii* infection can reactivate and cause cerebral inflammation (toxoplasmic encephalitis), resulting in seizures and death.

Cryptosporidiosis, an emerging infectious disease, causes severe diarrhea that can be fatal in immunocompromized individuals. Since 1976, when only five cryptosporidiosis cases were reported, the number of cases has risen dramatically to include reports from more than 90 countries and six continents [4,5]. The most severely affected individuals are AIDS patients, the elderly [6] and children in day-care centers [7], with 2–4% prevalence rates in day-care centers in the USA [5]. During day-care center outbreaks, attack rates of 13–90% are common [5]. The *Cryptosporidium* parasite is water-borne and often transmitted by a fecal–oral route via wastewater contaminated from

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infected animals. One of the most well-known outbreaks among immune-competent individuals occurred in Milwaukee in 1993, where >400,000 people were infected by *Cryptosporidium parvum* from the city water supply [8].

Treating infectious diseases with inhibitors of essential metabolic enzymes has proven to be an effective strategy. Dihydrofolate reductase (DHFR) is an essential enzyme in nucleic acid and amino acid synthesis in all cells and, as such, has been a widely recognized drug target for over 50 years. Inhibitors of DHFR are the basis of, for example, anticancer [9,10], antibacterial [11,12] and antiparasitic [13] therapies. In fact, inhibitors of DHFR in parasitic protozoa have been successful as antimalarial therapies since the late 1960s [14]. The safe and effective use of DHFR inhibitors in treating infectious disease has promoted continued research in this area.

Dihydrofolate reductase as a drug target

In most species, including humans, DHFR has a key role in the folate biosynthesis pathway (Figure 1) and is responsible for the generation of the DNA base, deoxythymidine monophosphate (dTMP). DHFR is also involved in the biosynthesis of purine nucleotides and the amino acids histidine and methionine.

There are several differences between the folate biosynthetic pathways in parasitic protozoa and in humans. Two of these differences will be highlighted here. (1) Parasitic protozoa possess an endogenous folate biosynthetic pathway that is susceptible to antifolate inhibitors, although *Plasmodium* and *Cryptosporidium* also have a salvage pathway to use exogenous folate [14,15]; humans do not have the ability to synthesize folates *de novo* and instead use a membrane-bound reduced folate carrier to bring dietary

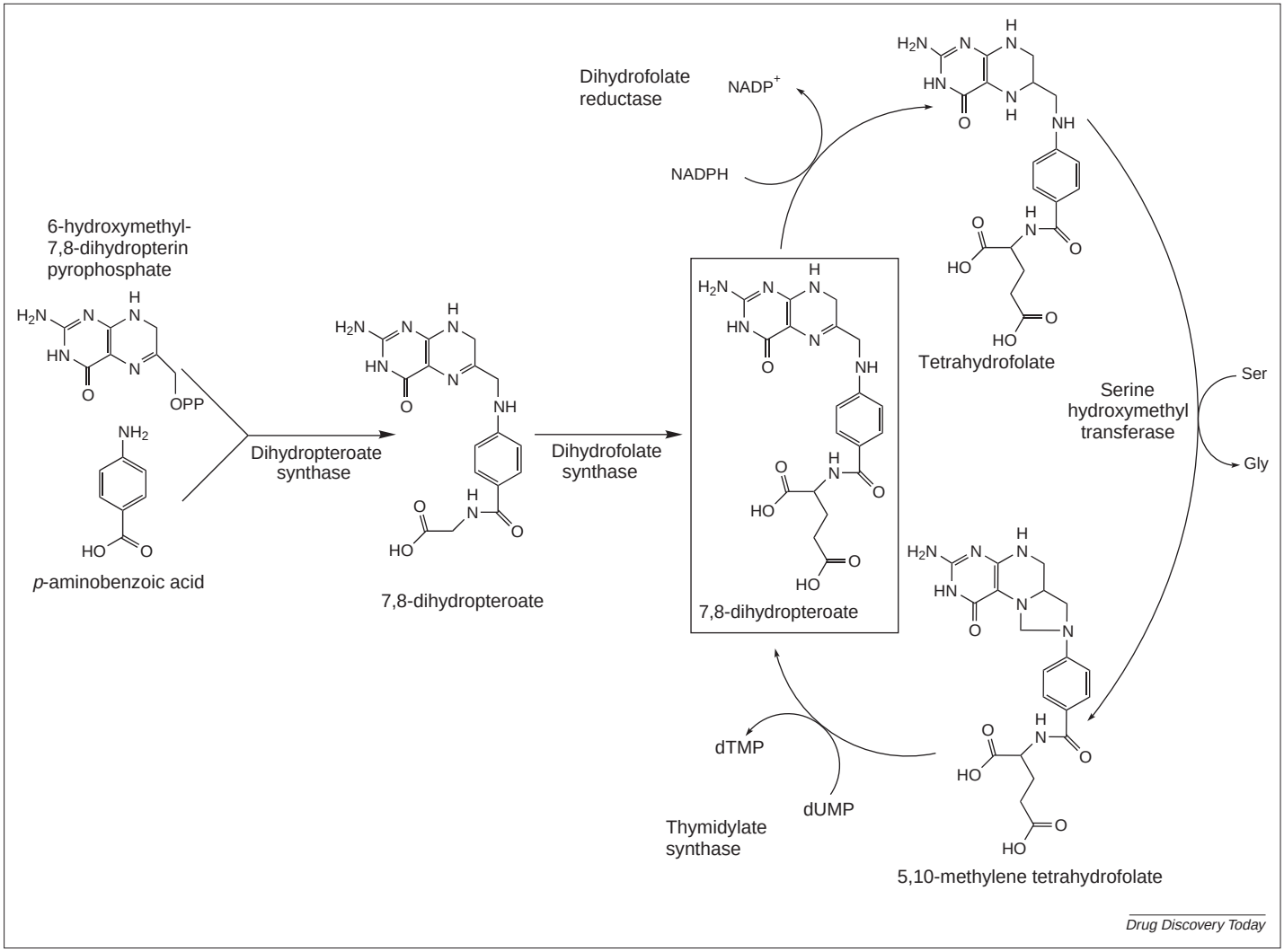

FIGURE 1

A section of the folate biosynthesis pathway in parasitic protozoa. Dihydropteroate synthase (DHPS) catalyzes the addition of *para*-aminobenzoic acid and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to form 7,8-dihydropteroate. Dihydrofolate synthase (DHFS) adds one glutamate moiety to 7,8-dihydropteroate to form 7,8-dihydrofolate. Folyl polyglutamate synthase (FPGS) can add additional glutamate moieties. Once dihydrofolate is formed, it enters the folate cycle. Dihydrofolate reductase (DHFR) reduces 7,8-dihydrofolate to form tetrahydrofolate with the concomitant oxidation of NADPH to NADP+. Serine hydroxymethyl transferase (SHMT) forms 5,10-methylene tetrahydrofolate by transferring the side chain of serine, forming glycine. Thymidylate synthase (TS) catalyzes the formation of dTMP from dUMP and 5,10-methylene tetrahydrofolate, forming dihydrofolate. In parasitic protozoa, DHFR and TS form a bifunctional enzyme. In mammals, the reduced folate carrier transports dietary folic acid into the cell and DHFR reduces the folic acid to tetrahydrofolate. Hence, mammals do not have DHPS or DHFS.

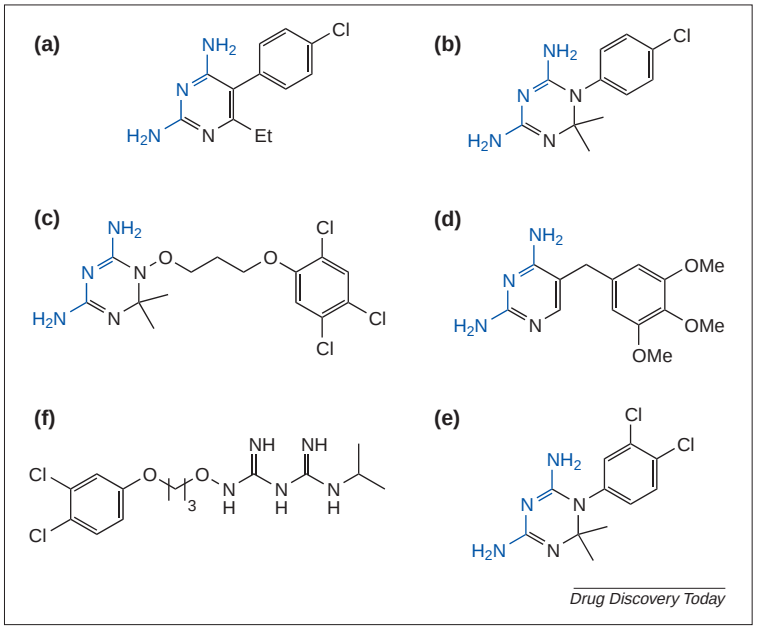

FIGURE 2

DHFR inhibitors. (a) Pyrimethamine, **(b)** cycloguanil, **(c)** WR99210, **(d)** trimethoprim, **(e)** chlorcycloguanil and **(f)** PS-15. The 2,4-diamino scaffold is shown in blue.

folic acid, which is then reduced to tetrahydrofolate (THF) by DHFR, into the cell. (2) Protozoa possess a bifunctional enzyme called dihydrofolate reductase-thymidylate synthase (DHFR-TS) in which DHFR and TS are two domains of a single homodimeric protein; in humans, DHFR and TS occur as two separate, monofunctional proteins.

The folate biosynthetic pathway in parasitic protozoa involves several enzymes; the reactions catalyzed by five of these are shown in Figure 1. Dihydropteroate synthase (DHPS: EC.2.5.1.15) catalyzes the addition of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) to *para*-aminobenzoic acid (*p*ABA) to produce 7,8-dihydropteroate. In *Plasmodium* and *Toxoplasma*, DHPS and hydroxymethyldihydropterin pyrophosphokinase (not shown in Figure 1), are located on the same polypeptide, forming another bifunctional enzyme. However, to date, analysis of the genome sequence of *Cryptosporidium parvum* [16] has not revealed the presence of a *dhps* gene in this parasite [15]. In a subsequent step, dihydrofolate synthase (DHFS: EC.6.3.2.12) adds a glutamate moiety to 7,8-dihydropteroate to form 7,8-dihydrofolate. To initiate the cycle in which dihydrofolate is regenerated, DHFR (EC.1.5.1.3) catalyzes the reduction of dihydrofolate, using the cofactor NADPH, to yield tetrahydrofolate (THF). The hydroxymethyl group of serine serves as the source of the final carbon in a reaction catalyzed by serine hydroxymethyl transferase (SHMT: EC.2.1.2.1), generating 5,10-methylenetetrahydrofolate. Ultimately, dihydrofolate is regenerated and dTMP is formed when TS (EC.2.1.1.45) catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) using 5,10-methylene tetrahydrofolate.

The catalytic role and the tertiary structure of DHFR are evolutionarily conserved across bacteria and mammals. Human cells also depend on DHFR for DNA replication, therefore, developing inhibitors that are not only potent but are also selective for the parasitic pathogen is of the ultimate importance. Fortunately, the sequence of the DHFR gene has significantly diverged throughout evolution, generating unique protein sequences in each organism, all of which are capable of the hydride transfer reaction. These differences in the active site have enabled the development of species-specific DHFR inhibitors, some of which are up to 2–3 orders of magnitude more potent against the bacterial or parasitic enzymes over the human enzyme. An example of a species-selective DHFR inhibitor is trimethoprim, which is ~50,000-fold selective for the bacterial DHFR over human DHFR [12].

Current DHFR inhibitors for parasitic protozoa

DHFR inhibitors are often the first-line therapies for malaria and toxoplasmosis; cryptosporidiosis exhibits some resistance to many DHFR inhibitors effective against other parasitic protozoa. A review of the current DHFR inhibitors employed as therapies for parasitic protozoa will be discussed in subsequent sections. Elucidating the binding mode of these inhibitors with DHFR from both sensitive and resistant strains enables the development of novel inhibitors with higher potency and selectivity.

Malaria

Pyrimethamine and cycloguanil (Figure 2) are potent, competitive inhibitors of *P. falciparum* DHFR with K_i values of 0.2 and 0.3 nM, respectively [17]. Pyrimethamine is usually administered in conjunction with sulfadoxine, a DHPS inhibitor (Fansidar®; Roche), to maximize the synergistic inhibition of both DHFR and DHPS (Figure 1). Cycloguanil is administered as a prodrug, proguanil, which is metabolized in the liver [18]. Proguanil can be administered alone or with atovaquone, an inhibitor of the cytochrome bc_1 complex (Malarone®; GlaxoSmithKline). Both pyrimethamine and cycloguanil share the 2,4-diamino scaffold (shown in blue in Figure 2), which enables hydrogen bonding to key catalytic residues in the DHFR active site. In cases of severe folate deficiency, leucovorin, a reduced form of folic acid, can be administered during treatment because mammalian cells can transport folic acid across the cell membrane, rescuing the function of human DHFR. *P. falciparum* can salvage leucovorin and folic acid, but the combination of the DHFR and DHPS inhibitors achieves therapeutic antiparasitic activity [14].

Plasmodium species that are resistant to pyrimethamine and cycloguanil have become a severe problem in malaria-endemic regions such as Southeast Asia [19]. It is estimated that, in some parts of the world, pyrimethamine has had only a five-year effective lifespan [20] and cycloguanil a 3-year lifespan [21]. Cloning and sequence analysis of DHFR from resistant strains of *P. falciparum* reveals that the DHFR gene can accumulate several key mutations that reduce the affinity of the inhibitors for the protein but

which continue to allow catalytic processing of the substrate [19,22]. For example, a point mutation at residue 108 changes the codon from serine to asparagine (S108N) and confers ten-fold resistance to pyrimethamine when compared to that of wild-type [23].

In a comparison of crystal structures of wild-type and mutant *P. falciparum* DHFR-TS, it was noted that the resistance mutations either caused steric interactions with the inhibitors or increased the volume of the active site, thus weakening the binding of the inhibitors [17]. Analysis of the crystal structure of *P. falciparum* DHFR-TS with the S108N mutation reveals that the *para*-chlorophenyl group of pyrimethamine lies close to the residue at position 108; an Asn at this position produces a severe steric interaction with this group [17]. Additional mutations at positions 51 (Asn to Ile), 59 (Cys to Arg), and 164 (Ile to Leu) confer increasing levels of resistance to pyrimethamine. A *P. falciparum* DHFR protein with all four mutations has ~900-fold resistance to pyrimethamine and 700-fold resistance to cycloguanil compared with those of wild-type parasites [23]. A comparison of the structures of *P. falciparum* DHFR-TS with the four mutations and the wild-type structure explains the effect of these multiple mutations: N51I causes a main chain displacement of residues 48–51, and I164L causes a shift in residues 164–167, thus expanding the volume of the active site and weakening binding [17]. DHFR mutations at position 16 (Ala to Val) and 108 (Ser to Thr) have been found to confer over 2000-fold resistance to cycloguanil [23]. One of the two methyl groups at the 6-position on cycloguanil appears to interfere with Val 16, decreasing affinity for the inhibitor [24]. Several field isolates of *Plasmodium* DHFR, with mutations in positions 108 as well as in position 164, have been shown to be resistant to both pyrimethamine and cycloguanil [25].

Two antifolates, chlorcycloguanil and WR99210 (Figure 2), have been developed to combat the pyrimethamine-resistant strains of *P. falciparum*. Chlorcycloguanil is administered as a prodrug, chlorproguanil, with dapsone (Lapdap™). The prodrug is metabolized in the liver to chlorcycloguanil with an extra *meta* chlorine group relative to cycloguanil. Lapdap, effective against the pyrimethamine-resistant triple mutant (N51I + C59R + S108N) [26,27], entered Phase III clinical trials in March 2000 in five African clinical sites and was found to have greater efficacy than pyrimethamine-sulfadoxine and to represent a useful alternative where pyrimethamine–sulfadoxine resistance is high [28]. WR99210, a diamino triazine antifolate, is effective against the quadruple mutant strain (N51I + C59R + S108N + I164L) [29,30] with a K_i value of 0.011 nM [17]. In a structural analysis of the quadruple mutant of *P. falciparum* DHFR-TS, Yuvaniyama *et al*. propose that the flexible side chain of WR99210 enables this compound to adopt a conformation that fits into the mutant active site, effectively inhibiting the enzyme [17]. Unfortunately, in clinical trials, oral administration of WR99210 led to gastrointestinal intolerance [31]. The biguanide prodrug, PS-15 [31] (Figure 2), is more easily absorbed than WR99210 from the gastrointestinal tract, relieving many of the side effects of WR99210, and is readily metabolized to WR99210 [32]. PS-15 is currently in preclinical trials and is expected to be effective in Southeast Asia where the quadruple mutant strain is prevalent [19].

Toxoplasmosis

Currently, the most effective treatment for toxoplasmosis is pyrimethamine–sulfadiazine (Daraprim®; GlaxoSmith-Kline). Pyrimethamine is a 0.25 μM inhibitor of *Toxoplasma* DHFR [33], and sulfadiazine is an inhibitor of dihydropteroate synthase (DHPS). The primary therapy (an initial dose of 200 mg pyrimethamine, followed by 75 mg day^{-1} and up to 8 g day^{-1} sulfadiazine) is administered for 3–6 weeks and then must be followed by maintenance therapy (25–50 mg day^{-1} pyrimethamine and 2 g day^{-1} sulfadiazine) for the remainder of the patient's life [34]. Sulfadiazine can induce severe allergic reactions in many patients; in a study of 29 patients, ~54% experienced severe allergic reactions [34]. For patients who are sensitive to sulfadiazine, a pyrimethamine–clindamycin (clindamycin belongs to the macrolide class of protein synthesis inhibitors) form of treatment is available. Again, leucovorin can be administered to rescue patients with severe folate deficiency.

Up to 10% of patients infected with *Toxoplasma* do not respond to pyrimethamine–sulfadiazine therapy, and another 10–20% of initial responders experience relapses during their treatment [35]. The inefficacy of treatment can in some cases be ascribed to sulfonamide resistance, conferred by a mutation at position 407 in the DHPS protein [35]. Although pyrimethamine-resistant strains of *T. gondii* have not been isolated in the field, such strains have been created in the laboratory by mutating the *T. gondii* DHFR-TS residues corresponding to those causing pyrimethamine resistance in *P. falciparum* [36].

Alternative treatments for toxoplasmosis include trimethoprim–sulfamethoxazole (TMP-SMX). Trimethoprim (Figure 2) is another selective DHFR inhibitor with low efficacy against human DHFR. Trimethoprim is advantageous for AIDS patients with toxoplasmosis because the TMP-SMX combination is already used as a prophylactic treatment for *Pneumocystis* pneumonia (PCP). Atovaquone is a second alternative for patients who are either intolerant or unresponsive to first-line therapies. Of all patients, 88% demonstrated some response to atovaquone [34].

Cryptosporidiosis

There is currently no effective therapy available for cryptosporidiosis. The reasons why DHFR inhibitors that are effective against other related parasitic protozoa are ineffective against *Cryptosporidium* are not clear, but could be due to: i) an inability of the inhibitor to bind the target protein in *Cryptosporidium*, ii) an inability to deliver the drug to the parasite; or iii) both.

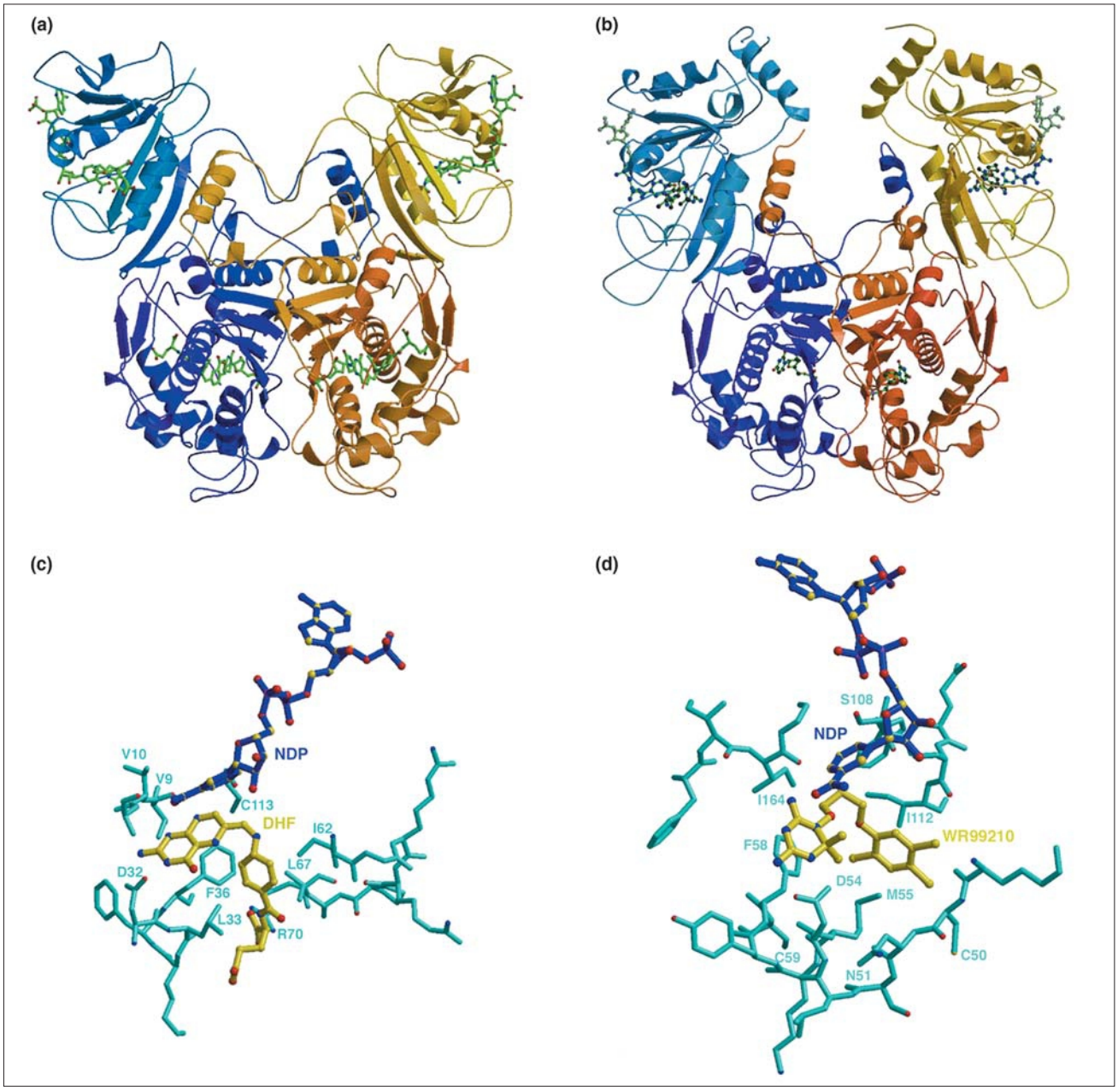

FIGURE 3

Crystal structures of DHFR-TS from (a) *Cryptosporidium hominis* and (b) *Plasmodium falciparum*. The enzymes are homodimers; one monomer is shown in blue and the other monomer is shown in yellow. The DHFR domains are light blue or yellow and the TS domains are dark blue or orange. Bound ligands are shown in green. A detailed view of the residues in the active site of the wild-type enzymes: **(c)** *C. hominis* DHFR-TS bound to DHF and NADPH; and **(d)** *P. falciparum* DHFR-TS bound to WR99210 and NADPH. The substrate, DHF, and the inhibitor, WR99210, are shown in yellow, NADPH (NDP) is shown in dark blue and protein residues are shown in cyan.

Experiments with purified *Cryptosporidium* DHFR-TS suggest that the enzyme has some inherent resistance to common DHFR inhibitors. Trimethoprim and pyrimethamine, usually effective at nanomolar concentrations against species of DHFR from *Toxoplasma*, *Plasmodium*, and bacteria such as *Escherichia coli* and *Mycobacterium avium*, are effective only at micromolar concentrations against *Cryptosporidium* DHFR [37]. At several key positions in the active site, *Cryptosporidium* DHFR incorporates smaller residues, thereby creating a larger active-site pocket relative to other DHFR enzymes that are inhibited by trimethoprim and pyrimethamine. Three of the resistance-determining residues in *Plasmodium* DHFR (N51I, S108T and I164L) are naturally found in *Cryptosporidium* DHFR (Ile 29, Thr 58 and Cys 113) [38]. *Plasmodium* DHFR with the I164L mutation shows decreased levels of van der Waals contact with the inhibitor; Cys 113 in *Cryptosporidium* DHFR decreases this contact even further. Although any of these individual substitutions might not substantially decrease the affinity for pyrimethamine or trimethoprim, the combination of the substitutions could explain the overall reduced affinity of antifolate DHFR inhibitors.

Finally, drug delivery to the *Cryptosporidium* protein target could also be difficult because *Cryptosporidium* occupies a unique niche in epithelial cells of the gastrointestinal tract. The *Cryptosporidium* sporozoite adheres to the epithelial cell until surrounded by microvilli, creating an intracellular, but extracytoplasmic niche.

Future development of DHFR inhibitors

The recent determination of the DHFR-TS structures from *Cryptosporidium hominis* [39] [Figure 3a and Protein Data Bank Identification (PDB ID): 1QZF, 1SEJ] and *P. falciparum* [17] (Figure 3b and PDB ID: 1J3I) reveal a novel architecture for the Apicomplexan bifunctional enzyme relative to the previously determined structure of DHFR-TS from *Leishmania major* [40], a protozoan from the kinetoplastid family. The structures also indicate the interactions between the *C. hominis* enzyme and its substrate, dihydrofolate, and the *P. falciparum* enzyme and the inhibitors, pyrimethamine and WR99210. The structure of the quadruple mutant (N51I, C59R, S108N, I164L) of *P. falciparum* DHFR-TS (PDB ID: 1J3K) bound to WR99210 has also yielded insight into the structural basis of pyrimethamine resistance and WR99210 sensitivity. The protein structures have led to several hypotheses for novel drug design, both in and away from the active site of DHFR. The following sections will elaborate on the overall structure of the enzymes, the interactions with the substrate and inhibitors, the structural basis of resistance and future structure-based drug design goals.

Structures and modeling

The structures of DHFR-TS from *Cryptosporidium* (Figure 3a) and *Plasmodium* (Figure 3b) show that the enzyme is a homodimer and that each monomer has DHFR, linker and TS domains. In both *Cryptosporidium* and *Plasmodium*, the overall folds of the DHFR and TS domains resemble those from several other eukaryotic species [41–43]. The linker domain, which connects the DHFR and TS domains, comprises 58 residues in *Cryptosporidium* and 89 residues in *Plasmodium*, and has an integral structural role in both enzymes. In the structure of *Cryptosporidium* DHFR-TS, the polypeptide forms the DHFR domain, crosses to the opposite monomer and forms a helix that packs against the active site of the DHFR domain of that monomer and then crosses back to form the TS domain [39]. In the *Plasmodium* DHFR-TS structure [17], the polypeptide also formed the N-terminal DHFR domain, the portion of the linker resolved in the electron density map formed the same helix as was observed for *Cryptosporidium*, packed against one DHFR monomer, and then extended to form the opposite TS monomer. It is presumed that the structure of *Toxoplasma* DHFR-TS will be homologous to that of *Cryptosporidium* and *Plasmodium* [39].

Both structures of DHFR-TS show that substrates and inhibitors are bound in the DHFR active site using many of the same interactions observed in other species of

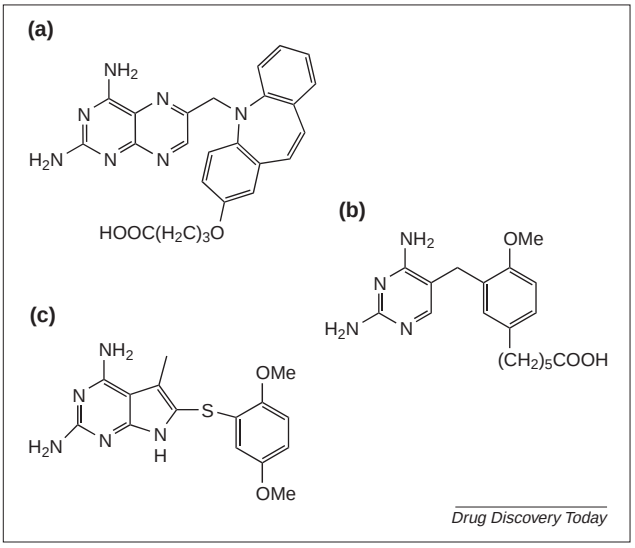

FIGURE 4

New DHFR inhibitors that are potent and selective for *Toxoplasma gondii* DHFR-TS. (a) 2,4-diamino-6-[2′-O-(3-carboxypropyl)oxydibenz[b,f]-azepin-5-yl] methylpteridine. **(b)** 2,4-diamino-5-[5′-(5-carboxypentyl)-2′-methoxy)benzyl]-pyrimidine. **(c)** 2,4-diamino-5-methyl-6-(2′5′-dimethoxyphenylthio)-pyrrolo[2,3-d]pyrimidine.

DHFR [41,44,45] (Figure 3c, 3d). The interactions between wild-type *P. falciparum* DHFR-TS and WR99210 [17] (Figure 3d) include two hydrogen bonds between Asp 54 and the 2,4-diamino groups on the pyrimidine ring. Phe 58, Ile 164 and Trp 48 form van der Waals interactions with the pyrimidine ring, and Ile 112, Met 55 and Trp 48 form van der Waals interactions with the (2,4,5-trichlorophenoxy) propyloxy substituent. Pyrimethamine binds the double mutant (S108N/C59R), although with 20-fold lower affinity than the wild-type enzyme, by forming two hydrogen bonds from Asp 54 to the 2,4-diaminopyrimidine and several van der Waals interactions between Phe 58 and Ile 164 and the pyrimidine ring and Ile 112, Ile 164 and Trp 48 with the *para*-chlorophenyl moiety. A steric interaction is observed between the Asn 108 mutation and the *p*-chlorophenyl group, providing a structural basis for pyrimethamine resistance in the mutated strains.

Earlier modeling predictions showed that mutations in the *P. falciparum* DHFR active site at residues 16 and 108 caused steric interactions for pyrimethamine and cycloguanil binding [46,47]. Kamchonwongpaisan *et al.* [48] used the crystal structure of *Plasmodium* DHFR-TS to design new cycloguanil and pyrimethamine derivatives with the purpose of avoiding steric interactions around residues 16 and 108. New inhibitors without the *para*-chloro group improved the inhibition of the quadruple mutant by 12-fold to 36-fold, and inhibitors with the *para*-chloro group moved to the *meta* position improved the inhibition of the quadruple mutant by 85-fold to 133-fold. In the case of Cyc derivatives, shifting the *para*-chloro to a *meta*-chloro and removing one of the methyl groups yields an inhibitor that is highly effective, with nanomolar inhibition levels, against both wild-type and mutant enzymes.

TABLE 1

Novel DHFR inhibitors against parasitic protozoa that are currently in development

DHFR inhibitor	Disease	Development stage	Refs
Lapdap (chlorcycloguanil/dapsone)	Pyrimethamine-resistant *P. falciparum*	Phase III clinical trials	[28]
PS-15	Pyrimethamine-resistant *P. falciparum*	Preclinical development	[31]
Compound 1[a]	T. gondii	Preclinical R&D	[49]
Compound 2[b]	T. gondii	Preclinical R&D	[50]
Compound 3[c]	T. gondii	Preclinical R&D	[51]

[a]2,4-diamino-6-[2′-O-(3-carboxypropyl)oxydibenz[b,f]-azepin-5-yl] methylpteridine;
[b]2,4-diamino-5-[5′-(5-carboxypentyl)-2′-methoxy)benzyl]-pyrimidine;
[c]2,4-diamino-5-methyl-6-(2′5′-dimethoxyphenylthio)-pyrrolo[2,3-days]pyrimidine.

The structure of *Cryptosporidium* DHFR-TS has also suggested new strategies for the development of potent and selective antifolates. Previously developed antifolates, such as trimethoprim, have been shown to be selective but not potent against the *Cryptosporidium* enzyme [37], while other antifolates, such as methotrexate, show high potency but low selectivity. There are several residue substitutions between *Cryptosporidium* and human DHFR enzymes, including Val 9 (Ile 7 in human DHFR) and Leu 33 (Phe 31 in human DHFR), which can be exploited for selectivity. Additional potency can be incorporated into *Cryptosporidium* DHFR inhibitors by increasing the bulk of the phenyl ring of trimethoprim, forcing contacts with the specific residues in the *Cryptosporidium* DHFR active site.

Although pyrimethamine–sulfadiazine has proven a useful therapeutic for toxoplasmosis, many patients have adverse reactions to sulfa compounds, driving the need to create new *Toxoplasma* DHFR inhibitors that do not require the co-administration of sulfa compounds. Similar to the case with *Cryptosporidium*, trimethoprim has been shown to be selective for the *Toxoplasma* enzyme, but is not potent enough to reduce parasitemia. Another antifolate, piritrexim, developed as a human anticancer drug, is potent but not selective. Attempts to combine the selectivity of trimethoprim and the potency of piritrexim have resulted in new compounds that display nanomolar inhibition and selectivity ratios in the range of 120-fold to 490-fold [49,50] (Figure 4, compounds a and b). A new thiopyrrolo pyrimidine scaffold yielded a compound with 46-fold selectivity against *T. gondii* DHFR over rat liver DHFR [51] (Figure 4, compound c). Without the tertiary structure of the complex, it is difficult to determine the basis of the increased potency and selectivity. However, compounds 1 and 2 are also selective for *Pneumocystis* DHFR, and modeling studies indicate that Lys 37 in *Pneumocystis* DHFR (a neutral glutamine in human DHFR) either forms an ionic bond or hydrogen bond with the terminal COOH group of the inhibitor [52]. The same lysine is present in *Toxoplasma* DHFR and might also be responsible for selectivity toward this enzyme.

Conclusion

DHFR is a validated drug target for therapeutics directed against the diseases of the parasitic protozoa: malaria, toxoplasmosis and cryptosporidiosis. Pyrimethamine and cycloguanil are two DHFR inhibitors that have been successful in the fight against malaria; pyrimethamine and trimethoprim have been successful against toxoplasmosis. Despite the continuing success of these compounds, however, there is a definite need to develop new DHFR inhibitors for sensitive and resistant strains of Apicomplexan parasitic protozoa (Table 1). DHFR inhibitors, especially chlorcycloguanil and WR99210, show promise against resistant strains of malaria parasites. New 2,4-diamino-pteridine and -pyrimidine inhibitors directed against *Toxoplasma* DHFR show both potency and selectivity and new *Cryptosporidium* DHFR inhibitors are in development using the information gained from the crystal structure.

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