DNA and RNA Synthesis: Antifolates

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1. Introduction

Tetrahydrofolate cofactors are essential for the synthesis of purines, certain amino acids, and thymidine. Most bacteria and plants produce these folate cofactors by de novo biosynthesis, although some bacteria and mammalian cells rely on the use of preformed folates and have salvage pathways for reduced folates, purines, and pyrimidines. Compounds that interfere with this pathway, antifolate agents, have found use in the clinic as antibacterials, antimalarials, and anticancer drugs. In the past decade, an intensive search for drugs that could be specifically used in a variety of opportunistic infections have been undertaken.

This review covers the major progress and developments in inhibitors of the enyzmes involved in folic acid biosynthesis from January 1995 till mid-2004. Outstanding comprehensive reviews on antifolates prior to this period include those of Hitchings and Smith,[1] Sirotnak,[2] Blakley,[3] and Rosowsky.[4] The reader is also reffered to other important reviews that emphasize particular aspects such as the selectivity of antifolates,[5] structure−activity relationships (SAR) of inhibitors in this area,[6,7] and general aspects of dihydrofolate reductase (DHFR) inhibitors.[8−12] Antifolates as antitumoral agents recently been reviewed by McGuire and Derouin.[13,14]

Starting from guanosine triphosphate, six enyzmes are involved (see Scheme 1, not all enzymes are shown) in the biosynthesis of tetrahydrofolic acid, and the crystal structures of all but one have been determined.[15] In addition, inhibitors of thymidylate synthase (TS)[16] and dihydropteroate synthase[17] (DHPS) have also been reported and will be dealt with in this review. In a broad sense, inhibitors of all these enzymes fall under the term "antifolates". However, because the overwhelming majority of antifolates are inhibitors of DHFR, very often the term "antifolates" is reduced to the inhibitors of this enzyme and these constitute the major part of this article.

Research efforts have concentrated on the discovery of safer or more potent compounds or both when compared with available antifolates in the corresponding therapeutic areas. In the past decade, these efforts have been reflected in the publication of well

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Ivan M. Kompis studied chemistry at the Slovak Technical University in Bratislava (former Czechoslovakia) and got his Ph.D. degree from the Slovak Academy of Sciences there in 1962. The years 1963–1964 were spent as a postdoctoral fellow with Prof. Edward Leete at the University of Minneapolis, MN, working on the biosynthesis of indole alkaloids. From 1968 to 1971, he was Assistant Professor at the Institute of Organic Chemistry, University of Zurich, Switzerland, with main interest in structure elucidation of natural products. In the years 1971–1995, he was working in Pharma Research of F. Hoffmann-LaRoche Ltd., Basel, Switzerland, and Nutley, NJ. In the last 10 years with Roche, he acted as the Head of the Department of Infectious Diseases. He is co-founder and former President of ARPIDA Ltd., a company devoted to research and development of anti-infectives, a member of SAB of KOSAN Biosciences Ltd. in Hayward, CA, and active as a consultant for medicinal chemistry.

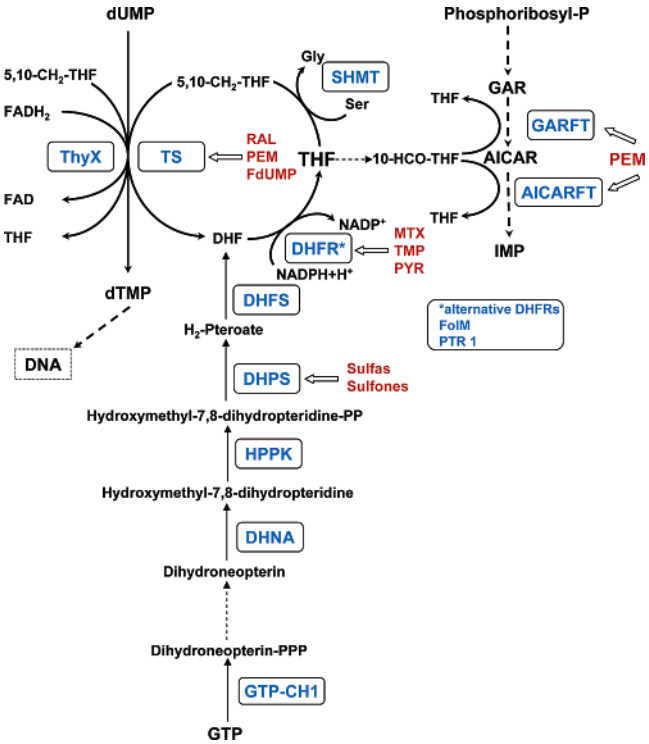
Khalid Islam obtained his Ph.D. in 1983 from Imperial College, University of London. During his Ph.D. and postdoctoral studies, he has worked on cellular mechanisms for the regulation of protein–protein interactions, and as an EMBO fellow (1985–1987), he worked on phosphorylation–dephosphorylation mechanisms. In 1987, he joined Marion Merrell-Dow where he coordinated multidisciplinary teams of biologists (biochemists, microbiologists, molecular and cell biologists) in drug discovery and preclinical development. From 1996 to 1999, he worked in Hoechst Marion Roussel (HMR), Paris, as a group leader in drug discovery and preclinical development of antibacterials and antifungals. In July 1999, he moved to Basel to join Arpida, where he is currently the President and Chief Executive Officer. He referees for several international journals and is a member of the editorial board of *Current Drug Discovery Technologies*. He holds several patents and has published over 75 articles in leading journals.

over 500 papers and the synthesis and evaluation of hundreds of compounds as potential inhibitors of the folate pathway enzymes. A number of new chemical entities, which are or can potentially be used in cancer chemotherapy or infectious diseases have been identified. Indeed, at least four antifolates are under development for the treatment of various cancers. Moreover, with the advance of the HIV epidemic and the concomitant emergence of opportunistic infections, in recent years a major effort has been dedicated to the search for inhibitors of folate pathway enzymes of causative agents for these infections.

Despite the fact that several other enzymes of the folate pathway have been characterized and their structures solved,[15] DHFR (E.C. 1.5.1.3), a key enzyme in folate utilization, and its inhibitors remain the focus of research in this area.

Rudolf Then received a diploma in Biology at the University of Mainz in 1966 and later obtained his Ph.D. in microbiology 1969. Aside from microbiology, he focused on biochemistry and pharmacology. After spending a short period as scientific assistant at the University of Mainz, he joined the antibiotic research group at F. Hoffmann-La Roche in Basel in 1970. There he spent most of his scientific career in research, interrupted by a short sabbatical in 1975 at the Roche Molecular Biology Institute in Nutley, NJ. His main research activities were the discovery and development of new antibacterial dihydrofolate reductase inhibitors, their mode of action, and synergy with sulfonamides, new β-lactam antibiotics, and β-lactamase inhibitors. He was always interested in the diverse mechanisms of resistance to these agents. DNA-gyrase inhibitors and screening assays for new antibiotics were other fields of his activities. He has published numerous articles in these fields and some chapters in text books. He is currently consultant for infectious diseases at Actelion Pharmaceuticals in Basel.

Scheme 1



2. Folate Pathway Enzymes

2.1. Function and Biochemistry

All living cells need tetrahydrofolate cofactors for the synthesis of purines, some amino acids, and especially thymidine. The biosynthesis of tetrahydrofolate from GTP is now well established,[1,2,18] and the antifolate target enzymes are outlined in Scheme 1. Although inhibitors of these target enzymes are the primary subject of this review, we have also included inhibitors of thymidylate synthase (TS) and serine hydroxymethyl transferase (SHMT) enzymes

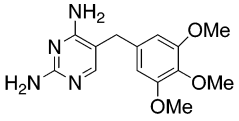
because these are intimately associated with folate biosynthesis in the thymidylate cycle. The general folate pathway can be accessed via the Internet at http://ca.expasy.org/cgi-bin/show_image?A7 and http://ca.expasy.org/cgi-bin/show_image?L2 or in the KEGG database, http://www.genome.ad.jp/kegg/pathway/map/map00790.html.

2.2. Dihydrofolate Reductase

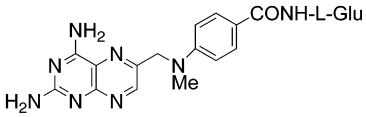
DHFR is by far the most intensively studied enzyme in the folate pathway. It is an essential and almost (vide infra) ubiquitous enzyme. It generates tetrahydrofolate (THF), various cofactors of which are involved in the transfer reactions of the one carbon unit used in the biosynthesis of nucleic and amino acids, including methylation of dUMP to dTMP. DHFR inhibitors act by halting synthesis of DNA, RNA, and proteins, thereby arresting cell growth. DHFR is an important target for drug development against cancer and a variety of infectious diseases caused by bacteria, protozoa, and fungi. The wealth of the knowledge acquired on inhibitors of this enzyme resulted relatively early—in the 1950s and 1960s—in the well-known anticancer, antibacterial, and antimalarial drugs, for example, methotrexate (MTX), trimethoprim (TMP), and pyrimethamine (PYR) (Chart 1). The recognition of the importance of these enzymes and the routine availability of protein crystallography led to an explosion of information of 3-D structures of DHFRs from human, protozoal, fungal, and bacterial sources with numerous ligands and cofactors bound in their active centers. Over 100 coordinates of 3-D structures of this enzyme with substrate, cofactor, and a wide variety of inhibitors are deposited in the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/).

In discussing perspectives of enzyme catalysis, S. Benkovic and S. Hammes-Schiffer have recently reviewed the kinetics of the DHFR reaction.[19] R. Kisliuk has studied the synergistic interactions in mammalian cells between anticancer antifolates, particularly inhibitors of DHFR, TS, and glycinamide ribonucleotide formyltransferase (GARFT)[20] and observed significant synergistic cytotoxicity in many human cell lines, dependent on folate levels and polyglutamation.

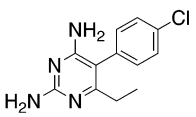
Chart 1. Reference Compounds



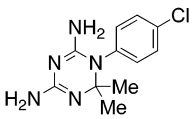
Trimethoprim (TMP)



Methotrexate (MTX)



Pyrimethamine (PYR)



Cycloguanil (CYC)

The folding mechanisms of human DHFR, DHFR from *Escherichia coli*, and DHFR from *Lactobacillus casei* have been studied. Despite less than 30% pairwise sequence identities, folding to the native state occurs via parallel folding channels, and conservation of the fast-, intermediate-, and slow-folding events provides convincing evidence for the hypothesis that evolutionarily related proteins achieve the same fold via similar pathways.[21]

2.2.1. Mammalian DHFR

In contrast to microbial DHFRs, mammalian DHFRs are highly conserved. A number of 3-D structures of DHFR complexes have been described.[22] The active site is somewhat larger than that of bacterial DHFRs and provides poorer specificity for contacts between TMP and surrounding residues.[23] In human DHFR, the pteridine ring of MTX binds in an inverse orientation as compared to the bound folate found in other DHFRs. TMP, which is remarkably species-specific, binds in a different conformation as compared to its orientation in the *E. coli* enzyme, and the trimethoxyphenyl group occupies the upper cleft of two hydrophobic pockets. The 4-amino group makes one hydrogen-bond, in contrast to two hydrogen bonds in the bacterial enzymes.[22]

Expression levels of DHFR play a significant role in MTX resistance in certain types of acute lymphoblastic leukemia (ALL) in children. Elevated levels correlate with MTX-resistance in T-cell ALL.[24] A study of the expression of hamster DHFR minigenes showed that expression in CHO cells was significantly higher in the presence of DHFR intron 1. The protein encoded by the intronless construct was also unstable, subject to lysosomal degradation, and had a shorter half-life, suggesting that the DHFR intron 1 plays an important role.[25]

2.2.2. Bacterial DHFR

Close to 60 3-D structures of bacterial DHFRs, either as apoenzymes or complexed with folate, NADPH, or various other ligands or inhibitors, are present in the protein data bank. The tight binding of TMP to the active site results from close fitting and optimal interatomic contacts between the drug and the enzyme. The X-ray structure of enzyme from the causative tuberculosis agent, *Mycobacterium tuberculosis*, was solved recently by Li and coworkers.[9] The binary complex with NADP and ternary complexes with NADP and different inhibitors were determined at 1.7−2.0 Å resolution. Despite only 26% sequence identity with human DHFR, the overall fold is similar, but there are significant differences that can be exploited for the design of specific inhibitors.

2.2.3. Other DHFRs

In recent years, the DHFR from *Pneumocystis carinii* has received significant attention because this pathogen is often associated with AIDS and other immunodeficiencies. The 3-D structure of this enzyme has been solved.[26] The active site of *P. carinii* DHFR is intermediate in size between those of *E. coli* and vertrebrate DHFRs. TMP has sufficient van der Waals interactions between the trimethoxybenzyl group and the enzyme or cofactor to stabilize the ligand in the "bacterial" mode of binding.[23]

The nomenclature for this organism has now been revised because this pathogen is now known to be species-specific. *Pneumocystis jiroveci* now refers to the pathogen found in human hosts, and *P. carinii* or *P. carinii* f. sp. *ratti* refers to the variants found in rats.[27] Most of the published studies on *Pneumocystis* DHFR, including the crystal structure, used the enzyme derived from the variant found in rats. [27]

Apart from *Pneumocystis*, where antifolates are clinically used both as therapy and prophylaxis, no antifolates are used as therapy against yeasts and filamantous fungi. Nevertheless, DHFR is considered a valid target for antifungal drug discovery and DHFR from *Candida albicans* has been crystallized.[28] Complexes with several 5-arylthioquinazolines, **3**, and NADPH were analyzed and two distinct modes of binding were reported. The most selective compounds were found to bind in an unusual mode, displacing the dihydronicotinamide portion of NADPH from its normal position within the enzyme active site.

2.3. Dihydropteroate Synthase

Dihydropteroate synthase (DHPS, E.C. 2.5.1.15), the target of sulfonamides and sulfones, has received less attention than DHFR. Nonetheless, close to 170 nucleotide sequences of the DHPS gene (*fol*P) from several organisms have been determined, including those from *E. coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *M. tuberculosis*, and *P. carinii*. More than 200 partial or complete protein sequences have been deposited, either for a monofunctional DHPS or as part of a larger, folic acid synthesis protein. The DHPS from *S. aureus* has been crystallized and the 3-D structure solved as the apoenzyme and a binary complex with the substrate analogue hydroxymethylpterin pyrophosphate at 2.2 and 2.4 Å, respectively.[29] In common with other eukaryotic DHPSs, the enzyme is a homodimer in solution and only a single molecule of the substrate analogue hydroxymethylpterin pyrophosphate is bound per dimer. The 3-D structure of *E. coli* DHPS was also solved in the same year.[30] Moreover, a 1.7 Å resolution crystal structure of the DHPS from *M. tuberculosis* complexed with 6-hydroxymethylpterin monophosphate is also available.[31] All three enzymes belong to the "TIM barrel" proteins with eight α-helices surrounding a central barrel composed of eight parallel β-strands. To date, about 10 3-D structures of DHPS or its complexes with ligands have been solved.

DHPS is usually expressed as a monomeric protein, located in the cytoplasm, although in certain organisms, for example, higher plants and fungi, it can be part of a bifunctional or trifunctional folate biosynthesis enzyme.[32]

2.4. Thymidylate Synthase

Thymidylate synthase (E.C. 2.1.1.45) drives the thymidylate cycle with the consumption of N^5,N^{10}-methylene tetrahydrofolate. The dihydrofolate generated has to be reduced by DHFR (see Scheme 1). The TS cycle is the sole de novo pathway for the synthesis of dTMP. Complete blockade of TS ultimately leads to "thymineless death.[33] As a key enzyme in general metabolism and an important target for anticancer agents, TS has been extensively studied. Reviews on its structure, mechanism, and inhibition,[34−39] its use as a target for chemotherapy,[40] or its function as a translational regulator[41] have been recently published. Close to 200 complete or partial sequences of TS genes and more than 100 3-D structures are currently deposited in the Protein Data Bank. The native enzyme is a symmetrical dimer of structurally similar subunits. Overall folding displays a series of eight α-helices, 10 strands of β-subunits, and several segments of coils that connect the secondary structural elements.[41] The TSs studied exhibit striking structural homologies, and so far there are no appropriate selective inhibitors known for TS from bacteria or protozoa. However, this situation could alter based on the recent discovery of thymidylate synthase complementing proteins in a number of bacteria that exhibit a different kinetic and molecular mechanism. These new proteins could be interesting targets for selective inhibitors (see also section 2.12.).[42,43]

2.5. Bifunctional DHFR−TS

Apicoplast parasites, such as *Plasmodium falciparum* (and other *Plasmodium* species such as *Pl. vivax*, *Pl. malariae*, *Pl. ovale*), *Toxoplasma gondii*, *Leishmania major*, *Trypanosoma cruzi*, or *Cryptosporidium parvum*, express a bifunctional DHFR−TS enzyme coded by a single gene, in contrast to the host, where both enzymes are separate gene products. The *Pl. falciparum* DHFR−TS, for example, is a polypeptide of 608 amino acids, of which the first 231 residues constitute the DHFR domain. [44] A junction region of 89 residues separates the DHFR domain from the TS domain, which is composed of 288 residues. This junction is absent in the bifunctional DHFR−TS enzyme of *Leishmania*.

As with the plasmodia, *Tr. cruzi*, the parasite that causes Chagas' disease, harbors a bifunctional DHFR−TS enzyme. Its crystal structure has not yet been solved, but a homology model has been used for inhibitor design.[45]

The DHFR−TS from *Babesia bovis*, an apicomplexan parasite of cattle, was recently sequenced and cloned.[46] It contains a moderately conserved 5′-end DHFR domain (190 aa), a nonconserved linker region (33 aa), and a highly conserved 3′-end TS domain (288 aa).

Several theoretical models have been used for inhibitor design.[47−50] These include homology modeling of the enzymes from both *Pl. falciparum* and *Pl. vivax*.[46]

Recently, crystal structures of the plasmodial DHFR−TS have been solved for the wild-type and a quadruple mutant complexed with NADPH, dUMP, and either PYR or WR99210.[51] Despite the presence of truncated fragments resulting from proteolysis, these studies provide a number of interesting insights on the junction region linking the two domains, the structure of the DHFR domain, and the possible roles of the two *Pl. falciparum*-specific insert regions, as well as on the binding and orientation of the inhibitors. The flexible WR99210 side chain adopts a conformation that can still bind to the mutant active site, in contrast to the more rigid cycloguanil or related structures. All this information would be useful in the design of new inhibitors able to overcome PYR resistance.[43]

The crystal structure of DHFR/TS from *Cryptosporidium hominis* (previously *Cr. parvum*) revealed a novel architecture of the bifunctional enzyme.[52] The unique linker domain with an 11 residue α-helix controls the relative orientation of the DHFR and TS domains, which is different in the apicomplexans (with *Cr. hominis*, *Toxoplasma*, *Plasmodium*) and the kinetoplastids (with *Leishmania* and *Trypanosoma*). The tertiary structure of the linker domain has therefore been used in the classification of protozoa.[51]

2.6. GTP-Cyclohydrolase I

GTP-Cyclohydrolase I (GTP-CH-I) (E.C. 3.5.4.16) catalyzes the first step in the synthesis of dihydroneopterin triphosphate and tetrahydrofolate from GTP in bacteria, plants, or animals. The reaction mechanism appears rather complex and poorly understood although a number of reaction mechanisms have been proposed.[53−55] The structures of the GTP-CH-I from *E. coli* and humans have been solved. These studies identified the key role of a zinc ion in human and bacterial GTP-CH-I and provide a much better understanding of the reaction mechanism.[54] In the rat enzyme, zinc binds to the conserved Cys-132, His-135, and Cys-203. In *Pl. falciparum*, the gene for GTP-CH-I is located on chromosome 12 along with the genes of other folate pathway enzymes.[56]

As the first enzyme in folate pathway, GTP-CH-I would constitute an interesting target for selective inhibitors. Although the amino acids involved in substrate binding and catalysis and the role of zinc seems to be identical in the *E. coli* and the human enzymes,[54] there are sufficient differences between these enzymes that could be exploited for the design of selective inhibitors. For example, the sequence identity between human and *E. coli* enzyme is only 37%, and the human enzyme lacks the N-terminal region. Similarly, in contrast to the bacterial enzyme, the mammalian enzyme, which plays a key role in the biosynthesis of tetrahydrobiopterin, is regulated by feedback inhibition. A ternary complex between GTP-CH-I, tetrahydrobiopterin, and an auxiliary protein (GFRP, GTP-CH feedback regulatory protein) is formed.[54]

2,4-Diamino-6-hydroxypyrimidine is a prototypic inhibitor of GTP-CH-1 and exerts a dual mechanism of inhibition.[57] At low concentrations, it competes with tetrahydrobiopterin and is part of the GFRP system, while at higher concentrations, it directly competes with the substrate GTP. It has been widely employed as a tool in the study of tetrahydrobiopterin, which is an essential cofactor of nitric oxide synthase and aromatic amino acid hydroxylases.[54] This compound was recently shown to exert a positive effect in rat postburn *Staphylococcus aureus* sepsis.[58] At present, we are not aware of any current efforts aimed at designing more potent and selective inhibitors of this enzyme for use as antimicrobial agents.

2.7. Dihydroneopterin Aldolase

Dihydroneopterin aldolase (DHNA) (E.C.4.1.2.25) catalyzes the conversion of 7,8-dihydroneopterin to 6-hydroxymethyl-7,8-dihydropterin. The enzyme from *S. aureus* has been purified after expression in *E. coli* and crystallized.[59] The X-ray structure at 1.65 Å resolution and the binding site of 6-hydroxymethyl-7,8-dihydropterin have been determined. The protein forms an octamer of 110 000 Da molecular weight. The crystal structure of the homo-octameric protein has been solved, and it has been shown that the folding topology, quaternary structure, and amino acid sequence is similar to that of the 7,8-dihydroneopterin triphosphate epimerase.[60] The vibrational structure of 7,8-dihydrobiopterin, an inhibitor of DHNA, has been studied by Raman difference spectroscopy[61] and the stereochemistry of the reaction by using deuterated buffer.[62] The gene for DHNA in *Pl. falciparum* has not yet been identified.[56]

Although repeatedly discussed as a potentially interesting target, only limited efforts have been directed toward the design of new DHNA inhibitors. A high-throughput screen carried out in the Roche group in the 1990s yielded a number of hits with moderate activity, but the potency of these could not be substantially improved. Highly functionalized 6-substituted pteridines were recently prepared as potential modulators of tetrahydrobiopterin activity or DHNA inhibitors, but no biological results have been reported.[63]

2.8. 6-Hydroxymethyl-7,8-dihydropterin Pyrophosphokinase

6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (E.C.2.7.6.3, HPPK) catalyzes the transfer of pyrophosphate from ATP to 6-hydroxymethyl-7,8-dihydropterin. Because this enzyme is absent in mammalian cells, it is a potential target for selective antimicrobial agents, but no useful inhibitors for this enzyme are currently known. The mechanism of HPPK-catalyzed pyrophosphoryltransfer and the crystal structure of the *E. coli* HPPK 3D-structure, in complex with one and two product molecules have been recently described.[64]

2.9. 7,8-Dihydroneopterin Triphosphate Epimerase

This epimerase is not shown in the Scheme 1 because no EC-number is available yet (A. Bacher, personal communication). The enzyme catalyzes the epimerization of carbon 2′ in the triphosphates of dihydroneopterin and dihydromonapterin. It can also slowly cleave the side chain in the position 6 of

several pteridines. Since a deletion mutant of *E. coli* exhibited normal growth properties, the physiological role of the *E. coli* epimerase remains unknown.[65] The enzyme from *E. coli* has been crystallized.[60]

2.10. Serine Hydroxymethyltransferase

Serine hydroxymethyltransferase (SHMT) (E.C. 2.1.2.1) is a pyridoxal-5′-phosphate-dependent enzyme.[66] SHMT reversibly interconverts serine and glycine with THF as the one-carbon carrier. SMHT exists as a dimer in prokaryotic organisms but forms a tetramer from obligate dimers in eukaryotic cells.[12,67] There are about 10 entries in the protein data bank for X-ray structures of SHMTs isolated from different bacteria, animals, or man. The enzyme is complexed either with glycine, serine, or 5-formylTHF. The genome analysis of *M. tuberculosis* indicated two putative SHMT enzymes, SHM1 and SHM2; recombinant proteins exist as homodimers under physiological conditions. In contrast to the usual stoichiometry of 2 mol of pyridoxal-5′-phosphate (PLP) per enzyme dimer, which applies to SHM2, only 1 mol of PLP is bound per enzyme dimer in SHM1.[68] The role of proline residues in the folding of SHMT has been studied with the *E. coli* enzyme.[69] The structure−function relationship in SHMTs was recently reviewed.[12]

Human SHMT is considered a target for anticaner drugs,[70] but there are currently no useful potent and selective inhibitors known. The triazine antifolate NSC 127755 has been found to inhibit also SHMT from myeloma cells with an IC_{50} of 50 nM.[71] Inhibition of SHMT, in addition to DHFR, may therefore contribute to its cytotoxic effects on tumor cells.

2.11. Multifunctional Folic Acid Synthesis Proteins

In some organisms, a number of enzymes in the folate pathway form a multifunctional protein. For example in *P. carinii*, the folic acid synthesis protein (Fas) contains dihydroneopterin aldolase, 6-hydroxymethyl-7,8-dihydroneopterin pyrophosphokinase, and dihydropteroate synthase.[72,73] Single amino acid substitutions in the FasAB, such as FasAB-Met23, result in a loss of DHNA activity and the ability to form stable tetramers.[74]

In *Pl. falciparum*, the dihydrofolate synthetase and folylpolyglutamate synthetase form a single protein,[56] as do hydroxymethyl dihydropterin pyrophosphokinase and DHPS.[75] The last two steps in purine biosynthesis in man are catalyzed by the bifunctional enzyme aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP cyclohydrolase; the crystal structure in complex with several inhibitors has been solved[76] (see Scheme 1).

2.12. Recent Discoveries in the Folate Pathway

The observation that certain bacteria, such as *Helicobacter pylori*, did not contain a ubiquitous enzyme like DHFR has for some time puzzled biologists (see http://www.genome.ad.jp/dbget-bin/get_pathway?org_name=hpj&mapno=00790). The explanation for this unusual finding has been provided recently. Myllykallio and co-workers,[77] in an elegant piece of postgenomic work, demonstrated that a number of pathogenic eubacteria, such as *Helicobacter pylori*, *Campylobacter jejuni*, or *Treponema pallidum*, and some archaebacteria lacked DHFR. These organisms also lack the classical thymidylate synthase gene, *thyA*. Instead, they express a new protein with thymidylate synthase function, named ThyX. ThyX and ThyA use different reductive mechanisms, ThyX being dependent on reduced flavin nucleotides, whereas ThyA uses tetrahydrofolate. Occasionally, both *thyA* and *thyX* were found in the same organism, for example in *M. tuberculosis*; the functional consequences remain unclear. Since there is no corresponding gene or protein in man, ThyX could be an attractive target for new and selective inhibitors for a number of important pathogens.[42,77,78]

Archaeabacteria usually perform C1-transfer reactions either by modified folates or in the absence of folates. By contrast to many archaea, *Haloferax volcanii*, an extremely halophilic archaeon, is significantly different in that it is sensitive to TMP. Search for a *dhfr* gene actually revealed two distinct *dfhr* genes, *hdrA* and *hdrB*. The *hdrB* gene is linked to the gene for TS in a single transcription unit, as in *Bacillus subtilis*. hDHFR-1 and hDHFR-2 share about 38.8% amino acid sequence identity and 56% identity at the nucleotide level. They differ considerably in stability and pH-optimum. The *hdrB* gene alone can support the growth of *H. volcanii* in minimal medium, whereas *hdrA* can support growth only in the presence of thymidine.[79] Another archaeon, *Thermus thermophilus*, also lacks a classical DHFR (no *dyrA* gene was detected in its genome). Instead, a dihydropteridine reductase (DH_{Tt}), related to other short chain dehydrogenase/reductases (SDR) is present. It is insensitive to inhibition by MTX and TMP and displays considerable DHFR activity (at 20% of the DHPR activity detected with $qPtH_2$). [80]

It has been known for some time that *folA*-containing deletion mutants of *E. coli* can grow in the presence of thymidine. This implied that another enzyme could probably carry out the de novo synthesis of tetrahydrofolate. A candidate for this function was identified in *E. coli* recently. Once more, this enzyme is a member of the SDR family, related to the trypanosomatid pteridine reductases, and is able to reduce dihydrobiopterin and dihydrofolate. This enzyme is resistant to TMP but sensitive to inhibition by MTX. The gene coding for this enzyme, *ydgB*, was renamed *folM*, and the protein called FolM (Table 1).[81] A BLAST search with the *folM* sequence as the query identified a number of bacteria, showing that this gene is widespread.

Trypanosomatid protozoans depend on exogenous pteridines or folates for growth, and these essential nutrients are accumulated by a specific folate and a specific biopterin transporter.[82] A broad spectrum pteridine reductase, PTR1, was recently discovered, which is able to reduce both pteridines and folate.[83] The enzyme is essential and related to SDR and forms tetramers from 30-kDa subunits. Compared to DHFR−TS, it is less sensitive to inhibition by MTX. The ability of different folate and pteridine substrates

Table 1. New Folate Pathway Enzymes

enzyme	E.C. number	host	function	ref
FolM	E.C. 1.5.1.34	*E. coli*, other bacteria	dihydropteridine reductase/dihydrofolate reductase; insensitive to TMP, sensitive to MTX	81
PTR1	E.C. 1.5.1.33	*Leishmania major*, *Crithidia*, other trypanosomatid protozoans;	broad spectrum pteridine reductase essential in pteridine salvage, reduces folates and pteridines; resistant to MTX	83, 84
QDPR	E.C. 1.5.1.34	*Leishmania major, T. brucei*, *T. cruzi*	regeneration of H4-biopterin	85
DH_{Tt}	E.C. 1.5.1.34	*Thermus thermophilus*	dihydropteridine reductase/dihydrofolate reductase; insensitive to TMP and MTX	80
hDHFR-2	E.C. 1.5.1.3	*Haloferax volcanii*	2nd DHFR besides hDHFR-1; can maintain THF-pool but not recycle thymidine	79
ThyX	E.C. 2.1.1.148	*H. pylori, Cam. jejuni*, *B. burgdorferi*, some archaea	alternative thymidylate synthase, using reduced flavin as reductant	77

to support growth of *Leishmania* correlated with their substrate properties for PTR1.[84] A separate enzyme, a quinoid-dihydropteridine reductase (QDPR), is found to maintain the H4-biopterin pool; it is resistant to common antifolates.[85]

Gene expression of several folate pathway enzymes in eukaryotes, such as DHFR, SHMT, and TS is controlled by translational autoregulation. Besides binding its cognate mRNA, TS is found to bind other mRNAs.[41] Regulation is different in DHFR from *Pl. falciparum* and man, and this contributes to selectivity for inhibitors.[86]

3. Impact of Bioinformatics

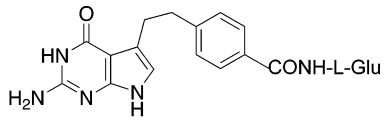
The number of sequenced genomes from bacteria, parasitic protozoa, and other human pathogens is constantly growing at a rapid pace. There are currently around 170 microbial genomes accessible in public databases, such as EMBL-EBI, GenomeNet, KEGG, or the NCBI Entrez Genome Data Base. This information allows easy comparison of the degree of conservation, the differences between taxa, and differences between host and parasite enzyme and thus greatly improves the drug discovery process. Detection of new enzymes and their function, as listed under 2.11 and 2.12, was greatly aided by applying these tools.

Most important information for drug discovery, however, is deduced from three-dimensional structures of the targeted proteins. They reveal information on inhibitor binding, conformational changes, enzyme−inhibitor−cofactor complexes, and exploitable differences between the parasite and host enzyme.

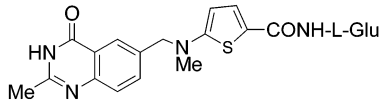
4. New Drugs and Drugs in Development

In recent years, DHFR as drug target for new antimicrobial agents has received little attention in big pharmaceutical companies. A number of academic institutions, however, actively pursue antifolate projects.[87] In addition to the established antifolate drugs (Chart 1), new antifolates have reached the market recently (Chart 2). Several new investigational drugs are at different stages of development

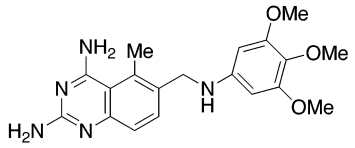
Chart 2. Marketed Antifolates



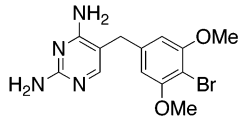
Pemetrexed (Alimta®)



Raltitrexed (Tomudex®)



Trimetrexate (TMX, NeuTrexin®)

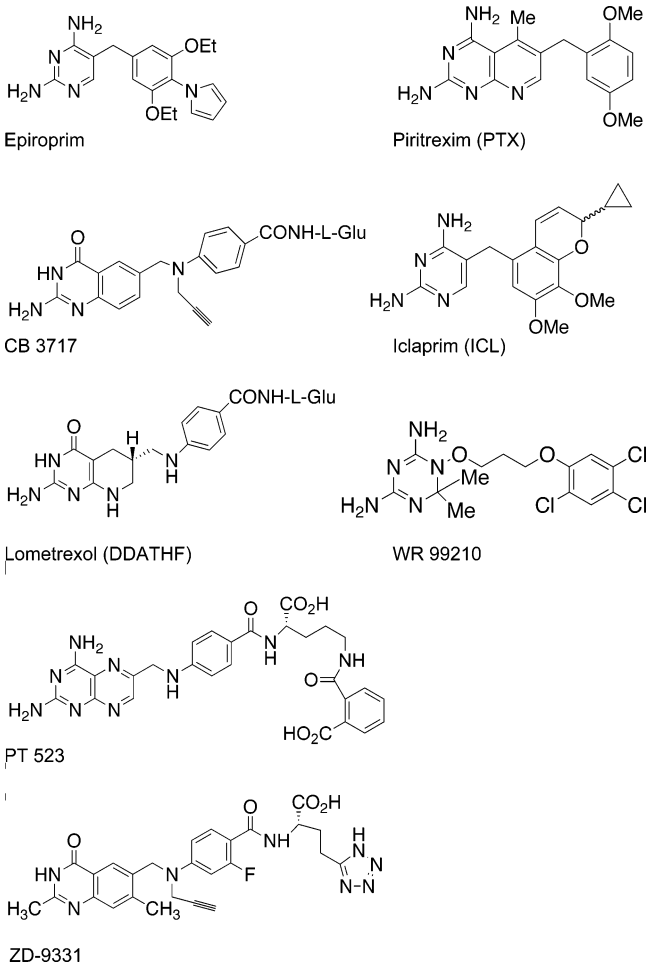


Brodimoprim

(Chart 3). The development status of several new drugs against malaria has been reviewed in 2003.[88] Antifolates in clinical development for treatment of cancer until 1997 have been reviewed by Takimoto.[89] More recent reviews on new antifolates in development include those by Purcell and Ettinger [90] and McGuire.[13]

Pemetrexed. Pemetrexed disodium (Alimta, LY231514) is a novel antifolate for use in oncology. Several tumor types were found to respond to pemetrexed in clinical trials, such as mesothelioma, non-small cell lung cancer, and colon, pancreatic, and breast cancers. Its properties have recently been reviewed.[91−94] It is called a multitargeted antifolate (MTA) because it inhibits de novo pyrimidine and purine pathways by TS, DHFR, glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase. It becomes fully active after polyglutamation, the polyglutamate being 60-fold more active against TS than

Chart 3. Investigational Drugs



the monoglutamate, whereas polyglutamation has no effect on its activity against DHFR. The compound was developed by Eli Lilly and approved in February 2004 by the FDA as the first treatment of malignant pleural mesothelioma, a condition usually associated with asbestos exposure. It is used in combination with cisplatin.[95]

Raltitrexed. Raltitrexed (Tomudex, ZD1694) is a selective TS inhibitor developed by AstraZeneca. It is transported into the cells by the reduced folate carrier where it is extensively polyglutamated. The compound has successfully completed clinical trials and was first launched in the U.K. in 1996 and is available in a number of countries for the intravenous treatment of colorectal cancer. Raltitrexed extends the range of tumors that are responsive to antifolates as MTX is ineffective in colon cancer. A. L. Jackman et al. have recently reviewed the development of Tomudex (ZD1694).[96]

Trimetrexate. Trimetrexate (NeuTrexin) has been approved for the treatment of *P. carinii* pneumonia in 1993. It is used as the glucuronate salt for intravenous application. Due to its inherent toxicity co-administration of leucovorin is mandatory (NeuTrexin product information).[97]

Piritrexim. Since 1998, piritrexim isethionate has been granted an orphan drug status for the treatment of infections caused by *P. carinii*, *T. gondii*, and *Mycobacterium avium-intracellulare*. Clinical trials in several cancers, such as MTX-resistant tumors or bladder carcinomas, are currently ongoing.[98,99]

ZD-9331. ZD-9331 is a nonpolyglutamable TS inhibitor in development by AstraZeneca for treatment of solid tumors. It is a potent inhibitor of various cancer cell lines at submicromolar concentrations.[100] In contrast to raltitrexed, it freely effluxes across the plasma membrane. It may have advantages over raltitrexed in ovarian cell lines expressing low folylpolyglutamate synthetase (FPGS) mRNA. Both intravenous and oral formulations are in clinical trials. The NDA submission is foreseen in 2004.

PT 523. A side chain modified analogue of aminopterin, N^{α}-(4-amino-4-deoxy-pteroyl-N^{δ}-hemiphthaloyl-L-ornithine (PT 523) has been synthesized in Rosowsky's group. It is 10−100-fold more potent than MTX against a large number of human cancer cell lines in culture. Its affinity for the transporter responsible for uptake of folates (RFC) is about 10-fold higher than that of MTX. Because PT 523 lacks a glutamate side chain, it is not a substrate for FPGS and cannot be polyglutamated once it enters the cell. The compound is in Phase I/Phase II clinical trials.[101,102]

Lometrexol. Lometrexol (DDATHF) is an antipurine antifolate that selectively inhibits GARFT. It is a good substrate for FPGS. It is in clinical studies as an anticancer agent, for example, against melanomas, renal cell carcinomas, and other cancers. Due to high accumulation after polyglutamation, it exhibits considerable systemic toxicity, which could be reduced by oral coadministration of folic acid or folinic acid.[103] This and other similar compounds have been reviewed by Purcell and Ettinger.[90]

Brodimoprim. This close analogue of trimethoprim was developed for single therapy for respiratory tract infections.[8,11] In contrast to TMP, it has a long elimination half-life of about 30 h, allowing once daily treatment. The compound has been commercialized in 1993 but did not find wide acceptance. In 2000, it was withdrawn from the market.

Iclaprim. Iclaprim (ICL, AR-100), a novel diaminopyrimidine, resulted from a program aimed at new broad-spectrum DHFR inhibitors with increased potency against Gram-positive bacteria at F. Hoffmann-LaRoche in Basel. The compound has been licensed and is under development by ARPIDA Ltd. ICL exhibits excellent activity against staphylococci, including most of the TMP-resistant variants.[104] The antimicrobial properties and mode of action of ICL[91] have been first presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy in 2002 and 2003 and later published.[105] The agent is targeted as monotherapy for serious hospital infections, particularly those by methicillin-resistant staphylococci. ICL also demonstrates good activity against respiratory tract pathogens, for example, pneumococci, including penicillin-resistant strains. In 2003, it successfully completed Phase II clinical trials for complicated skin and skin structure infections in hospitalized patients.

Epiroprim. A diaminopyrimidine with attractive properties for the treatment of Gram-positive infections or infections by several opportunistic proto-

zoa,[106,107] epiroprim is one of the most active DHFR inhibitors against *Mycobacterium ulcerans* and exerted synergism when combined with dapsone.[108] Epiroprim is also synergistic with dapsone against *Mycobacterium leprae* in vivo.[109] To the best of our knowledge, there is no development work ongoing with this compound.

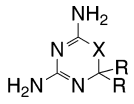
Pyrimethamine/Dapsone (Maloprim). This combination is used together with chloroquine for prophylaxis against malaria in certain areas with a high risk of chloroquine resistance.[110]

Chlorproguanil/Dapsone (Lapdap). This synergistic combination of a DHFR inhibitor, chlorcycloguanil, and a DHPS inhibitor, dapsone, has undergone clinical trials in Africa and has been approved in the U.K. in 2003. The development of a fixed triple combination, chlorproguanil/dapsone/artesunate, is under way.[88]

WR 99210. The DHFR inhibitor WR 99210 has been developed as a promising antimalarial agent as far back as 1973. Clinical evaluation of this drug has been hampered by its gastrointestinal intolerance.[111]

5. Inhibitors of Folate Pathway Enzymes

All DHFR inhibitors exhibiting IC_{50}'s in the micromolar range or less contain the 2,4-diamino-1,3-diaza pharmacophore **1**. Therefore, a primary classification



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of antifolates according to their structures appears impractical. The authors have therefore opted to organize their efforts primarily on the search for inhibitors of a potential target, medical indication, or pathogenic organisms and only then in terms of the structure of the compounds. Therefore throughout the current review, after the description of the general aspects of screening and methodology, the new antifolates will be presented according to their targeted medical utility.

In the literature, a distinction is frequently made between classical and nonclassical antifolates. "Classical" antifolates are structural analogues of the substrate folic acid and thus bear an acidic moiety in the distal part of the molecule. They are, as a rule, substrates for folylpolyglutamate synthetase (FPGS). "Nonclassical" antifolates are lipophilic analogues of the substrate molecule, lacking the glutamate portion, or its surrogates, of the folate molecule. They are, therefore, not substrates for FPGS.

5.1. Screening and Methodology

It seems to be an economic and logical procedure during design and synthesis of new antimicrobial antifolates to test them against multiple enzymes rather than a single enzyme or organism. This approach has been used by the Roche group, evaluating potential new antifolates against the wild-type and resistant *E. coli* and *S. aureus* DHFR, the *P. carinii* DHFR, a pneumococcal DHFR, and human DHFR for determining the selectivity.[112] Similar approaches have been used by other groups, for example, A. Rosowsky, et al., using DHFRs of *T. gondii*, *P. carinii*, and *M. avium*. Simultaneous testing of new inhibitors was greatly improved with an elegant genetic assay in which the DHFR from *Saccharomyces cerevisiae* has been replaced by the DHFRs from *Pl. falciparum*, *T gondii*, *P. cariniii*, *Cryptosporidium parvum*, or humans.[113,114]

DHFR from *M. tuberculosis* is also considered an important target for drug design.[115] Since *M. tuberculosis* is a slowly growing microorganism and dangerous to handle, a simple in vitro screen with *Sac. cerevisiae* expressing the *M. tuberculosis* DHFR has been used for the selection of new DHFR inhibitors. Recombinant wild-type and mutant *Pl. falciparum* DHFR, derived from a synthetic gene of the DHFR domain of the bifunctional *pf*DHFR−TS, has been used for the screening of new inhibitors with activity against *Pl. falciparum*.[116]

5.2. Antibacterials

In recent years, only limited efforts have been directed at DHFR inhibitors for common bacteria. Kuyper et al. have synthesized conformationally restricted analogues of trimethoprim, designed to mimic the conformation of the drug observed in its complex with bacterial DHFR. The restriction has been achieved by linking the 4-amino group with the methylene of TMP by one- and two-carbon bridges. Of the three analogues prepared, 2-amino-4-methyl-5-(3,4,5-trimethoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine shows good activity and inhibits four bacterial DHFRs with a potency similar to TMP. However, it is significantly less selective than the reference compound.[117] Efforts aimed at identification of selective and potent DHFR inhibitors for Gram-positive bacteria, particularly *S. aureus*, yielded iclaprim, which is much more active than TMP against both wild-type and TMP-resistant *S. aureus* strains (see Chart 3, Investigational Drugs). The development of even more potent and selective inhibitors failed, largely because of poor solubility and high protein binding of these inhibitors.[87]

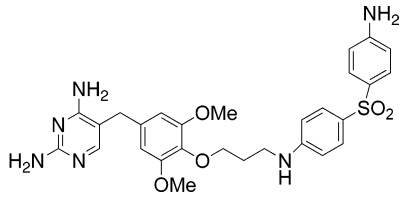
The design of antifolates with activity against protozoal pathogens, often results in interesting activities against the *Mycobacterium avium* complex (MAC).[118] *M. avium* as a target organism for new DHFR inhibitors has also been pursued in a novel approach to use pharmacophores in a series of 2,4-diamino-5-deazapteridines.[119]

Mycobacterium tuberculosis and particularly the multiresistant strains pose emerging threats to public health in many countries, and new drugs are urgently needed. No DHFR inhibitor is currently used for tuberculosis treatment. Epiroprim has recently been evaluated against various *M. tuberculosis* strains and been found to exhibit weak activity. It exhibits synergy with isoniazid (INH) in INH/rifampicin-sensitive strains and prevents development of INH resistance. More potent inhibitors of the *M. tuberculosis* DHFR would be needed to become useful chemotherapeutic agents. The triazine DHFR inhibitor WR99210 has been shown to exhibit rea-

sonable in vitro activity against *M. tuberculosis*, as well as against other mycobacteria. This structural class has been further pursued using a genetically modified *Sac. cerevisiae* strain for screening. [115] The selectivity of WR99210, however, is not sufficient for use as an antimicrobial agent because it shows poor gastrointestinal tolerance. A series of analogues have been synthesized and tested and show activity that is comparable to the lead molecule.

The three-dimensional structure of the *M. tuberculosis* DHFR complexed with several inhibitors has been solved, and the enzyme has been characterized.[9,120] Although the general fold of the protein is essentially the same as that of the human enzyme, a number of differences were detected, which could be exploited for inhibitor design.

A diaminopyrimidine covalently linked to dapsone, named K-130, has been shown to potently inhibit the

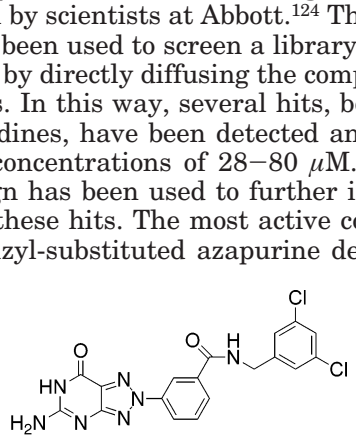


K-130

DHFR from *Mycobacterium lufu*, a model organism, and to exhibit good in vitro and in vivo activity against *M. lufu*, and *M. leprae.*[121]

A series of 2,4-diamino-5-deazapteridine derivatives[122,123] exhibit significant activity against recombinant *M. avium* DHFR with IC_{50}-values in the nanomolar range and correlating in vitro activities with minimum inhibitory concentrations (MICs) below 0.13 μg/mL. Many of these possess selectivity ratios of >100 against the human DHFR. However, these compounds are significantly less potent against *M. tuberculosis* in vitro. Although these data are encouraging, an improved selectivity ratio would be required for therapeutic applications.

A new approach for the discovery of inhibitors of DHNA as potential antibacterial agents has been undertaken by scientists at Abbott.[124] The *S. aureus* DHNA has been used to screen a library of ~10 000 compounds by directly diffusing the compounds into the crystals. In this way, several hits, both purines and pyrimidines, have been detected and inhibited DHNA at concentrations of 28−80 μM. Structure-based design has been used to further improve the potency of these hits. The most active compound, a dichlorobenzyl-substituted azapurine derivative, **2**,



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exhibited an IC_{50} of 68 nM against the enzyme. None of the compounds, however, inhibit common bacteria in vitro, including hypersusceptible strains of *E. coli* and Acr$^-$ efflux pump mutants of *E. coli* and

Table 2. Inhibition of DHFR and in Vitro Antifungal Activity of Reference Compounds[127]

compd	DHFR IC_{50} (μM) *C. albicans*	DHFR IC_{50} (μM) human	selectivity ratio	*C. albicans* MIC (μg/mL)
TMP	50	490	10	>50
PYR	5.0	2.6	0.5	>50
TMX	0.04	<0.001	<0.03	>50
PTX	0.004	0.002	0.005	>10
3a	0.05	>10	>200	1.0
3b	0.13	70	540	>50
3c	0.03	3.1	100	0.25
3d	0.05	2.0	40	0.25
3e	0.06	0.32	5.0	2.5
3f	0.057	0.82	14	1.0
3g	0.008	2.0	250	0.10

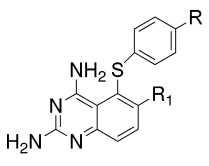
Haemophilus influenzae. It has been suggested that these compounds were not potent enough to compete successfully with the intracellular substrate levels, but the reasons for this lack of in vitro activity remain to be explained.

In contrast to earlier findings recent studies on the mechanism of action of sulfonamides in yeast indicate that sulfa-analogues formed via DHPS are active and compete with dihydrofolate. The precise in vivo target of sulfa-dihydropteroate remains to be determined, but this finding may lead to novel antifolate inhibitors.[125,126]

5.3. Antifungals

So far, no effective antifungal agent based on inhibition of DHFR is used in therapy. The known inhibitors are neither sufficiently potent nor sufficiently selective.

The search for DHFR inhibitors as antifungal agents has been undertaken in the former Wellcome Research Laboratories.[127] Several compounds belonging to the class of 5-(arylthio)-2,4-diamino-quinazolines have been identified as potent inhibitors of *C. albicans* DHFR. The compounds are up to 540-times less active against human DHFR, and most of the selected compounds are also good inhibitors of *C. albicans* cell growth (Table 2). The most selective inhibitor, compound **3b**, though showing a good

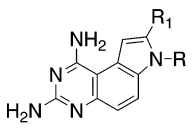


3

Compd	R	R_1
3a :	cyclohexyl	H
3b :	morpholino	H
3c :	Br	H
3d :	F	H
3e :	NH_2	H
3f :	*t*-Bu	OEt
3g :	*t*-Bu	H

selectivity index of 540 shows a poor MIC of >50 μg/mL against *C. albicans*.

A series of 7,8-dialkylpyrrolo[3,2-*f*] quinazolines, **4**, have been evaluated as inhibitors of *C. albicans* and



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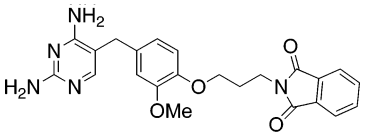
Compd	R	R1
4a :	H	*i*-Pr
4b :	H	*t*-Bu
4c :	*i*-Pr	Me
4d :	$CHEt_2$	H
4e :	$C(Me)_2$	Me
4f :	$CHEt_2$	Me

Table 3. Biological Data for 7,8-Dialkylpyrrolo[3,2-*f*] Quinazolines[117]

compd	*C. albicans* DHFR K_i (nM)	human DHFR K_i (pM)	*C. albicans* MIC (μg/mL)
4a	0.16	10	0.05
4b	0.12	<2.0	0.05
4c	0.22	6.4	0.025
4d	0.22	4.5	0.001
4e	0.0071	0.4	0.025
4f	0.030	0.3	0.025

human DHFR and for growth inhibition of fungal and human cells.[117] Several compounds displayed exceptional, albeit nonselective, affinity for *C. albicans* DHFR and cell growth inhibition. The values for six compounds, **4a**−**4f**, out of 21 tested are presented in Table 3. The level of growth inhibition did not correlate with the inhibition of DHFR. Compound **4f** is also a potent inhibitor of *P. carinii and T. gondii* DHFR and has also been tested in vivo against P388 leukemic cells and fungal and *P. carinii* infection in mice.

In an attempt to exploit the synergism observed when both DHFR and DHPS are inhibited, new 2,4-diaminopyrimidines and 4′-substituted 4-aminodiphenyl sulfones have been prepared and tested against enzyme systems from *C. albicans.*[127,128] A representative compound, HH-136, inhibits *C. albicans* DHFR with an IC_{50} of 0.031 μM. For at least some of the derivatives, the differences in the activities against enzyme and whole cells are in part due to active efflux systems.



HH-136

5.4. Inhibitors of Opportunistic Pathogens

5.4.1. Dihydrofolate Reductase Inhibitors

Since the outbreak of the AIDS epidemic and the recognition that opportunistic infections are the premier cause of morbidity and mortality, major efforts have been undertaken from the early 1990s onward to find novel, potent, and selective inhibitors of the DHFRs of the causative organisms. Two groups, namely, those of Andre Rosowsky and Aleem Gangjee, a selected list of their publications in this area can be found in refs 128 and 129, made major contributions to the developments in this area.

The frequently targeted pathogens are the fungus *Pneumocystis carinii* the protozoa *Cryptosporidium parvum*, *Leishmania ssp.*, *Trypanosoma cruzi*, and *Toxoplasma gondii.* Some inhibitors of DHFR from the bacterial pathogens *Mycobacterium avium* and *M. tuberculosis* have also been reported.

Low doses of co-trimoxazole are used for the treatment and prophylaxis of *Pneumocystis* pneumonia, the most common opportunistic infection.[130] One of the active ingredients, TMP, is a selective but rather weak inhibitor of *P. carinii* DHFR. It is, therefore, used in a combination with sulfamethoxazole. An alternative treatment with piritrexim, trimetrexate, or both,[129] both potent inhibitors of the target enzyme[128] but lacking selectivity for the mammmalian DHFR, requires concomitant and expensive rescue therapy with leucovorin. Thus, the goal of the research efforts in this area is the design and synthesis of antifolates for use against one or more of the abovementioned organisms that are potent enough not to require co-administration of a sulfa drug, as is the case with TMP, and selective enough not to require leucovorin rescue.

The compounds synthesized have been, as a rule, tested for their inhibitory activities against *P. carinii* and *T. gondii* and rat liver DHFRs and less frequently against DHFRs from other organisms mentioned above. Because the procedures for the determination of inhibitory activities are standardized, the IC_{50} values, although extracted from different sources as summarized in the tables, are comparable. In the following part, representative structures of the inhibitors are presented. Within these groups, the values of the selected compounds with the best potency, selectivity, or both are shown in the tables.

The IC_{50} values and selectivity indices of the reference compounds used throughout this section are presented in Table 4. The values shown in Table 4 reflect the previously mentioned problem of potency versus selectivity. In addition, MTX and other inhibitors with an acidic side chain fail to enter the bacterial cell.[124,127,128]

Studies performed in a rat model of dual infection with *P. carinii* and *T. gondii* in which TMP combined with sulfamethoxazole and PYR in vitro and in vivo have shown that the biguanide PS-15 and epiroprim were effective against both *P. carinii* and *T. gondii.*[133]

Table 4. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, Rat, and Human DHFRs by the Reference Compounds

	IC_{50} (μM)					selectivity		
compd	*P. carinii*	*T. gondii*	rat liver	*M. avium*	human	rl/*pc*	rl/*tg*	rl/*ma*
PTX[b]	0.031	0.004	0.001	0.011[c]		0.05	0.1	5.4[c]
TMX[b]	0.042	0.016	0.003	0.01[c]		0.07	0.3	5.3
TMP[b]	12	2.7	133	0.3[d]	>300	14	65	610
ICL[d]	2.4				>300	>125[a]		
MTX[e]	0.011	0.022	0.006		0.022	20[a]	1	
epiroprim[f]	2.6	0.47	170	0.041		12.8	70.6	4100
PYR[g]	3.65	0.39	2.30			0.63	5.9	
pemetrexed[h]		0.0002			0.002			

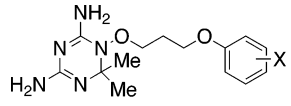
[a] Human. [b] Reference 202. [c] Reference 158. [d] Reference 105. [e] Reference 164. [f] Data on file, F. Hoffmann-La Roche AG. [g] Reference 138. [h] Reference 16.

Table 5. Inhibition of *P. carinii*, *T. gondii*, and Rat DHFRs with Compounds 6

	IC_{50} (μM)			selectivity ratio		
compd	*P. carinii*	*T. gondii*	rat liver	rl/*pc*	rl/*tg*	ref
PYR	3.65	0.39	2.3	0.63	5.9	138
6a	20.4	1.2	6.1	0.3	5.1	155
6b	12.9	16.4	11.4	0.9	0.7	155
6c	3.0	1.4	3.1	1.0	0.5	155
6d	0.84	0.41	1.2	1.4	2.9	155

Several reviews dealing with partial aspects of these efforts have been published.[84,134,135]

Substituted Monocyclic 2,4-Diaminopyrimidines and 4,6-Diamino-1,2-dimethyl-1-(X-phenyl)-*s*-triazines. In a quantitative structure−activity relationship (QSAR) study, the inhibitory activities of 60 2,4-diaminopyrimidines and 4,6-diamino-1,2-dimethyl-1-(X-phenyl)-*s*-triazines, **5**, versus purified,

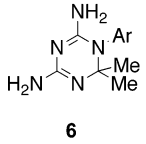


5

Compd	X
WR 99210:	2,4,5-Cl_3
5a :	2,4.Cl_2
5b :	2,3-C_4H_4
5c :	4-Br

recombinant *pc*DHFR were analyzed, and their activities were compared with inhibition of DHFRs from different sources, including recombinant human DHFR.[136] Although it is difficult to draw definitive conclusions from this and a similar type of work,[137] the study indicates that such comprehensive analysis could be helpful in identifying potent and potentially selective antifolates as therapeutic agents.

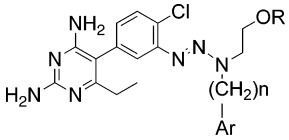
Ten previously untested 1-aryl-4,6-diamino-1,2-dihydro-*s*-triazines, **6**, were assayed. Both their activity and selectivity were poor (Table 5).



6

Compd	Ar
6a :	2,5-$(OMe)_2$Ph
6b :	3,5-$(OMe)_2$Ph
6c :	2-(6-chloronaphtyl)
6d :	2-(5,7-dichlonaphtyl)

SAR data of a series of triazenyl-pyrimethamine derivatives, **7a**−**7d**, exhibited IC_{50} values against

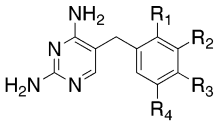


7

Compd	R	n	Ar
7a :	H	3	Ph
7b :	Ac	1	Ph
7c :	H	2	Ph
7d :	Ac	2	Napth-1-yl

*pc*DHFR in the low micromolar range (Table 6). Compound **7a** (TAB) is one of the most selective inhibitors of the *P. carinii* enzyme.[138] The crystal structure and modeling studies on *pc*DHFR−cofactor complexes with TAB have been reported.[139] The overall structure of the ternary complex is similar to that observed for other antifolates. The most notable feature of the binding orientation of TAB in this complex is that its binding is disordered in such a way that there are two alternative positions for the binding of the benzyl and acetyloxy groups.

In an effort to discover more active and selective antifolates, a series of 2,4-diamino-5-(2′-methoxy-5′-substituted)-benzylpyrimidines containing a carboxyl group at the distal end of the 5′-substituent have been synthesized and tested. Based on the analysis of the structure of *pc*DHFR, compounds **8a**−**8h** have



8

Comp	R_1	R_2=R_3	R_4
8a :	OMe	H	OCH_4COOH
8b :	OMe	H	$(CH_2)_5COOH$
8c :	OMe	H	$C{\equiv}(CH_2)_2CO_2H$
8d :	OMe	H	$C{\equiv}C(CH_2)_3CO_2H$
8e :	OMe	H	$C{\equiv}CH_2CO_2H$
8f :	OMe	H	OCH_2(4′-carboxyphenyl)
8g :	H	OMe	$OC{\equiv}C(CH_2)_3CO_2H$
8h :	H	OMe	$OC{\equiv}C$(4′-carboxyphenyl)

been designed such that their ω-carboxyl group could interact with the basic residues of Arg-75 and Lys-37 in the active site of the enzyme.[128,140,141] These compounds are about 1 order of magnitude less

Table 6. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, and Rat DHFR by Substituted 2,4-Diaminopyrimidines

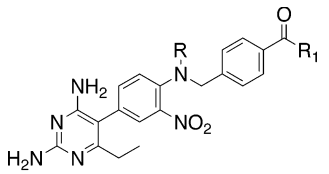
	IC_{50} (μM)				selectivity ratio			
compd	*P. carinii*	*T. gondii*	*M. avium*	rat liver	rl/*pc*	rl/*tg*	rl/*ma*	ref
PTX	0.013	0.004	0.001	0.003	0.26	0.76	5.4	128
7a (TAB)	0.17	0.69		19.4	114	28		138
7b	0.68	0.69	3.9	7.35	10.87	3.66	2.01	138
7c	0.26	2.01		7	27			138
7d	0.053			0.28	5.36	1.4		138
8a	0.054	0.11	0.058	4.6	85	42	79	128
8b	0.15	0.0084	0.016	4.1	27	490	260	128
8c	0.13	0.097	0.004	4.0	31	41	910	128
8d	0.028	0.032	0.008	2.2	79	69	280	128
8e	0.87	0.072	0.041	25	28	340	590	128
8f	1.20	2.0	0.060	21	17	11	340	128
8g	0.001	0.034	0.002	5	5000	150	2100	141
8h	1.2	0.042	0.082	88	73	210	11000	141
9a	0.27	0.0018		0.029	0.01	1.61		142
9b	0.27	0.0031		0.038	0.01	1.23		142
9c	0.53	0.037		0.008	0.01	2.03		142
9d	0.58	0.0043		0.060	0.01	1.4		142
9e	0.10	0.0062		0.013	0.13	2.1		142

Table 7. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, Rat Liver, and Human DHFRs by 2,4-Diaminopyrimidines with Fused Five-Membered Rings

	IC_{50} (μM)					selectivity ratio			
compd	*P. carinii*	*T. gondii*	*M. avium*	rat liver	human	rl/*pc*	rl/*tg*	rl/*ma*	ref
PTX	0.013	0.0043	0.0006	0.003		0.026	0.077	0.55	146
10a	0.6	11.6		12.3		18.9	1.1		144
10b	7.7	45.4		137		17.8	3.02		144
10c	0.90	0.70		1.3	0.45	1.44	1.85		143
10d	0.035	19.8		0.4	0.22	11.4	0.02		143
10e	>4.0	>4.0		>37	>26				143
10f	8.3	>3.9		25.6	>25				143
11a	1.8	0.14		0.51		0.28	3.64		145
11b	1.3	0.14		0.22		0.17	1.57		145
11c	7.5	26		10		1.4	0.39		152
12a	45.7	1.70		156		3.4	92		148
12b	35.3	1.4		14.4		0.4	10.3		148
12c	0.038	0.21		0.044		1.2	0.21		148
12d	0.044	0.15		0.06		1.36	0.40		147
12e	11.1	2.60		16.7		1.50	6.42		147
12f	29.0	3.3	14	9.6		0.3	0.3	0.69	146
12g	72	14	67	52		0.72	0.72	0.78	146
12h	0.77	0.037	0.067		0.26	5.4	2.60	35	146
13	19.5	6.7		252		13	38		149

potent against *pc*DHFR when compared with PTX but exhibit higher selectivity (Table 6). Against *tg*DHFR several compounds show both higher potency and selectivity than the reference compound. Analogues of TMP and brodimoprim, designed with the same rationale,[131,132] have been shown to be inactive in vitro and in vivo against *E. coli* and *L. casei*, respectively. For recently synthesized compounds, it it remains to be seen whether they are taken up into intact cells.

Several derivatives of 2,4-diamino-5-[4′-(substituted)-3′-nitrophenyl]-6-ethyl-pyrimidines, **9a**−**9e**, have been

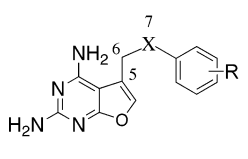


9

Compd	R	R_1
9a :	H	OH
9b :	Me	OH
9c :	H	NH_2
9d :	H	NHMe
9e :	H	NH-L-Glu

synthesized and evaluated as inhibitors of *P. carinii* and *T. gondii* DHFR.[142] The compounds exhibit potent inhibitory activity against the latter enzyme, whereas all are relatively weak inhibitors of the former one (Table 6). These compounds exhibit little or no selectivity based on their activity on the rat liver enzyme.

Bicyclic 2,4-Diaminopyrimidines with Fused Five- and Six-Membered Rings. Antifolates **10a**−**10f** containing a furo[2,3-*d*] pyrimidine ring system have been synthesized as potential dual inhibitors



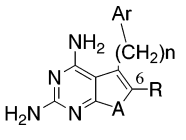
10

Compd	X	R
10a :	S	2-naphthyl
10b :	NH	2-phenyl-Ph
10c :	NH	4-CO-L-Glu
10d :	NMe	4-CO-L-Glu
10e :	NH	3,4,5-$(OMe)_3$
10f :	NH	3,4,5-Cl_3
10g :	CH[a]	4-CO-L-Glu
10h :	CH_2	4-CO-L-Glu
10i :	CHMe	4-CO-L-Glu
10j :	CHEt	4-CO-L-Glu

[a]6,7-dehydro

of DHFR and TS.[143] The compounds have been tested against *P. carinii* and *T. gondii* DHFR, as well as against recombinant human and *L. casei* DHFR (Table 7). The classical analogues **10c** and **10d** have also been evaluated as inhibitors of TS, glycinamide ribonucleotide formyltransferase, and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase. They are inactive against these enzymes. Nonclassical 2,4-diamino-5-(substituted)furo[2,3-*d*] pyrimidine antifolates with a variation X in the bridge region and an appended substituted aromatic ring have been designed such that the aromatic ring could specifically interact with Phe-69 of *pc*DHFR. Compounds **10a** and **10b** show significant potency and selectivity for the target enzyme. The X-ray structure of **10a** with *pc*DHFR has further confirmed the design rationale.[144]

The synthesis and inhibitory properties of (*R*,*S*)-2,4-diamino-5-[(3,4,5-trimethoxyphenyl)alkyl]-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidines **11a** and **11b** as



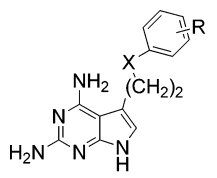
11

Compd	A	n	Ar	R
11a[a] **:**	CH_2	2	3,4,5-$(OMe)_3$-Ph	H
11b[a] **:**	CH_2	3	3,4,5-$(OMe)_3$-Ph	H
11c :	S	1	3,5-Cl_2-(4-pyrrolo)	Br

[a]5,6-dihydro

anologues of TMP have been reported. Unlike TMP, both compounds were better inhibitors of rat liver enzyme than of microbial enzymes[145] (Table 7).

Classical and nonclassical 2,4-diamino-5-(substituted)methyl)pyrrolo[2,3-*d*] pyrimidines **12a** and **12e**



12

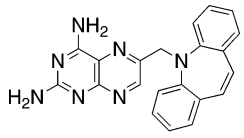
Comp	N	X	R
12a :	1	NH	2,5-$(OMe)_2$
12b :	1	NH	3,4-Cl_2
12c :	1	NH	CO-L-Glu
12d :	1	NMe	CO-L-Glu
12e :	1	S	3,4-$(OMe)_2$
12f :	1	-	3,4-Cl_2
12g :	1	-	3,4,5-$(OMe)_3$-Ph
12h :	2	-	3,4,5-$(OMe)_3$-Ph

Table 8. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, and Rat Liver DHFRs by 2,4-Diaminopteridines and 2,4-Diaminoquinazolines

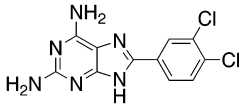
	IC_{50} (μM)				selectivity ratio			
compd	*P. carinii*	*T. gondii*	*M. avium*	rat liver	rl/*pc*	rl/*tg*	rl/*ma*	ref
14a	0.082	0.028		0.32	3.9	11.4		154
14b	0.2	0.033		1.1	5.5	33.3		154
14c	1.1	1.0		2.7	2.5	2.7		154
14d	2.6	0.46		27	10	59		154
14e	0.41			5	12			155
14f	0.28			2.3	8.2			155
14g	1.6			5.9	3.7			155
14h	12			19	1.6			155
14i	5.3			7.9	1.5			155
14j	0.14			0.8	5.7			155
15a	0.092	0.013	0.09	0.028	0.3	2.2	3.1	146
15b	0.12	0.0064		0.012	1	1.9		155
15c	0.15	0.017	0.007	0.042	0.28	2.5	6	146
16a	4.6	0.054		0.29	0.06	5.4		156
16b	0.095	0.007		0.038	0.40	5.43		156
16c	0.30	0.015		0.26	0.9	17.3		156
16d	0.114	0.017		0.071	0.62	4.20		156
16e	0.502	0.01		0.011	0.22	11.0		156
16f	0.171	0.022		0.067	0.39	3.05		156
16g	0.10	0.023		0.047	0.50	2.04		156

with various substitution patterns of the phenyl ring have been prepared to better understand the effect of the bridge variations, in particular of *N*-methylation on one hand and the effect of introducing of 4′-L-glutamate substitution on the inhibitory potency on the other hand. These studies show that *N*-methylation does not greatly influence the parameters measured but the introduction of a glutamate moiety increases the potency by several orders of magnitudes (Table 7). The substances with amine and sulfide groups in the bridge region are almost equipotent.[146−148]

Compound **13** is the most active compound of a the



PT 653

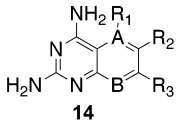


13

series of 22 2,6-diamino-8-substituted purines designed as TMP analogues in which rotation around the two flexible bonds of TMP, linking the pyrimidine ring and the side chain phenyl ring, was restricted by incorporation into a purine ring system. It is as potent as TMP with a selectivity ratio of 13 for *P. carinii* and 38 for *T. gondii* DHFR.[149] (Table 7)

Structural data for human and *pc*DHFR inhibitor complexes have been corroborated in V. Cody's group for a number of antifolates including TMP, MTX, and folate,[150] as well as for a number of novel classical and nonclassical furopyrimidine antifolates[144,151] and TAB.[139] Analysis of quinazoline and pyrido[2,3-*d*] pyrimidine N9−C10 reversed bridge antifolates in complex with $NADPH^+$ and *pc*DHFR[152] and with PT 653[153] has also been performed. Taken together, these data show that despite a number of residue changes compared with human DHFR, the key structural features in the active site of *pc*DHFR are well conserved. The volume in the active site is slightly smaller in *pc*DHFR than in the human enzyme. These small changes can enhance binding affinity and, consequently, the selectivity for one enzyme over the other.[139]

Several novel substituted diaminopteridines have been identified and tested against *P. carinii* in vitro.[154] Two of them, **14f** (GR 92754) and **14g**,

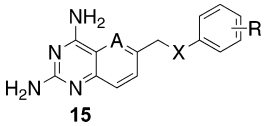


14

14a : A = B: N R_1: H $R_2 = R_3$: $CH_2CH(Me)_2$
14b : A = B: N R_1: H $R_2 = R_3$: $CH(Me)_2$
14c : A = B: N R_1: H $R_2 = R_3$: $(CH_2)_3Ph$
14d : A = B: N R_1: H $R_2 = R_3$: $CH_2C_6H_{11}$
14e : A = B: CH R_1: F $R_2 = R_3$: H
14f : A = B: CH R_1: Br $R_2 = R_3$: H
14g: A = B: CH R_2: I $R_1 = R_3$: H
14h: A = B: CH R_3: Br $R_1 = R_3$: H
14i : A = B: CH R_1: H $R_2 = R_3$: Me
14j : A = B: CH R_1: Cl R_2: Br: R_3: H

antagonize the uptake of a folate precursor, *p*-aminobenzoic acid, and are at least 10−100 times more active than TMP in this assay. The inhibition and selectivity data are shown in Table 8.

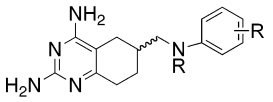
Twenty-eight 2,4-diamino quinazolines **14** substituted with alkyl, halogen, or alkoxy groups, eight 2,4-diaminopteridines, nine 4,6-diamino-1,2-dihydro-*s*-triazines, and five 1,3-diamino-7,8,9,10-tetrahydropyrimido[4,5-*c*]isoquinolines have been evaluated as inhibitors of *P. carinii* and *T. gondii* DHFR enzymes. Generally speaking, these compounds, as well as compounds **15**, exhibit a modest selectivity, for example, compound **14d** (Table 8).[146,155]



15

Compd	A	X	R
15a :	CH	-	3,4-$(OMe)_2$
15b :	CCl	NMe	3,4,5-$(OMe)_3$
15c :	CH	-	3,4,5-$(OMe)_3$

Twenty 6-substituted 2,4-diaminotetrahydroquinazolines **16** have been designed, synthesized as their

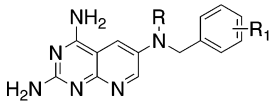


16

Cmpd	R	R_1
16a :	H	3,4,5-$(OMe)_3$
16b :	Me	3,4,5-$(OMe)_3$
16c :	Me	2,5-$(OMe)_2$
16d :	Et	2,5-$(OMe)_2$
16e :	H	2,6-Cl_2
16f :	Me	4-Cl
16g :	Me	2,3-C_4H_4

racemic mixtures, and biologically evaluated. N-Substitution was conducive to potency. As shown in Table 8, the compounds have been significantly more potent and selective against *T. gondii* DHFR, and compound **16c** shows also an exceptionally high inhibitory activity, $IC_{50} = 5.4 \times 10^{-8}$ M, against the growth of *T. gondii* cells in culture. Selected analogues have been evaluated as inhibitors of tumor cells in culture.[156]

2,4-Diamino-6-(benzylamino)pyrido[2,3-*d*]pyrimidine antifolates **17**, lacking a 5-methyl substitution,



17

Compd	R	R_1
17a :	Me	H
17b :	H	3,4,5-$(OMe)_3$
17c :	Me	3,4,5-$(OMe)_3$
17d :	Me	2,4,5-$(OMe)_3$
17e :	Me	3,5-$(OMe)_2$
17f :	Me	2,5-$(OMe)_2$
17g :	H	4-CONH-L-Glu
17h :	Me	4-CONH-L-Glu
17i :	H	2,5-$(OMe)_2$

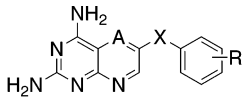
which has been shown to be important for increased human DHFR activity, have been synthesized and tested. They contain a reversal of the C9−N10 bridge present in folates and most antifolates. C9-Methylated compounds **17a** and **17c**−**17f** are the most potent inhibitors in this series (Table 9). Compound

Table 9. Inhibition of *P. carinii*, *T. gondii*, Human, and Rat Liver DHFRs by 2,4-Diamino-6-(methylamino-substituted)pyrido[2,3-*d*]pyrimidines[157]

	IC_{50} (μM)				selectivity ratio		
compd	*P. carinii*	*T. gondii*	human	rat liver	rl/*pc*	rl/*tg*	h/*tg*
17a	0.068	0.032	0.7	0.014	2.1	4.4	20
17b	14.1	0.35	76	3.3	0.23	9.4	192
17c	0.061	0.014		0.033	0.5	2.4	
17d	0.079	0.026	0.53	0.030	0.4	1.2	20.4
17e	0.076	0.031	2.9	0.072	0.9	2.3	100
17f	0.084	0.0028	8.5	0.057	0.7	9.0	304
17i	3.8	0.31		0.35	0.09	1.1	

17f with 2,5-dimethoxy-substitution of the phenyl ring shows a selectivity of 9.0 when compared to rlDHFR and a selectivity of 304, when compared to recombinant human DHFR. Compound **17f** when tested in vivo for the inhibition of *T. gondii* trophozoites in mice demonstrated a distinct prolongation of survival of infected animals.[157] Compounds **17g** and **17h** have also been evaluated as antitumor agents.[152] Three crystal structures of a 5-methyl-6-*N*-methylanilino pyridopyrimidine antifolate complex with hDHFR have been determined and analyzed.[158]

A series of 2,4-diamino-6-(substituted)pyrido[2,3-*d*]pyrimidines **18** with variations in the X bridge



18

Compd	A	X	R
18a :	C-Me	NH	3,4,5-$(OMe)_3$
18b :	CH	CH_2	3,5-$(OMe)_2$
18c :	C-Me	NMe	3,4,5-$(OMe)_3$
18d :	CH	NMe	2,5-$(OMe)_2$
18e :	CH	NH	2,5-Cl_2
18f :	CH	NH	3,4,5-Cl_2
18g :	CH	S	H
18h :	CH	S	3,4-C_4H_4
18i :	CH	NH	3,4,5-$(OMe)_3$
18j :	CH	NMe	3,4,5-$(OMe)_3$
18k :	CH	NEt	3,4,5-$(OMe)_3$
18l :	CH	CH_2	3,4,5-$(OMe)_3$
18m:	CH	CH_2	4-OMe
18n :	CH	CH	3,4,5-$(OMe)_3$
18o :	C-Me	NH	2,5-$(OMe)_2$
18p :	C-Me	NH	2-OMe-5-CF_3
18r :	C-Me	NH	2,5-Me_2
18s :	C-Me	CH_2	2,5-$(OMe)_2$
18t :	C-Me	S	2,3-C_4H_4
18u :	C-Me	NH	2,3-C_4H_8
18v :	C-Me	NH	2,5-(*i*-Pr)$_2$
18w:	C-Me	NH	2,5-$(OEt)_2$
18x :	C-Me	NMe	2,5-$(OEt)_2$
18y :	C-Me	CH_2	3,5-$(OMe)_2$
18z :	C-Me	S	3,4-$(OMe)_2$

connecting the pyridopyrimidine part of the compound with the distal phenyl moiety (**18a**−**18t**) have been prepared.[144,146,158−161] Compounds **18o** and **18u**−**18z** shown in the Table 10 represent examples with the highest specificity ratio for hDHFR versus MAC DHFR. The N-Me analogue **18j** exhibits the best balance of potency and selectivity against both *tg*DHFR and *pc*DHFR, whereas the ethylene bridge has a detrimental effect on the potency (Table 11). The 10-deaza analogues are generally less potent and selective than compounds with an amino group in this position. The activity and selectivity of the 5,6,7,8-tetrahydro derivative of **18l** (data not shown) against *pc*DHFR was markedly decreased.[159]

The highest potency and selectivity, especially against *T. gondii*, is found in the group of 2,4-diamino-6-(substituted)pyrido[2,3-*d*]pyrimidines and is lower for the other heterocyclic types.

The compound **18s** is over a 1000-fold more selective than PTX and inhibits *T. gondii* cell growth with $IC_{50} = 0.1$ μM. Several other compounds (e.g., **18r**

Table 10. Inhibition of *Mycobacterium Avium* Complex and Human DHFR by 2,4-Diamino-5-methyl-6-(substituted)pyrido[2,3-*d*]pyrimidines[123]

	IC_{50} (nM)		
compd	MAC	human	selectivity ratio
18o	1.1	1100	909
18u	1.0	7300	7300
18v	1.9	710	374
18w	0.84	2300	2738
18x	1.4	1000	714
18y	1.5	990	660
18z	4.5	1200	293

Table 11. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, and Rat DHFR by 2,4-Diamino-6-(substituted)pyrido[2,3-*d*]pyrimidines

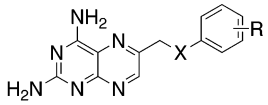
	IC_{50} (μM)				selectivity ratio			
compd	*P. carinii*	*T. gondii*	*M. avium*	rat liver	rl/*pc*	rl/*tg*	rl/*ma*	ref
18a	0.013	0.001		0.0076	0.6	0.85		158
18b	0.210	0.036	0.04	0.37	1.8	10.3	9	146
18c	0.013	0.0009		0.008	0.6	8.9		144
18d	0.084	0.006		0.057	0.7	9		144
18e	0.44	0.3		6.9	15.7	23		144
18f	0.35	0.98		4.6	13	4.7		144
18g	1.30	0.47		1.9	1.46	4.04		161
18h	0.17	0.09		0.022	1.29	2.44		161
18i	0.038	0.3		1.9	1.3	6.3		159
18j	0.042	0.009		0.28	1.2	31		159
18k	1.5	0.049		0.12	0.63	3.2		159
18l	0.24	0.2		1.14	0.23	5.7		159
18m	0.19	0.25		0.26	0.9	1.0		159
18n	5.0	1.4		12.9	2.58	9.2		159
18o	0.011	0.014		0.01	0.94	0.73		160
18p	0.044	0.022		0.02	0.5	1		160
18r	0.03	0.016		0.12	4.0	7.5		160
18s	0.34	0.0079		0.77	2.3	97.5		160
18t	0.29	0.03		0.55	0.03	18.3		160

Table 12. Inhibition of *P. carinii*, *T. gondii*, and Rat DHFR by 2,4-Diamino-6-substituted-pteridines[160]

	IC_{50} (μM)			selectivity ratio	
compd	*P. carinii*	*T. gondii*	rat liver	rl/*pc*	rl/*tg*
19a	9.5	0.77	246	25.9	319
19b	6.2	6.9	22.9	3.7	3.3
19c	3.9	0.21	0.47	0.1	2.2
19d	21	10.6	21	1	2.2
19e	7	1	1.9	0.27	2

with $IC_{50} = 0.01$ μM) demonstrate even lower values in the same test (Table 11).

A large group of 2,4-diaminopteridines with a two atom bridge and an aryl group attached to the 6-position of the heterocyclic moiety **19** (Table 12),



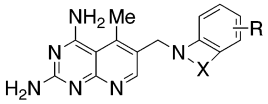
19

Compd	X	R
19a:	S	H
19b:	NH	2,5-$(OMe)_2$
19c:	NMe	2,5-$(OMe)_2$
19d	NH	2-OMe-5-CF_3
19e:	NH	3,4,5-$(OMe)_3$

37 2,4-diamino-6-(substituted)pyrido[2,3-*d*]pyrimidines, and four 2,4-diaminoquinazolines have been synthesized and evaluated.[160,161]

Seventy-seven 2,4-diamino-5-methyl-6-(substituted)pyrido[2,3-*d*]pyrimidines **18** have been evaluated in vitro as potential drugs against *Mycobacterium avium* complex (MAC).[123] The activities of the compounds were assessed against recombinant MAC DHFR and human DHFR. Most of these derivatives show good activity against three strains of MAC with MICs ranging from 0.13 to 1.3 μg/mL.

Another series of 2,4-diamino-5-methyl-6-substituted-pyrido[2,3-*d*]pyrimidines **20** have been de-



20

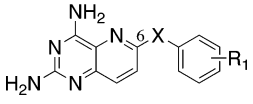
Compd	X	R
20a:	CH_2CH_2	4-OMe
20b:	CH_2CH_2	5-OMe
20c:	CH=CH	4-OMe
20d:	-	2,5-$(OMe)_2$-4-pyrrolo

Table 13. Inhibition of *P. carinii*, *T. gondii*, and Rat DHFR by Compounds 20 and 21

	IC_{50} (μM)			selectivity ratio		
compd	*P. carinii*	*T. gondii*	rat liver	rl/*pc*	rl/*tg*	ref
20a	0.29	0.048	0.015	0.52	3.1	161
20b	0.25	0.057	0.17	0.68	3.0	161
20c	0.57	0.077	0.47	0.83	6.1	161
20d	0.35	0.033	0.23	0.7	7.0	161
21a	0.086	0.019	0.002	0.21	0.95	260
21b	0.023	0.001	0.0004	0.17	0.45	260
21c	0.56	0.063	0.52	0.93	8.25	261
21d	0.12	0.044	0.052	0.43	1.18	261
21e	2.00	0.13	0.52	0.26	4	262
21f	0.36	0.048	0.086	0.23	1.8	204
21g	1.40	0.1	0.43	0.31	4.3	263
21h	0.13	0.047	0.026	0.20	5.5	263
21i	0.02	0.98	0.32	1.5	0.33	263

signed and synthesized as conformationally restricted analogues of TMX so that the side chain nitrogen is incorporated in an indoline or indole system. Compound **20d** and its congeners have been designed to investigate the role of the pyrrolo-substitution of the phenyl ring, a group present also in epiroprim. Conformational restriction in the form of an indoline or an indole ring did not result in analogues with better potency or selectivity when compared with the previously synthesized open chain analogues (Table 13). Among these compounds, **20c** also inhibits the growth of *T. gondii* cells in culture.[161]

A novel easy access to 2,4-diaminopyrido[2,3-*d*]pyrimidines **21** has been developed.[146] In this study, 13 previously untested 2,4-diamino-6-(substituted benzyl)quinazolines have also been evaluated as



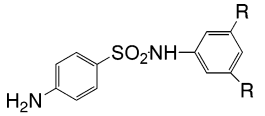
21

Compd	X	R
21a.	S	3,4-$(OMe)_2$
21b:	NMe	3,4-$(OMe)_2$
21c:	CH_2S	4-OMe
21d:	CH_2NMe	2,4,6-Cl_3
21e:	CH_2S	H
21f:	CH_2S	3,4-C_4H_4
21g:	CH_2NH	2,5-$(OMe)_2$
21h:	CH_2NMe	3,4,5-$(OMe)_3$
21i:	CH_2NMe	3,4-Cl_2

inhibitors of DHFR isolated from major opportunistic pathogens.[260−263] All the compounds tested are less active against *P. carinii*, *T. gondii*, and *M. avium* DHFR than the reference compounds PTX and MTX (Table 13). The modest gain in selectivity was achieved at the cost of decreased potency.[146]

5.4.2. Dihydropteroate Synthase Inhibitors

Sulfonamides are well-known inhibitors of the folate pathway enzyme DHPS(EC 2.5.1.15) and have been used clinically for over 60 years. Today, the best clinical utility as antibacterial agents is their combination with TMP or PYR. The combination of TMP with sulfamethoxazole (SMZ) has also found use against nonbacterial pathogens such as *P. carinii*, *T. gondii*, and *Pl. falciparum*. The sulfonamides used in these combinations have, however, very modest potency against the target enzyme. Recently, Chio et al.[17] reported the discovery of a class of sulfonamides **22**, which are active in culture models against



22

22a :	F
22b :	Cl
22c :	Br
22d :	I

P. carinii and T. gondii albeit not better than standard agents against the DHPS enzyme (Table 14).

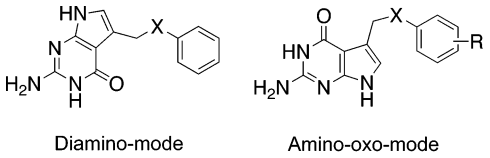
Table 14. Inhibition of Dihydropteroate Synthases from *T. gondii*, *P. carinii*, *M. avium*, and *E. coli*[17]

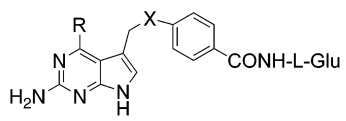
	IC$_{50}$ (μM)			
compd	*T. gondii*[a]	*M. avium*[a]	*P. carini*[ib]	*E. coli*[a]
SMZ	7.30	1.8	0.23	5.8
sulfadiazine	2.83		0.62	
22a	32.9	1.2	1.95	
22b	111	1.2	0.58	
22c	33.4	0.82	0.99	5.6
22d	66.5	2	4.05	

[a] Substrate (PABA) $c = 11$ μM. [b] Substrate (PABA) $c = 2.2$ μM.

5.4.3. Inhibitors of Thymidylate Synthase (TS) and Multitargeted Antifolates (MTA)

Classical and nonclassical analogues **23** of the multitargeted antifolate pemetrexed have also been tested as inhibitors of TS and DHFR as potential agents against opportunistic infections.[162]



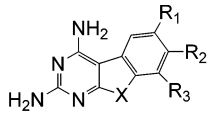


23

Compd	R	X
23a :	OH	CH_2
23b :	OH	-
23c :	Me	CH_2

Pemetrexed is on the market as an antitumor agent. The 2,4-diaminopyrimidine moiety is considered mandatory for potent DHFR inhibitors. The activity of pemetrexed, which contains a 2-amino-4-oxopyrimidine ring, is unusual. One possible explanation is that one considers an alternate model of DHFR, in which the pyrrole NH of this and similar compounds mimics the 4-amino group of the 2,4-diaminopyrimidine ring system. Indeed, Gangjee et al. have demonstrated that 4-methyl analogue **23c** does bind in an alternate mode to DHFR.[162] Among the synthesized classical analogues, the replacement of a CH_2 group by a NH did not result in an improved activity against either TS or DHFR. Within the group of 10 nonclassical inhibitors with the same structural change as described above, the 2,4-dichloro-substituted compound is the most potent of all analogues tested.[16]

Tricyclic 2,4-Diaminopyrimidines with Fused Five- or Six-Membered Rings. Twenty-one conformationally restricted PYR analogues **24** with

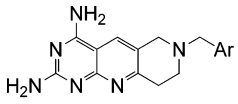


24

Compd	X	R_1	R_2	R_3
24a :	CH_2	H	H	Cl
24b :	$(CH_2)_2$	Me	Me	H
24c :	CH=CH	Cl	H	H
24d :	$(CH_2)_3$	Cl	Cl	H

substituents at different positions of the phenyl ring have been synthesized and tested as DHFR inhibitors. Heterocyclic systems studied include indeno[2,1-*d*]pyrimidines, benzo[*f*]quinazolines, and benzo[3,4]-cyclohepta[2,1-*d*]pyrimidines. Neither the potency nor the selectivity of these compounds is substantially better than that of the reference compound (Table 15). Computer-simulated docking into the active site pocket of *P. carinii* and human DHFR suggests that the rotationally restricted tricyclic structures are at a disadvantage relative to PYR in that the torsional relief between the chlorine atoms and the critical serine and threonine residues in the active site is prevented by the bridge.[163]

Novel classical and nonclassical, partially restricted, linear tricyclic 5-deaza antifolates represented by the compounds **25a**−**25d** have been syn-



25

Compd	Ar
25a :	Ph
25b :	3,5-$(OMe)_2$Ph
25c :	2,4-Cl_2Ph
25d :	4-Ph-CO-*L*-Glu

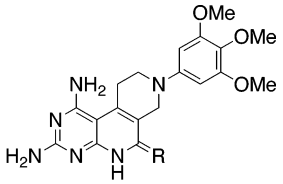
thesized and tested against DHFR from different sources and for antitumor activity.[164] The nonclassical analogues show moderate, but better than that of PTX, selectivity against DHFR from pathogenic microbes compared to recombinant human DHFR, which supports the idea that the removal of 5-methyl group of PTX, along with the restriction of the side chain, can translate into selectivity for DHFR from pathogens (Table 15).

Seven novel tricyclic pyrimido[4,5-*c*][2,7]naphtyridones and the corresponding naphtyridines **26** have been synthesized as conformationally restricted analogues of the inhibitors of DHFR as antitumor or

Table 15. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, Rat Liver, and Human DHFR by Tricyclic Compounds

	IC$_{50}$ (μM)					selectivity ratio			
compd	*P. carinii*	*T. gondii*	*M. avium*	rat	human	rl/*pc*	rl/*tg*	rl/*ma*	ref
28a	>30			2.8		<0.1			155
28b	1.0			0.062		<0.1			155
28c	>30			0.18		<0.1			155
28d	6.8			0.45		<0.1			155
27a	>9.0	1.4	>15	15			>10		166
27b	22.6	13.1	>50	50.9		2.3	3.9		166
27c	24.1	22.3	>20	20.6		0.9			166
27d	40.5	31.7	>81.2	81.2		2.0	2.5		166
27e	10.9	21.5	0.97	85.8		7.9	4.0	88	166
25a	16	1.4			32	2[a]	25.6[b]		164
25b	14	2.9			2.3	1.48[a]	2.7[b]		164
25c	15	2.7			7.35	0.32[a]	2.1[b]		164
25d	2	0.9			133	3[a]	6[b]		164
24a	7.2	4.7		19		2.6	4		163
24b	0.59	0.038		0.036		0.06	0.95		163
24c	0.12	0.011		0.016		0.13	1.5		163
24d	1.3	0.006		0.005		<0.01	0.83		163

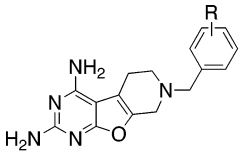
[a] Ratio rh/*pc*. [b] Ratio rh/*tg*.



26a : R = O
26b : R= H_2

antiinfectious agents or both. The tricyclic compounds are about 2 orders of magnitude less potent inhibitors than their bicyclic analogues when tested against *P. carinii* and *T. gondii* DHFR. The activity and lack of selectivity of these compounds lends further credence to the idea that the inappropriate orientation of the substituted phenyl ring in the compounds **26a** and **26b** is responsible for their inhibitory properties.[165]

Seven nonclassical and one classical antifolate have been designed as conformationally restricted analogues of TMX. They show moderate inhibitory activity against the pathogen DHFRs (Table 15). Compound **27e** was 88-fold more potent against *M. avium*

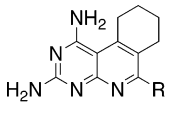


27

27a : H
27b : 3,5-$(OMe)_2$
27c : 3,4-Cl_2
27d : 2,6-Cl_2
27e : 4-NHCO-L-Glu

DHFR than against rat liver DHFR.[166]

Examples of angular tricyclic 1,3-diamino-7,8,9,10-tetrahydro-pyrimido[4,5-*c*]isoquinolines **28** have been



28

Compd R

28a : H
28b : Me
28c : Et
28d : Ph

prepared and tested as inhibitors of rat liver and *pc*DHFR. Neither analogue exhibits the desired selectivity[155] (Table 15).

In an attempt to summarize the results of the different substance types, it can be stated that substituted monocyclic 2,4-diaminopyrimidines in general exhibit high selectivity but rather modest inhibitory potency. The group of 2,4-diaminopyrimidines with fused five- or six-membered rings contains the most potent compounds, but unfortunately, they lack selectivity; they are almost equally active against mammalian and target pathogen organisms. Conformationally restricted tricyclic analogues of the above-mentioned types are practically devoid of inhibitory activities.

Several 3-D structures of DHFRs from the targeted organisms mentioned above with different ligands are available and have been studied intensively. Hundreds of compounds designed using this knowledge have been synthesized and tested. Despite these efforts, today we are still not in a position to understand the subtle differences in the active centers of the enzyme to the extent that would enable us to rationally design a compound that would be both a potent and a selective inhibitor of the enzyme of one or more targeted opportunistic pathogens.

5.5. Antimalarials and Other Antiprotozoal Agents

Malaria each year kills about 2 million children and debilitates over 500 million individuals worldwide, and its incidence continues to increase.[167] Although the need for antimalarial agents is acute, in recent years very few new antifolates have been designed, synthesized, and tested against *Plasmodium* spp., the causative agent of malaria. The research in this field has been concentrated almost exclusively in academia, becuase most, if not all, big pharmaceutical companies terminated their engagement in tropical diseases in the past decade.

The medical need for new antimalarial drugs, resistance, and drug development efforts have been reviewed by Ridley in 2002.[168] Debaert summarized the recent results in this field,[169] and structure-based approaches have been discussed by Brady and Cameron.[170] The research efforts have concentrated on the mechanisms of resistance from malarial DHFR–

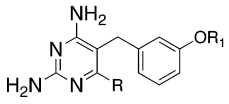
Table 16. Inhibition of Trypanosomal, Leishmanial, and Human DHFRs[a]

	K_i (nM)				
compd	*Le. major*	*Tr. cruzii*	*Tr. brucei*	human	ref
TMP	120 (12)	1000 (1.3)	10 (134)	1380	178
PYR	250 (0.49)	98 (1.2)	11 (11)	120	178
MTX		0.038 (4.7)		0.179	180
29a	97 (25)	1130 (2.1)	24 (100)	2400	178
29b	48 (83)	710 (0.57)	19 (21)	400	178
29c	150 (6.2)	220 (4.2)	3.6 (257)	930	178
29d	160 (8.5)	23 (60)	8.8 (156)	1400	178
29e	65 (15)	200 (4.9)	11 (88)	1000	180
30a		0.107 (18.4)		1.97	180
30b		0.131 (11.1)		1.45	180
30c		0.183 (8.9)		1.63	180

[a] The selectivity is shown in parentheses.

TS,[44,48,171−176] the biology of the parasite, and epidemiology of its resistance.[177]

I. H. Gilbert's group, in collaboration with others, explored the SAR of 2,4-diamino-5-benzylpyrimidines as inhibitors of trypanosomal and leishmanial DHFR, starting with a lead compound **29a**, which was

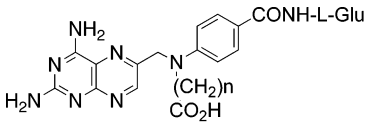


29

Comp	R	R_1
29a :	H	C_8H_{17}
29b :	H	C_7H_{15}
29c :	C_2H_5	C_4H_7
29d :	H	iC_3H_7
29e :	H	CH_2Ph

reported to have a 100-fold selectivity for the leishmanial enzyme (Table 16). The best compounds show some selectivity for parasite over human DHFR. They are surprisingly more active against *Tr. brucei*, given the high structural similarity between the *Tr. cruzi* and *Tr. brucei* enzymes. The compounds have also been tested against the clinically relevant forms of the intact parasite. Among others, compound **29a** is also active in vivo against *Tr. brucei*.[178]

Knighton and co-workers created a homology model of *Tr. cruzi* DHFR using the published structure of *Leishmania major* DHFR.[179] Based on the differences in the active site of these enzymes and the binding mode of MTX, compounds **30a**−**30c** have been de-

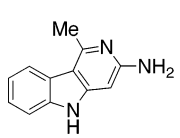


30

Compd	n
30a :	3
30b :	4
30c :	5

signed as inhibitors of *Tr. cruzi* DHFR. None of the compounds are either significantly selective for the parasite enzyme (Table 16) or active in vitro against amastigote stage of the parasite.[180]

Computational screening of commercially available compounds has been performed using a 3-D structural model of the DHFR domain of the bifunctional DHFR−TS of *Pl. falciparum*.[181] Twenty-one compounds identified in this screen have been also assayed for their inhibitory activities. Two of these, **31** and **32**, inhibit the recombinant *Pl. falciparum* domain with K_i values of 8.7 and 0.54 μM, respectively. These results support the validity of the model



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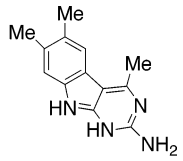


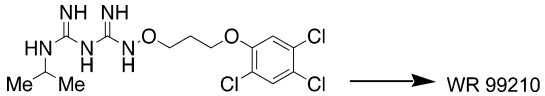
32

and the docking experiments. However, compound **31** has previously been reported to be mutagenic in bacteria and carcinogenic in animals.

Another molecular docking strategy aimed at discovery of compounds that are active against *Pl. falciparum* DHFR is described by Rastelli et al.[50] Twelve compounds, *N*-hydroxyamidines, pyrimidines, triazines, urea, and thiourea-derivatives, unrelated to known antifolates, were identified as micromolar inhibitors of the wild-type and resistant mutant *pf*DHFR harboring the widespread single, double, triple, and quadruple mutations of this enzyme. In agreement with the design, they bind with similar affinity to the wild-type and mutated DHFRs. Insights into how these inhibitors bind to their targets is presented.

An inspiration and a fresh start for the design and discovery of novel folate inhibitors was provided by the publication of the 3-D structure of *Pl. falciparum* DHFR−TS, the target for the clinically established antimalarial drugs pyrimethamine and cycloguanil. The structure reveals insights into the nature of inhibitor WR 99210 binding in complex with adenine dinucleotide phosphate and 2′-deoxyuridylate in drug resistance and autologous gene repression, all of which influence species-specific drug sensitivity.[44] The historical background and importance of this work for further developments in the area are outlined in the paper of Rathod and Phillips.[182]

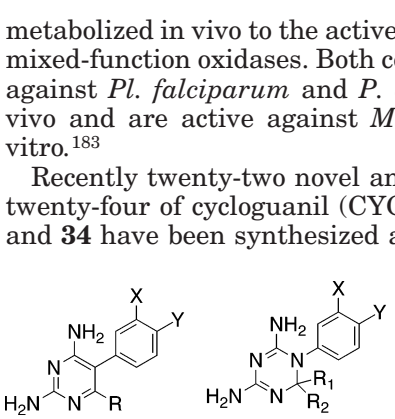
PS-15 is a prodrug form of WR 99210. It is



PS −15

metabolized in vivo to the active form by microsomal mixed-function oxidases. Both compounds are active against *Pl. falciparum* and *P. carinii* infections in vivo and are active against *M. avium* complex in vitro.[183]

Recently twenty-two novel analogues of PYR and twenty-four of cycloguanil (CYC) represented in **33** and **34** have been synthesized and tested as inhibi-



33 : X,Y : H,Cl, Me, OCH_2O
R : Et, $(CH_2)_3CO_2Me$, $(CH_2)_3Ph$, $(CH_2)_3OR$, etc.

34 : X,Y : H, Cl
R_1 : H,Me ; R_2 = alkyl, aryl

tors of *Pl. falciparum* DHFR carrying triple and quadruple mutations responsible for antifolate re-

sistance.[184] The inhibitors were designed to avoid steric clashes in the active site of the mutant enzymes. Several compounds show inhibition constants at a low nanomolar level against the mutant enzymes. A number of these inhibitors have also been shown to have good antiplasmodial activity against resistant strains of *Pl. falciparum* in vitro with IC_{50} values at low micromolar level and relatively low toxicities. The properties of the compounds demonstrate the feasibility of developing antifolates against mutated targets in *Pl. falciparum*.

5.6. Anticancer Antifolates

Although the number of antifolates synthesized as potential anticancer agents in the last 10 years has been smaller than those synthesized for antimicrobial targets, the success rate of these efforts is impressive. Several new targets—TS, FPGS, GarFTase—have been exploited, and novel chemical entities have progressed to clinical development or reached the market (section 4). The current state of the research and development of anticancer antifolates has been reviewed recently.[13]

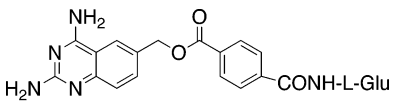
5.6.1. Classical Inhibitors of DHFR

MTX and TMX are highly potent but nonselective DHFR inhibitors in use. MTX is a mainstay in single or combination chemotherapy of lymphoblastic leukemias and other cancers. It is also considered a "gold standard" in the treatment of rheumatic arthritis and is used to treat psoriasis.[185−190]

DL-4,4-Difluoroglutamic acid and DL-γ,γ-difluoromethotrexate have been synthesized. Their evaluation revealed that the former compound is a poor alternate substrate for FPGS and the latter is neither a substrate nor an inhibitor of human FPGS.[191]

The stereospecific synthesis of methotrexate analogues containing L-threo-(2*S*,4*S*)-4-fluoroglutamic acid and DL-3,3-difluoroglutamic acid has been reported. The compounds do not act as substrates for FPGS and inhibit human DHFR at a similar level as MTX.[192]

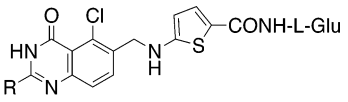
In recent years, MTX has also been used as a therapeutic agent in the treatment of patients with Crohn's disease, a chronic inflammatory bowel disease. Classical DHFR inhibitors resembling the MTX structure were synthesized as potential drugs for this indication. They contain ester bridges in the central parts of the MTX molecule, and the pteridine ring system has been substituted by a quinazoline. The compounds inhibit rat DHFR in the low nanomolar range. Compound **35** is also active in vivo in the



35

corresponding disease model. However, the mechanism of action is not well understood, and the results cannot be fully explained by DHFR inhibition or by inhibition of lymphocyte cell proliferation.[193]

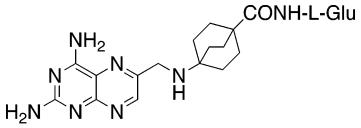
The synthesis of two thiophene analogues of 5-chloro-5,8-dideazafolic acid has been reported. Compounds **36a** and **36b** were tested as inhibitors of



36a : R= NH_2
36b : R= Me

tumor cell growth in culture, and their IC_{50}'s against CCRF-CEM human leukemic lymphoblasts are 1.8 and 2.1 μM, respectively.[194]

Compound **37**, in which the bicyclo[2.2.2]octane



37

ring system replaces the phenyl ring of the *p*-aminobenzoate moiety of aminopterin has been synthesized and tested for antifolate activity. It is ineffective against L1210 DHFR and three tumor cell lines. In contrast to many classical DHFR inhibitors bearing appropriate aromatic ring systems in the side chain, the compound negates the stoichiometric binding to the target enzyme.[195]

Gangjee et al. have studied the effect of C9-methyl substitution and C8−C9 conformational restriction on the antifolate and antitumor activity of classical 5-substituted 4-diaminofuro[2,3-*d*]pyrimidines.[196] Compound **10i** with a 9-methyl group shows increased inhibitory potency against recombinant human DHFR, as well as against the growth of CCRF-CEM tumor cells in culture. Conformationally restricted analogues are significantly less active. The analogues with the C−C bridge are also good substrates for human FPGS, indicating that FPGS is relatively tolerant to conformations in the bridge region.

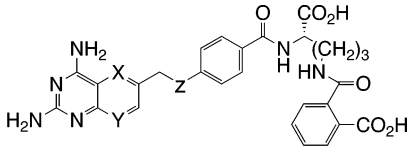
With the design and synthesis of **10j**, the effect of homologation of a C9-methyl to an ethyl on DHFR inhibition and antitumor activity was investigated. The extension doubles the inhibitory potency against hDHFR (IC_{50} = 0.21 μM) when compared with its lower homologue and is 4-fold more potent than the C9−H analogue **10h**. It also demonstrates increased growth inhibitory potency against several human tumor cell lines in culture with GI_{50} values of $<1.0 \times 10^{-8}$ M and is a weak inhibitor of rhTS. Compounds **10i** and **10j** are efficient substrates of human FPGS. Further evaluation of the cytotoxicity of the latter compound in MTX-resistant CCRF-CEM cell lines and metabolite protection studies implicated DHFR as the primary intracellular target. The authors conclude that alkylation of the C9 position in the C8−C9 bridge of the classical 5-substituted 2,4-diaminofuro-[2,3-*d*]pyrimidine is highly conducive to DHFR and tumor inhibitory activity, as well as FPGS substrate efficiency.[197]

Two new analogues of a nonpolyglutamable antifolate PT 523, which is currently in clinical development, have been synthesized.[198] Compounds **39a** and

Table 17. Cell Growth Inhibition and DHFR Binding by Compounds 39 and 40

compd	cell growth IC_{50} (nM)	DHFR binding K_i (pM)	ref
PT 523	1.5 ± 0.39	0.33 ± 0.04	101, 102, 198
39a	0.69 ± 0.04	0.21 ± 0.005	198
39b	1.3 ± 0.35	0.60 ± 0.02	198
39c	0.64 ± 0.04	0.014 ± 0.005	199
39d	0.53 ± 0.07	0.35 ± 0.06	199
40a	0.63 ± 0.08		201
40b	1.2 ± 0.25		201
40c	54 ± 4.9		201
40d	1.2 ± 0.22		201
40e	4.4 ± 1.1		201

39b were tested in a 72 h growth inhibition assay against cultures of CCRF-CEM human leukemic lymphoblasts (Table 17). The activities are comparable to those of PT 523 and the previously studied analogues **39c** and **39d**.[199] However, they are more active than aminopterin despite the fact that they cannot form γ-polyglutamated metabolites as classical antifolates with a glutamate side chain.



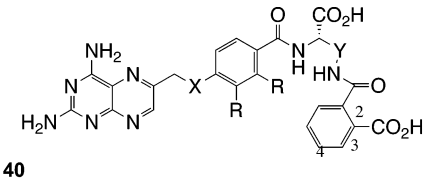
39

Compd	X	Y	Z
PT 523 :	N	N	NH
39a :	CH	CH	CH_2
39b :	N	N	CHPg[a]
39c :	CH	CH	NH
39d :	N	N	CH_2
39e :	CMe	N	NH

[a] Pg= E/Z propargyl

The consequences of changes in the ring B of PT 523 on the inhibition of hDHFR and growth inhibition of a large panel of tumor cell lines, performed also at NCI, have been reported and results with the **39e** are discussed in detail. [200]

Synthesis of a series of analogues of PT 523 with modifications in the side chain, *p*-aminobenzoyl moiety, or C9−C10 bridge has been reported.[198,201] The growth inhibition values of the selected compounds **40a**−**40e** are shown in the Table 17. The shift



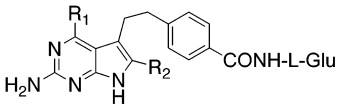
40

Compd	X	R	Y
40a :	CHMe	H	$(CH_2)_3$
40b :	CHEt	H	$(CH_2)_3$
40c :	$C(Et)CO_2Me$	H	$(CH_2)_3$
40d :	NH	$(CH=CH)_2$	$(CH_2)_3$
40e :	NH	H	CH_2SCH_2

of the terminal ortho-carboxyl group to the meta or para position has a detrimental effect on the activity.

Replacement of ornithine in PT 523 by L-2,4-diaminobutanoic acid or lysine affects the binding to hDHFR but results in a loss of activity against some carcinoma cells in culture. 3,5-Dichlorosubstitution in the *p*-aminobenzoic moiety decreases neither DHFR binding nor cytotoxicity.[202]

Compounds **41** have been designed as antitumor



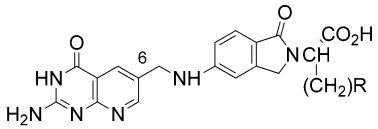
Compd	R_1	R_2
41a :	Me	H
41b :	Et	H
41c :	Et	Et

41

agents acting as dual inhibitors of TS and DHFR. Compared to pemetrexed, inhibitory potency against human DHFR of compounds **41b** and **41c** is 1 and 2 orders of magnitude lower that that of the reference compound, respectively.[203] Both **41b** and **41c** are more potent than pemetrexed against *E. coli* TS. Against human TS, **41b** is 7-fold less potent than pemetrexed and **41c** shows similar inhibitory activity as pemetrexed. In contrast to **41b**, which is an efficient substrate of human FPGS, **41c** is substantially less active. Compound **41b** shows GI_{50} values in the nanomolar range against more than 18 human tumor cell lines in the standard NCI preclinical in vitro screen.

Many compounds, designed as inhibitors of DHFR of opportunistic pathogens, such as **10c**, **10d**,[143] **15**,[156] or **18**,[204] have also been tested against a variety of tumor cell lines in culture. Others have been evaluated in the in vitro screening program of the National Cancer Institute (e.g., **17** and **18**).

5-Deazafolate analogues with a rotationally restricted glutamate or ornithine side chain, **42**, have



42

Compd	R	
42a:	COOH	
42b:	CH_2NH_2	
42c:	COOH	(6*R*,6*S*)-tetrahydro
42d:	CH_2NH_2	(6*R*,6*S*)-tetrahydro

been synthesized and tested as substrates for FPGS and as inhibitors of the growth of CCRF-CEM cells.[205] Whereas compounds **42b** and **42d** are potent inhibitors of rhFPGS, compounds **42a** and **42c** are exceptionally efficient FPGS substrates. All four compounds are inactive in the CCRF-CEM cell growth assay.

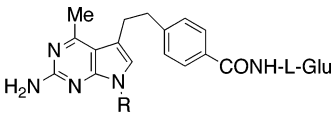
5.6.2. Inhibitors of Thymidylate Synthase

The potential of TS inhibitors in cancer therapy has been recently reviewed by McGuire et al.,[206] N. L. Lehman,[207] and Ackland et al.[208] The inhibitory concentrations of compound considered as references for inhibition of TS are given in the Table 18.

Table 18. Inhibitory Concentrations (IC_{50}, M) of the Reference Compounds against Isolated TS[204]

compd	*rec*	*rpc*	rh
pemetrexed	1.1×10^{-4}	5.7×10^{-5}	5.7×10^{-5}
raltitrexed	8.0×10^{-6}		1.0×10^{-6}
CB 3717	5.8×10^{-8}	5.0×10^{-8}	1.5×10^{-7}
MTX	1.8×10^{-4}		3.6×10^{-5}

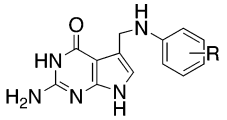
Compounds **43** have been designed as dual inhibitors of TS and DHFR and as antitumor agents.[162] The



43a : R = H
43b : R = CH_2Ph

X-ray crystal structure of the ternary complex of **43a**, DHFR, and NADPH shows that the inhibitor binds in a "2,4-diamino mode", where the pyrrolo-nitrogen mimics the 4-amino moiety of 2,4-diaminopyrimidines. The compounds have been evaluated as inhibitors of *L. casei*, *E. coli*, rat, and human TS, along with reference compounds pemetrexed, ZD 1694, and PDDF. Compound **43a**, an excellent substrate for FPGS, is similar in potency to ZD 1694 and 4.4-fold more active than pemetrexed. The activities against DHFR from the above-mentioned sources, as well as against CCRF-CEM human leukemia and FaDu squamous cell carcinoma have also been reported.[162]

Compounds **44** are in the bridge C8−C9 isosteric



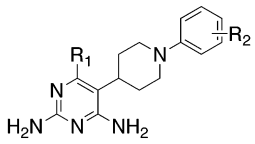
44a: R = *p* - CONH-L- Glu

with MTA pemetrexed. Both classical and nonclassical analogues have been prepared and tested as antitumor agents and agents against opportunistic infections. The compounds are poor inhibitors of *P. carinii* DHFR and possess similar potency as TMP against *T. gondii* DHFR. The nonclassical analogues are also inactive against TS. Compound **44a** marginally (IC_{50} = 46 μM) inhibits human TS, but it is a potent inhibitor of several cell carcinoma lines.[204]

5.6.3. Nonlassical Inhibitors of Folate Enzymes

With TMX and PTX in clinical development, the search for nonclassical antifolates as anticancer agents slowed considerably. Compounds synthesized for other target indications have often been evaluated in basic screens either at NCI or at the particular institution for their anticancer activities.

A series of 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines **45a** and 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidines **45b** have been synthesized and evaluated for their in vitro cytotoxicity.[209] The studies revealed that the former compounds are more cytotoxic than their 2,4-diamino-6(5*H*)-oxopyrimidine counterparts. Among 2,4,6-



45

Compd	R_1	R_2
45a :	NH_2	various
45b :	OH	various
45c :	NH_2	2-F
45d :	NH_2	4-Cl

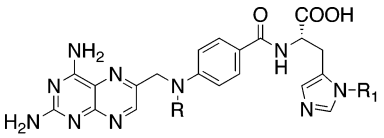
triaminopyrimidines, for which DHFR is the major target, **45c** and **45d** are more potent than MTX in inhibiting the growth of H23/0.3 cell line.

5.6.4. Inhibitors of Folylpolyglutamate Synthetase

Folylpolyglutamate synthetase (FPGS) (E.C.6.3.2.17) plays a critical role in endogenous folate metabolism, as well as in the cellular pharmacology of classical antifolates and, more specifically, in the therapeutic selectivity of these drugs and the development of resistance. It is responsible for the conversion of naturally occurring folates (and antifolates) to their poly(γ-glutamate) derivatives, forms required for intracellular retention of folates and the preferred substrates for most folate-dependent enzymes. Because of its role, several studies have been performed to identify the structural requirements for binding of folates and folate antagonists to the active site of the enzyme. The developments in the area till 1999 and the definitions of polyglutamable and nonpolyglutamable inhibitors of types A and B have been reviewed by A. Rosowsky.[210] Recent advances in the chemistry and biology of folyl-poly(γ-glutamate) synthetase substrates and inhibitors have been reviewed by Gangjee et al. in 2002.[211]

Surprisingly, suramine, a substance outside of the class of known FGPS inhibitors, is reported to be a potent inhibitor of human FPGS.[212] The effects of suramine on growth of CCRF-CEM cells and a MTX-resistant subline, expressing low levels of FPGS activity, suggest that inhibition of folate metabolism could be involved in the mechanism of action of suramine.

Folate and MTX analogues **38a**−**38c**, with L-



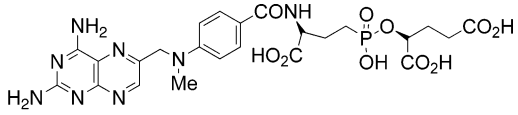
38

Compd	R	R_1
38a :	Me	H
38b :	Me	CH_2CO_2H
38c :	H	H

histidine in place of L-glutamate, have been synthesized as potential inhibitors of FPGS. No significant inhibition of the target enzyme by these compounds is observed.[213]

Partially restricted tricyclic antifolates **25d** and **27e** are reasonable substrates for FPGS but virtually inactive against CCRF-CEM human leukemia cells. The compounds and their congeners have also been evaluated in the NCI preclinical antitumor screening program.[164,166]

Compound **46** and its congeners have been designed as mechanism-based inhibitors of FPGS where a phosphonate moiety mimics the tetrahedral intermediate in the ligation reaction. They do not act as



46

substrates but are potent and competitive inhibitors of this enzyme.[214]

5.6.5 Inhibitors of Other Enzymes

DHFR and TS are established targets for anticancer agents. As mentioned in section 4, a number of newer inhibitors are known to inhibit more than one enzyme in the folate pathway or related reactions. Thus, pemetrexed is an inhibitor of DHFR and TS, as well as glycinamide ribonucleotide formyltransferase. The latter is a crucial enzyme in purine biosynthesis. The finding that 5,10-dideazatetrahydrofolate (lometrexol, see Chart 3) is an effective inhibitor of GARFT and an efficacious antitumor agent establishes purine biosynthesis as a viable target for anticancer agents. Specific inhibitors of essential enzymes in purine synthesis, such as GARFT or AICARFT, for example, conformationally restricted 10-formyl-tetrahydrofolate analogues have been synthesized.[215,216] This topic is not further addressed in the present review.

6. Resistance to Antifolates

Resistance to DHFR inhibitors or inhibitors of DHPS in bacteria, protozoa, fungi, or cancer cells can be caused by a variety of mechanisms. Point mutations in the target enzyme that alter the binding of the inhibitor thereby leading to resistance are frequently found in Gram-positive bacteria, in protozoa, and in other parasites. Resistant plasmid-borne bypass enzymes are the main cause of resistance in Gram-negative bacteria. Almost 20 different TMP-resistant DHFR bypass enzymes have been found in Gram-negative bacteria and a detailed discussion of their characteristics, their genetic location, and their epidemiology is beyond the scope of this article. Mechanisms of TMP resistance in bacteria have been studied intensively for years, and there is a wealth of information available.[217−220] Considerable new information has been acquired, however, in recent years on antifolate resistance in protozoa, particularly in malaria parasites, aided by the application of modern biochemical and genomic tools.[221] We therefore focus on the latter.

6.1. Bacteria

Streptococcus pneumoniae is a major human pathogen, causing upper and lower respiratory tract infections. Penicillin-resistant strains are now prevalent, and many of these strains are co-resistant to TMP and sulfonamides. A number of amino acid changes in the *Str. pneumoniae* DHFR have been reported, but only a single point mutation, isoleucine 100 to leucine (Ile100Leu), can lead to high TMP resistance.[222,223] Similarly, a single amino acid substitution, that is, phenylalanine 98 to tyrosine (Phe98Tyr), was found to be responsible for TMP resistance in *S. aureus*.[218] X-ray crystallography with the ternary complex of the Phe98Tyr DHFR with folate−NADPH showed that the mutation resulted in a loss of a hydrogen bond between the 4-amino group of TMP and the carbonyl oxygen of Leu-5. This mechanism is predominant in both transferable plasmid-encoded and nontransferable chromosomally encoded resistance.[224]

The effect of mutations on the interactions between dimers of the R67 plasmid-encoded DHFR from *E. coli* have been investigated. The native enzyme is a tetramer.[225]

Numerous point mutations in DHPS have been described in many species, conferring resistance to sulfonamides and sulfones.[217,226] For high level, transferable sulfonamide resistance, mainly in Gram-negative bacteria, two genes, *sul1* and *sul2*, have been found.

6.2. Protozoa

Pl. falciparum, the most important causative agent of malaria, has acquired resistance to many of the established agents. The consequences of parasite resistance for the dynamics of malaria spread and public health have been comprehensively reviewed recently.[227−229] The mechanism of antifolate resistance was reviewed by Warhurst.[230] Mutations in the DHFR domain of the bifunctional DHFR−TS enzyme have been associated with antifolate resistance. Several recent reviews describe single, double, or multiple mutations[44,228,231−235] in the gene. The prevalent single mutation is Ser108AsN, which confers a moderate level of resistance to PYR and CYC. Higher resistance levels are observed with double mutations, such as Cys59Arg and Ser108Asn, triple mutations, Asn51Ile, Cys59Arg, and Ser108AsN or Cys59Arg, Ser108Asn, and Ile164Leu, and the quadruple mutant, Asn51Ile, Cys59Arg, Ser108Asn, and Ile164Leu. The latter is highly resistant to both agents. One double mutant, Ala16Val and Ser108Asn, confers CYC resistance only. The Ser108Asn mutation could be specifically identified in field isolates with PCR-based fluorescent probes.[236]

A single Asp54Glu mutation in the *pf*DHFR domain greatly decreases the catalytic activity of the enzyme and affects both K_m values for substrate and K_i values for PYR, CYC, and WR99210[237] (Table 19).

Resistance to CYC was found to increase in several African countries, as monitored by the Ser108Asn mutation, from 19.8% in 1995 to 43.6% in 1997.[238]

Fixed trimethoprim−sulfamethoxazole (5:1) combination (co-trimoxazole) is widely used in Africa for prophylaxis against opportunistic infections in HIV-infected individuals. Pyrimethamine−sulfadoxine and co-trimoxazole select for antifolate resistance in *Pl. falciparum*, and there is cross-resistance between these two agents.[239]

Drug resistance alleles for both mutated *dhfr* and *dhps* genes are frequently found in *Pl. falciparum* isolated from many parts of the world where resistance is common, that is, in African countries, Java, Indonesia, or South America.[240,241]

Resistance to pyrimethamine−sulfadoxine in Africa is mostly due to point mutations at codons 108,

Table 19. Inhibition Kinetics and in Vitro Sensitivity of *Pf*DHFR/TS and Parasites[44]

	inhibition constant (K_i, nM)			antiplasmodial activity (IC_{50}, μM)		
DHFR type	PYR	CYC	WR99210	PYR	CYC	WR00210
wild-type	0.2	0.3	0.011	0.08	0.037	0.00057
Cys59Arg/Ser108Asn	9.8	6.2	0.02	30.9	2.4	0.0023
Asn51Ile/Cys59Arg/ Ser108Asn/Ile164Leu	283	254	0.037	>100	>100	0.018

51, and 59 of *dhfr* and codon 437, 540, or both of *dhps*. In contrast to South East Asia and South America, the Ile164Leu mutation in *dhfr* was not detected until recently.[242]

Point mutations in the gene for *dhps* of *Pl. falciparum* are responsible for resistance to sulfadoxine, dapsone, and other sulfonamides. Mutations at codons 436, 437, 540, 581, and 613 of *dhps* were observed in isolates from Kenya, but the poor correlation between genotype and in vitro resistance suggests that additional factors contribute to resistance; these include the folate content of the medium and utilization of exogenous folates.[243]

Expression of mutated *pf*DHPS in *E. coli* unequivocally demonstrates that these enzymes confer resistance to sulfonamides and sulfones.[244]

DHFR and DHPS genotypes, analyzed in 70 *Pl. falciparum* isolates, correlate with resistance to PYR, TMP, sulfadoxine, and sulfamethoxazole.[245]

Genotyping of pyrimethamine−sulfadoxine-resistant *Pl. falciparum* has been recently performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technique conveniently identified single nucleotide polymorphisms (SNPs) occurring at position 16, 51, 59, and 108 of the *pfdhfr* gene, which are associated with PYR resistance.[246]

Similarly, as in *Plasmodium* species, mutations in the DHFR−TS protein have been found to be responsible for resistance to pyrimethamine of *Toxoplasma gondii*, Arg-59 and Asn-108 (*Pl. falciparum* numbering system). Arg-36 and Ser-83 in *T. gondii* do not exhibit significant fitness defects in vitro but exhibit a 1.8% fitness defect per generation in mice. The high-level PYR resistant mutant Arg-59 and Ser-223 exhibits a >2.8% fitness defect both in vitro and in vivo. This high cost of mutation is assumed to be responsible for the fact that this mutation is not observed in the field.[247]

There is very little information about the prevalence and mechanism of antifolate resistance in *Toxoplasma*. Several mutations in the *T. gondii* DHFR−TS generated in vitro have been shown to confer resistance to PYR, namely, Trp25Arg, Leu98Ser, and Leu134His.[248]

6.3. Fungi

Sulfonamides are more active than TMP in the treatment of *Pneumocystis carinii* pneumonia (PCP), and TMP is a moderately active inhibitor of the *P. carinii* DHFR with an IC_{50} of 43 μM.[86] A considerable body of information on the mechanism of resistance to DHFR inhibitors and sulfonamides has accumulated in the past few years. This has been made possible through the use of modern genetic tools to study this "difficult to grow" pathogen.

Mutations in the DHFR of patient isolates of *P. carinii* have been detected repeatedly. Amino acid substitutions Ala67Val and Cys166Tyr have been found in two patients in Japan.[249]

Genotyping of DHPS from *P. carinii* from AIDS patients revealed several mutations, the most frequent being Thr55Ala and Pro57Ser, which are located in the sulfa-binding site and may occur singly or as a double mutation in the same isolate. Although these mutations often lead to failures of prophylaxis with co-trimoxazole, therapy is often successful.[250,251] These mutants are still infrequent but are increasing and linked to prior sulfonamide prophylaxis.[252]

Two case reports published in 1994 and 2002 show failure of co-trimoxazole prophylaxis or therapy with concomitant administration of leucovorin (5-formyltetrahydrofolate), which is sometimes given to reduce the incidence of neutropenia.[253,254] This suggests that *P. carinii* might be able to use exogenous folates.

6.4. Cancer Cells

Various mechanisms of resistance to antifolates in cancer cells have been described and recently reviewed.[255,256] Alterations in transport, efflux, polyglutamation, and hydrolase activities are the major determinants for MTX resistance. Increased *dhfr* gene copy number, mutations in DHFR, and changes in transcriptional regulation are additional resistance mechanisms. These mechanisms are responsible for a considerable failure rate in the treatment of acute lymphoblastic leukemia in pediatric patients. Similarly, increased levels of TS, decreased uridine monophosphate kinase (UMPK), or changes in thymidine phosphorylase or dihydropyrimidine dehydrogenase may be responsible for resistance to 5-fluorouracil or its derivatives.

Alterations in membrane transport are an important mechanism of resistance that affects a number of antifolates.[257]

For pemetrexed, mutations in the reduced folate carrier leading to a decrease in the activity of FPGS and an increase in the activity of γ-glutamylhydrolase or of TS have been described as resistance mechanisms.[92] Loss of FPGS activity is a dominant mechanism of resistance to polyglutamation-dependent novel antifolates in human leukemia cell sublines.[258]

A number of multiple mutants of murine DHFR have been constructed and analyzed for stability and resistance to MTX and trimetrexate. The K_i values of the Phe31Ala/Phe34Ala mutant are >10 000-fold higher for MTX than wild-type values, but only 13.5-fold higher for trimetrexate.[259]

7. Conclusion

Inhibition of the folate pathway enzymes in the past decade continued to be an area of intensive efforts, both in academia and in industry. The ubiquitous nature of these enzymes provided the basis to target several indication areas. Thus, in addition to antifolates aimed at combating bacterial pathogens, especiallly those involved in opportunistic infections, fungi, protozoa, and, in particular, cancer cells remain an area of high interest. Antifolate research has become an exercise field for scientists from different disciplines to demonstrate the power of modern methodologies to contribute to better understanding of the basic processes in the folate biosynthesis, in the utilization of folates, and in resistance mechanisms to antifolates. The combination of X-ray crystallography of numerous enzymes from different biological sources, molecular modeling, and skilled synthetic work resulted in design and synthesis of many hundreds of antifolates and to the identification of almost a dozen of new investigational drugs. A number of these drugs have reached the market. New discoveries in the folate pathway, greatly aided by the application of genomic and proteomic tools, not only improved our general understanding of this key pathway in all living cells, its conservation, and its modifications but also offer new possibilities for drug discovery.

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