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2 # Perturbation of genome integrity to fight pathogenic microorganisms

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Abstract

Background: Resistance against antibiotics is unfortunately still a major biomedical challenge for a wide range of pathogens responsible for potentially fatal diseases.

Scope of Review: In this study, we aim at providing a critical assessment of the recent advances in design and use of drugs targeting genome integrity by perturbation of thymidylate biosynthesis.

Major Conclusion: We find that research efforts from several independent laboratories resulted in chemically highly distinct classes of inhibitors of key enzymes within the routes of thymidylate biosynthesis. The present article covers numerous studies describing perturbation of this metabolic pathway in some of the most challenging pathogens like *Mycobacterium tuberculosis*, *Plasmodium falciparum*, and *Staphylococcus aureus*.

General Significance: Our comparative analysis allows a thorough summary of the current approaches to target thymidylate biosynthesis enzymes and also include an outlook suggesting novel ways of inhibitory strategies.

Highlights

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32   •   critical assessment of approaches against enzymes in thymidylate biosynthesis
33   •   detailed assessment of perturbation strategies to inhibit various families of dUTPases,
34   thymidylate synthases and dihydrofolate reductases
35   •   focus on the biomedically most challenging pathogens
36   •   identification of novel strategies by e.g. proteinaceous inhibition

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Abbreviations

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42   CH_2THF: 5,10-methylene tetrahydrofolate, DCD: dCTP deaminase, DCD-DUT:bifunctional
43   dCTP deaminase − dUTPase, DUT: dUTPase, DHF: dihydrofolate, DHFR: dihydrofolate
44   reductase, DHFR-TS bifunctional dihydrofolate reductase − thymidylate synthase, FAD:
45   flavin adenine dinucleotide, SHMT: serine hydroxymethyltransferase, THF: tetrahydrofolate,
46   TK: thymidine kinase, TS, ThyX: classical and flavin-dependent thymidylate synthase,
47   TMPK: dTMP kinase, Ugi: uracil-DNA glycosylase inhibitor, UNG: uracil-DNA
48   N-glycosylase. Abbreviations of organisms are listed in Table 1.

Background

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50 Despite huge efforts in antimicrobial drug design, numerous microbes still present excessive

51 biomedical challenge all around the world, in less developed and highly developed countries

52 alike. Major reasons for this unfortunate situation include several factors. Among these, high

53 mutation rate in many pathogenic organisms that may be an inherent characteristic of bacterial

54 species (e.g. presence of error-prone polymerases in *Mycobacterium tuberculosis* may lead to

55 resistant strains [1]). Also, the over-excessive use of antibiotics drives the development of

56 resistance. In this respect it is important to note that overuse of antibiotics in animal

57 agriculture is a major hazard especially since these are frequently the very same chemical

58 compounds that are used in human medicine. Antibiotics in the livestock and poultry feed

59 presents a low-level exposure over long periods of time that strongly contribute to the

60 appearance of resistant microbial strains. Wide availability of antibiotics is also a concern.

61 Although in some countries, there is an increasingly cautious attitude towards prescribing

62 antibiotics in minor illnesses, still, in many situations, prescriptions are easy to obtain for

63 larger amounts of antibiotics, sometimes even as over-the-counter-drugs, as well. In addition

64 to all these, patients carrying pathogenic microbes may easily spread the infection among

65 different populations as traveling is largely facilitated *via* flights and other means of transport.

66 The most efficient way to overcome the problems of microbial resistance against drugs is to

67 focus on new pathways, to discover novel molecular targets such that the strains that became

68 resistant against the "old" drugs could not rely on the already developed resistance

69 mechanism. Despite the general agreement in these questions, the number of novel antibiotics

70 with a new molecular mechanism of action developed over the last decades is very low. This

71 is perhaps at least partially, but maybe mostly, due to the fact that the development of

72 antibiotics is not among the most profitable actions for a drug company. Development of new

73 drugs is a very complex and costly project, with inherent problems relating to unexpected

74 side-effects when a pathway or protein is targeted that has not been previously among those

75 affected by drugs in clinical use. Approval of novel drugs is also a lengthy and expensive

76 process. These factors are deterrent for large pharmaceutical companies and contribute to the

77 present suboptimal situation.

78 No matter how few truly novel antimicrobial drugs have been introduced in the clinical use

79 during the last years, the basic research background is much flourishing. Even a quick and

80 superficial view on the number and variety of studies published in this context strongly argues

81 for the high momentum in the academic research on these fields. The present study attempts

82 to cover most of the recent developments, focusing on investigations related to inhibition of

83 enzymes involved in thymidylate biosynthesis as a novel target in multiple pathogenic

84 microorganisms (Table 1).

Genome integrity and thymidylate biosynthesis – why it is a promising field for novel drug candidates?

87 For an effective drug candidate, the target protein has to be essential for the given organism.

88 Microbe-specific enzymes are traditionally considered to be the best targets, however, in

89 many instances, enzymes where homologues are also present in the host organism still may be

90 good targets for design of antimicrobial diseases. Hence, the wealth of information gained on

91 more complex eukaryotic systems, e.g. in cancer research may also be used in the research

92 against infectious microbes. The common denominator in these issues may be found when we

93 focus on the joint characteristics of infectious microbes and cancer cells – namely, during

94 acute phases of the disease, these cells are all multiplying very fastly and drugs interfere with

95 this increased division cycle. In the present study, we focus on thymidylate biosynthesis that

96 is part of the preventive DNA repair pathways and responsible for maintaining genome

97 integrity (Figure 1). Perturbation of this metabolic pathway was proven to be an important

98 pharmaceutical strategy for both cancer and microbial cells.

99 Thymidylate biosynthesis shows several characteristics different from the biosynthesis of

100 other nucleic acid building blocks (Figure 1). Most importantly, the *de novo* biosynthesis of

101 the thymine base is realized at the monophosphate nucleotide level from dUMP, provided

102 mostly by the dUTPase enzyme, through the catalytic action of thymidylate synthases (TS or

103 ThyX). The methyl group transfer to the 5'-position in the pyrimidine ring requires complex

104 biochemical catalysis. Thymine containing nucleotides can also be regained by salvage

105 pathways through thymidine kinase. The rather limited and constrained routes in thymidylate

106 biosynthesis, together with its essentiality for DNA biosynthesis designates the enzymes

107 within these pathways as key targets for development of antimicrobial drugs, while the human

108 homologues of these enzymes are also very important entities in anticancer chemotherapy.

109 Nevertheless both thymidylate synthase and dihydrofolate reductase enzymes are heavily

110 studied primary enzymatic targets of antibacterial/antiparasite therapy [2,3]. However, there

111 are other important drug targets amongst the folate pathway enzymes, for example

112   dihydropteroate synthase. Due to the indirect connection of these enzymes to dTTP

113   biogenesis, detailed presentation of these are out of the scope of this review.
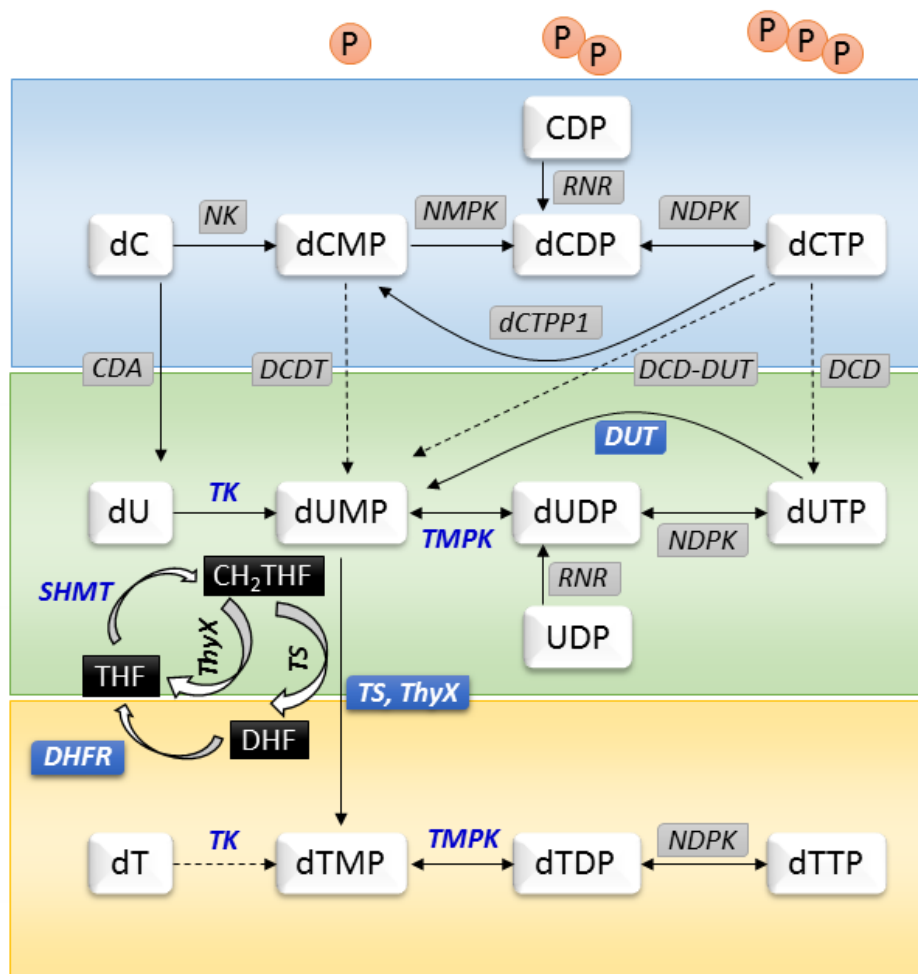
114   Below, we will summarize in a comparative manner the current advances in application of

115   enzymes involved in thymidylate biosynthesis as drug targets in several pathogens that still

116   present major biomedical challenge (Table 1).

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118   **Table 1 List of organisms and their human relevance included in this study**

Organism (Abbreviation)	Human relevance	Section
Bacillus anthracis (Ba)	etiologic agent of anthrax	DHFR, TMPK
Campylobacter jejuni (Cj)	gastric pathogen	DUT
Candida glabrata (Cg) *Candida albicans (Ca)*	systemic infection agent	DHFR
Cryptococcus neoformans	causative of fungal meningitis and encephalitis	TS
Cryptosporidium hominis, (Ch) *Cryptosporidium parvum (Cp)*	gastric pathogen	DHFR-TS, TK
Enterococcus faecalis (Ef)	causative of urinary tract infections and endocarditis	TS, DHFR
Helicobacter pylori (Hp)	gastric pathogen	ThyX
Klebsiella pneumoniae (Kp)	causative of urinary and respiratory tract infections	DHFR
Lactobacillus casei (Lc)	*Lc*TS is a model protein of classical TSs	TS
Leishmania donovani (Ld)	causative of visceral leishmaniasis (black fever)	DHFR-TS
Leishmania major (Lm)	causative of cutaneous leishmaniasis	DUT, DHFR-TS, TK
Mycobacterium tuberculosis (Mt)	causative of tuberculosis	DUT, DHFR ThyX,
Paramecium bursaria chlorella virus-1 (PBCV)	*PBCV*-ThyX is a model protein of ThyX	ThyX
Plasmodium falciparum (Pf) *Plasmodium vivax (Pv)* *Plasmodium ovale (Po)*	causatives of malaria	DUT, DHFR-TS
Pneumocystis carinii (Pc) *Pneumocystis jirovecii (Pj)*	causatives of *Pneumocystis* pneumonia	TS, DHFR
Pseudomonas aeruginosa (Pa)	causative of urinary tract and blood infections	TMPK
Staphylococcus aureus(Sa)	causative of skin and respiratory infections	TS, DHFR, TMPK
Staphylococcus epidermidis (Se)	causative agent of endocarditis, infects medical catheters and prostheses	TS
Streptococcus mutans (Sm)	major aetiological agent of dental caries	DHFR
Toxoplasma gondii (Tg)	causative of toxoplasmosis	DHFR-TS
Trypanosoma brucei (Tb)	causative of African sleeping sickness (African trypanosomiasis)	DUT, DHFR-TS, TK
Trypanosoma cruzi (Tc)	causative of Chagas disease (American trypanosomiasis)	DUT, DHFR-TS, SHMT

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Figure 1. Summary of the key steps in pyrimidine biosynthesis. Enzymatic reactions that are not ubiquitous are represented by dotted lines. Traditional antiparasitic drug target enzymes of the thymidylate biosynthesis are shown with white lettering on blue background, recently verified target enzymes are denoted with blue lettering. Abbreviations are as follows: CDA: cytidine deaminase, CH_2THF: 5,10-methylene tetrahydrofolate, DCD: dCTP deaminase, DCD-DUT:bifunctional dCTP deaminase – dUTPase, DCDT: dCMP deaminase, dCTPP1: dCTP pyrophosphatase 1, DUT: dUTPase, DHF: dihydrofolate, DHFR: dihydrofolate reductase, NDPK: nucleoside-diphosphate kinase, NK: nucleoside kinase, NMPK: nucleoside monophosphate kinase, RNR: ribonucleotide reductase, SHMT: serine hydroxymethyltransferase, THF: tetrahydrofolate, TK: thymidine kinase, TS, ThyX: classical and flavin-dependent thymidylate synthase respectively, TMPK: dTMP kinase.

1. dUTPase inhibition

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135   The role of the dUTPase enzyme, catalyzing the hydrolysis of dUTP into dUMP and

136   inorganic pyrophosphate is dual: on the one hand, it provides the dUMP precursor for dTTP

137   *de novo* biosynthesis, while on the other hand, the enzyme keeps the level of cellular dUTP at

138   a low value such that to prevent incorporation of uracil moieties into DNA [4]. This

139   preventive action has great significance due to the suboptimal specificity of most DNA

140   polymerases that will incorporate either dUMP or dTMP against the dAMP, simply depending

141   on the cellular availability of dUTP versus dTTP. There are two major families of dUTPases:

142   the trimeric dUTPases are especially widespread, while the dimeric dUTPases are found in

143   some parasites and some phages (Figure 2) [4,5]. The overall structures as well as active site

144   close-ups for these two dUTPase families are shown in Figure 2. In addition to the dUTP

145   substrate, these active sites provide a specific environment for the catalytic water molecule

146   that initiates nucleophilic attack at the α- or β-phosphorus atom (for trimeric, and dimeric

147   dUTPases, respectively). Divalent metal ions also play an important role in the catalysis: in

148   trimeric dUTPases, Mg(II) ion is present coordinating to the triphosphate chain, whereas in

149   dimeric dUTPases, two divalent metal ions are involved in the catalytic steps.

150   Considering that the human dUTPase also belongs to the trimeric family, the dimeric enzyme

151   family (present in some parasites) perhaps provides a more straightforward dUTPase target,

152   with potentially less side effects due to the large differences in the pathogen and host enzymes

153   (cf Table 2). Such efforts singled out very effectively the dUTPases from *Leishmania*,

154   *Trypanosoma* and *Campylobacter* species. Essentiality of dUTPases in these organisms was

155   investigated and established in *Trypanosoma brucei* by RNAi [6]. In these cases,

156   dissimilarities between the dUTP binding sites of the pathogen and the human enzymes

157   presented a clear-cut starting point for development of drug candidates presumably showing

158   less side effects [7–9]. Inhibitor design was performed by analyzing the high-resolution 3D

159   crystal structures based on either small scale rational design or large scale *in silico* screening

160   [10].

161   With regard to the trimeric family of dUTPases, two major pathogens were at the focus of

162   relevant research efforts in targeting dUTPase (Table 2). Namely, *Mycobacterium*

163   *tuberculosis* and *Plasmodium falciparum*, the causative agents of tuberculosis and malaria,

164   respectively. In both of these organisms, the significance of the dUTPase enzyme in

165   thymidylate biosynthesis is strongly enhanced due to the lack of salvage pathways [4,11]. The

166   3D structures of *M. tuberculosis* and *P. falciparum* dUTPases were published in 2004 and

167   2005, respectively [12,13]. These structures formulated the basis for further studies. A quick

168   and reliable dUTPase activity assay developed for *M. tuberculosis* dUTPases facilitated

169   further high-throughput searches for inhibitor molecules potentially targeting species-specific

170   segments in *M. tuberculosis* dUTPases [14]. An integrated approach starting from the ZINC

171   molecule database and using more than 2 million drug-like compounds in a virtual screening

172   provided a short list of promising candidates [15,16]. Importantly, this study utilized an

173   innovative novel concept that can be possibly termed as "allosteric drug design". Namely, in

174   order to avoid potential side effects, the protein surface that was targeted in this study

175   constituted by an essential species-specific segment of the mycobacterial dUTPase [17], in the

176   vicinity of the highly conserved active site. The best performing drug candidates were

177   successfully tested in *in vitro* assays and animal models (guinea pig) as well [15]. This

178   approach showed a model case for a highly potential integrated method in drug design starting

179   from the already available drug compound library.

180   Similar high throughput searches were carried out in compound libraries of several thousands

181   of candidate molecules for *Plasmodium falciparum* dUTPases [18]. The resulting

182   considerations focused on the important observation that the uracil ring is of utmost

183   importance in binding of ligands to the active site, whereas more variation is allowed at the 3'

184   and 5' substituents [19]. Selectivity of the drugs against the pathogen and the human enzyme

185   was also tested and the best performing drug candidates showed more than 200-fold

186   selectivity [20]. This result is suggested to be further combined with transfer factors that may

187   facilitate accumulation of the drugs within the parasite. Ongoing research in this field very

188   recently focused on QSAR models facilitating design of novel compounds [21].

189   In spite of the vast number of studies and approaches there is still much to do in this field to

190   arrive at a clinically tested drug candidate that may be the focus of further processes

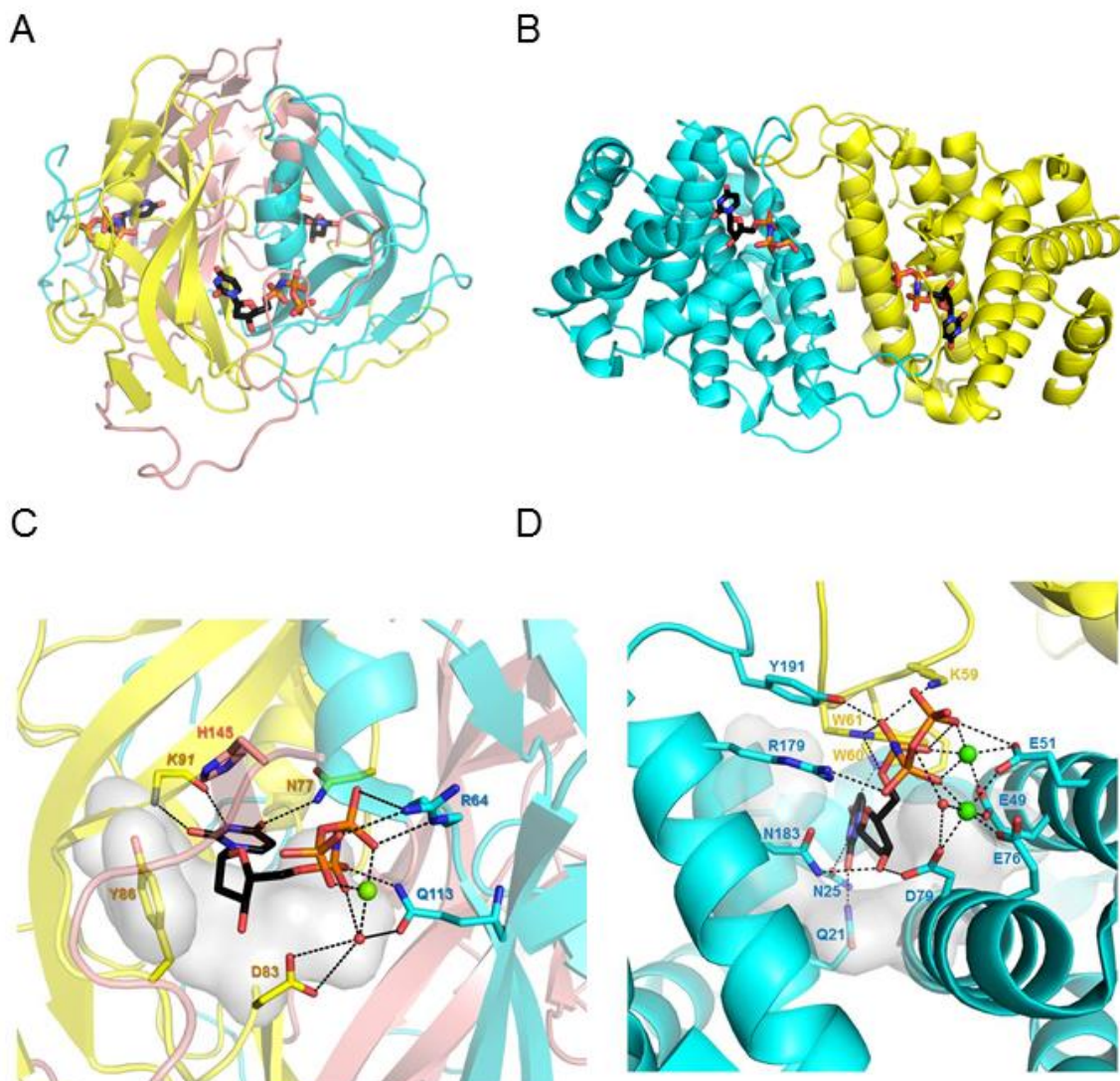191   potentially resulting in approval of dUTPase inhibitors as antimicrobial drugs.

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Figure 2. Comparison of trimeric and dimeric dUTPases. A) Cartoon representation of the trimeric *Mycobacterium tuberculosis* dUTPase (PDB ID:2PY4, [14]), chains colored by yellow, salmon and cyan. Substrate analogue dUPNPP shown as sticks with atomic coloring (carbon: black, oxygen: red, phosphorus: orange, nitrogen: blue) **B)** Cartoon representation of the dimeric *Leishmania major* dUTPase (PDB ID:2YAY, [7]), chains colored by yellow and cyan, substrate analogue dUPNPP shown as sticks with atomic coloring (carbon: black, oxygen: red, phosphorus: orange, nitrogen: blue). **C)** Active center of *Mt*Dut (PDB ID:2PY4, [14]), colored as in panel A, residues important for substrate binding and catalysis are shown as sticks with atomic coloring (carbon: colored by chain, oxygen: red, nitrogen: blue), catalytic magnesium and water as green and red spheres, respectively. Substrate analogue dUPNPP shown as sticks with atomic coloring as in Panel A. Dashed lines represent hydrogen-bonding interactions. **D)** Active center of *Lm*Dut (PDB ID:2YAY, [7]) colored as in Panel B, residues important for substrate binding and catalysis are shown as sticks with atomic coloring (carbon: colored by chain, oxygen: red, nitrogen: blue), catalytic calciums and water as green and red spheres respectively. Substrate analogue dUPNPP shown as sticks with atomic coloring as in Panel B. Dashed lines represent hydrogen-bonding interactions.

211   **Table 2.** Recent results on the inhibition of dUTPases

Organism	Year	Summary	Ref.
Leishmania major	1997	Identifying *Lm*Dut. Lysates of *E. coli* showed significant dUTPase activity increase after orthologous expression *Lm*Dut gene.	[22]
	2000	Verification of dimeric form of *Lm*Dut. dUDP and dUTP are both substrates of *Lm*Dut (dUDP is inhibitor of trimeric dUTPases).	[23]
	2001	Kinetic parameters of *Lm*Dut for dUTP hydrolysis are comparable to that of *h*Dut. Dissimilarities in the binding of dUDP and dUMP as compared to the human enzyme suggest differences in the structure of the active sites.	[24]
	2011	Crystal structure of *Lm*Dut in complex with substrate analogues, product dUMP, and a substrate fragment (dU). Very tight ligand binding pocket modifications at the uracil or ribose rings might perturb binding of an inhibitor. The presence of a single phosphate or similarly charged group is important to induce ligand binding conformation of *Lm*Dut.	[7]
Trypanosoma cruzi	2004	Crystal structure of *Tc*Dut. Major differences between the substrate binding pocket of dimeric and trimeric dUTPases provides potential for selective inhibitor design. It was observed for the first time that ligand binding induces large conformational change in case of dimeric dUTPases.	[8]
	2006	Inhibitor design based on *in silico* docking. No *in vivo* and *in vitro* effects of the compounds. Protein flexibility has to be taken into account.	[10]
Trypanosoma brucei	2008	The dimeric *Tb*Dut is a nuclear enzyme and down-regulation of its activity by RNAi proved that *Tb*Dut is indispensable for efficient cell cycle progression and DNA replication in *T. brucei*	[6]
	2013	Conditional Dut knockout without adding thymidine caused impaired proliferation and lethality in *T. brucei*. Adding uracil, uridine or deoxyuridine could not rescue this phenotype. dUTPase has major role in the provision of pyrimidine nucleotides in kinetoplastids.	[25]
	2013	Crystallographic and NMR studies revealed that similarly to *Cj*Dut in case of *Tb*Dut nucleophilic attack also occurs on the β-phosphate of the substrate. Unlike in the trimer enzymes in case *Tb*Dut one of the divalent metal ions plays direct role in catalysis.	[26]
Campylobacter jejuni	2004	The crystal structure of *Cj*Dut. Mg^{2+} is important in enzymatic action. It was shown for the first time, that nucleophilic attack occurs on the β-phosphate in contrast to the trimeric enzymes, where it happens at the α-phosphate. Ligand binding causes large conformational change so as in the case of *Tc*Dut [8].	[9]
	2009	Difference in inhibition constants as compared to the trimeric dUTPase enzymes permits the design of specific inhibitors of *Cj*Dut.	[27]
	2011	Crystal structure of *Cj*Dut with dUPNPP substrate analogue contains only two metal ions at the active site, while in the dUPNP complex three of those	[7]

		were indentified. Glu49 is flipped away from the active site in the presence of the triphosphate, and no longer coordinates any of the metal ions.	
Mycobacterium tuberculosis	2004	Crystal structure of *Mt*Dut reveals that its binding pocket is similar to that of *h*Dut, hampering the design of *Mt*Dut specific inhibitors. Tris molecule in the trimer channel interface might be an inhibitor lead.	[12]
	2008	Identification of a bifunctional dCTP deaminase dUTPase in *M. tub*. Lower similarity to *h*Dut and broader substrate specificity than *Mt*Dut marks this enzyme a possible target for chemotherapy.	[28]
	2008	Introducing a highly sensitive fluorescent label to follow the *Mt*Dut enzymatic reaction. Structure and activity of H145W *Mt*Dut is not altered.	[14]
	2011	Molecular modeling of *Mt*Dut nucleotide binding, based on activation energies from QM-MM modeling hydrolysis is slower than product release.	[29]
	2012	The dUTPase enzyme is essential in *Mycobacterium smegmatis*. Mycobacteria-specific loop has no major effect on *Mt*Dut activity *in vitro*, but a loop-specific function seems to be essential within the *in vivo* model *M. smegmatis*.	[17]
	2015	Virtual screening of several million small molecules against the species-specific surface loop of *Mt*Dut was performed. An optimized hit was conjugated to a phagocytosis stimulating tuftsin peptide derivative and encapsulated into PLGA nanoparticles. *In vivo* efficacy of this formulation was verified in guinea pig model.	[15]
	2016	The prevention of DNA uracilation and the regulation of dNTP balance are decoupled in Mycobacteria and separately achieved by Dut and Dcd:dut enzyme functions, respectively.	[30]
Plasmodium falciparum	2005	Development of selective inhibitor leads against *Pf*DUT with antiparasite activity. Crystal structure of inhibitor bound *Pf*Dut.	[13]
	2005	Selective, nontoxic, drug-like inhibitor lead design against *Pf*Dut. Analogues of dUMP with variety of substituents at the 5'- and 3'-positions. Effectivity is not sufficient against *Leishmania* and *Trypanosoma* parasites.	[31]
	2006	Acyclic uracil derivatives with similar or better antiplasmodial properties then those in Ref [31] especially with regards to selectivity. K_i of best active compound was 0.2 μM.	[32]
	2007	Study of *Pf*Dut ligand binding. No significant conformational changes upon binding are inferred based on ITC measurements.	[33]
	2009	Tritylated uracil acetamide derivatives containing amide bond between the β-C and N-1 of uracil ring were found to be weak inhibitors of the *Pf*Dut.	[34]
	2010	Study on *Pf*Dut and *h*Dut kinetics. Specific product inhibition of the Plasmodium dUTPase compared to the human enzyme, was caused by the substituent at the C-5 position of the uracil ring.	[35]
	2011	*In vitro* HTS for *Pf*Dut inhibitors in a 3086 item compound library of commercially available non-proprietary compounds did not identify any hits.	[18]
	2011	Modification on the uracil ring of the tested compounds impaired inhibitory	[36]

		effect of those on *Pf*Dut. While there is room for variation of the 5'-trityl group and the 3'-substituent.	
	2011	Testing β-branched acyclic uridine analogues as *Pf*Dut inhibitors. K_i of the best inhibitor was 0.5 μM. This showed more than 200-fold selectivity compared to *h*Dut and EC_{50} = 0.61 μM growth inhibition of *P. falciparum*.	[20]
	2011	Mutational analysis of residues important in binding of uracil based inhibitors containing trityl groups at the 5'-position. F46A mutation of *Pf*Dut leads to an increase in K_i values while K96A mutation has an opposite effect.	[19]
	2011	Testing lower lipophilicity and molecular weight diphenyl substituted inhibitors of *Pf*Dut. Slightly decreased activity against both dUTPase and parasite than the corresponding trityl derivatives was observed.	[37]
	2013	Characterization of novel conformationally restrained amide derivatives to overcome entropic disadvantages of former *Pf*Dut inhibitors. Inhibitors showed similar or greater potency but lower selectivity (<40x) in cellular assays, than the previous drug candidates against *Pf*Dut.	[38]
	2015	2D- and 3D-QSAR model using the LQTA-QSAR methodology on a series of *Pf*Dut inhibitors with high predictive power facilitates the design of new compounds with higher antimalarial bioactivities.	[21]

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2. Inhibition of thymidylate synthases

Thymidylate synthase enzymes can be divided to two distinct categories: the so-called "classical" (TS) and flavin-dependent (ThyX) enzymes (Figure 3). These two types of thymidylate synthase enzymes share no mechanistic and structural similarity, however they perform the same enzymatic function (an interesting parallel to the two families of dUTPases, trimeric and dimeric). The flavin-dependent ThyX catalyzes FAD mediated methyl group addition to dUMP from 5,10-methylene tetrahydrofolate (CH_2THF), resulting in dTMP and tetrahydrofolate in prokaryotes [39]. Recovery of CH_2THF from the reaction products is performed by serine hydroxymethyltransferase. In contrast, in case of the classical thymidylate synthases (TSs), which are the product of gene *tyms* in eukaryotes and gene *thyA* in prokaryotic systems (often referred as ThyA), the methyl group is directly transferred from CH_2THF to dUMP and the resulting dihydrofolate is recycled through the consecutive action of dihydrofolate reductase and serine hydroxymethyltransferase. The "classical" enzymes can be divided into two subgroups since some of those are bifunctional DHFR-TSs (Figure 3), although the TS domains of the bifunctional enzymes are highly similar to monofunctional TSs in terms of structure and catalytic mechanism. As a consequence of key importance of both kinds of thymidylate synthases in thymidine biosynthesis, those are subjects of extensive inhibitor design programs.
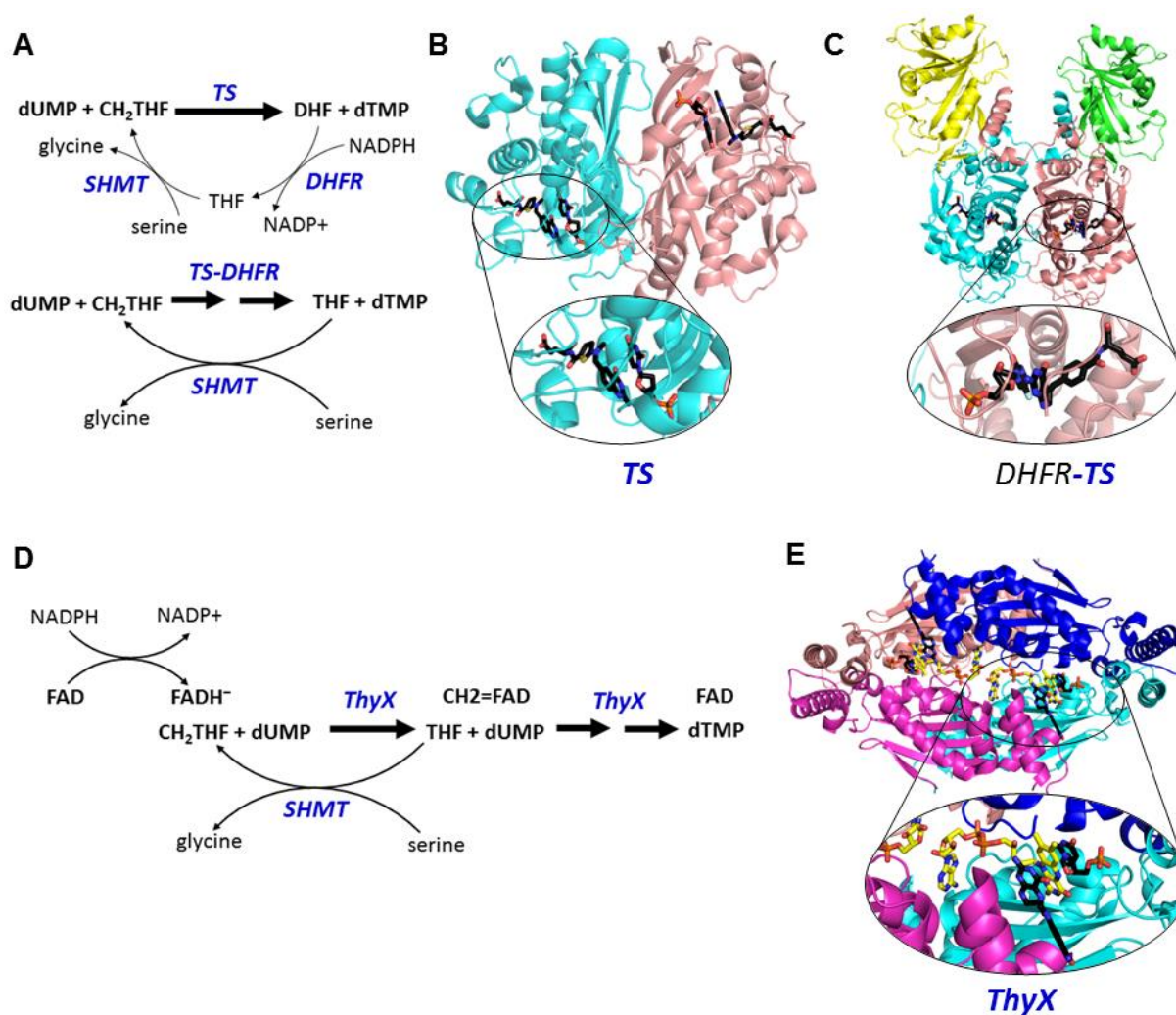
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Figure 3. Comparison of catalytic mechanism and structure of different thymidylate synthases. A) Classical thymidylate synthase reaction cycle **B)** Structure of *M. tuberculosis* classical thymidylate synthase – *Mt*TS (PDB ID: 4FOX) Protein is in cartoon representation, monomers colored cyan and salmon. dUMP and folate analogue inhibitor raltitrexed represented as sticks with atomic coloring (carbon: black, oxygen: red, phosphorus: orange, nitrogen: blue). Close up enlightens the ligand binding orientation. **C)** Structure of *Ch*DHFR-TS (PDB ID: 4Q0D). Protein is in cartoon representation, TS domains with cyan and salmon, the corresponding DHFR domains with yellow and green. 5F-dUMP, and folic acid analogue 2XB (from Ref [40]) represented as sticks with atomic coloring (carbon: black, oxygen: red, phosphorus: orange, nitrogen: blue). Close up enlightens ligand binding orientation. **D)** ThyX mechanism according to ref. [39]. **E)** *Thermotoga maritima* ThyX (PDB ID: 4GT9) Protein is in cartoon representation, subunits of the tetramer colored cyan, blue, magenta and salmon. dUMP, CH$_2$THF substrates and FAD cofactor represented as sticks with atomic coloring (substrate carbon: black, cofactor carbon: yellow, oxygen: red, phosphorus: orange, nitrogen: blue) To ease visualization, substrates from only two substrate binding pockets are shown. Close up enlightens ligand binding orientation of one of the active sites.

2.1. Inhibitors of classical thymidylate synthases

Although the structure of the "classical" thymidylate synthases is highly conserved, several drug design programs still attempt to exploit the slight differences between pathogenic and human enzymes to design selective inhibitors of parasite TSs (Table 3).

Comparing the structures of TS enzymes from bacteria and human it can be concluded that the conformation adopted by the enzymes upon binding of dUMP and folate-analog inhibitors is analogous [41]. The difference in the degree of active site closure could be a key to discriminate bacterial TSs against eukaryotic TSs and may provide basis for the design of species-specific non-folate analogues [41]. Towards this end *Lactobacillus casei* thymidylate synthase was studied as a model for TS enzymes from pathogens such as *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus anthracis,* since the dimer interface loop region of these proteins is highly similar and both contain a small domain of fifty amino acids (residues 90-139 in *Lc*TS), which is not present in the *E. coli* and human TSs [42–44].

First studies identified phenolphthalein and its derivatives as *Lc*TS inhibitors with micromolar activity [45–47]. Extending the phthalimidic core resulted in 1,8-, 2,3- naphthalein compounds binding to *Lc*TS at sub-micromolar concentrations while showing no detectable affinity for the human enzyme [48]. Several of these derivatives also showed selective antibacterial activity against other bacteria, such as *S. aureus, Streptococcus* and *Cryptococcus neoformans* in cell culture assays [49]. Nonetheless, crystal structures of complexes of *C. neoformans* and *E. coli* enzymes with one of the best active compounds did not provide a clear explanation for the origin of selectivity [50].

In parallel to these studies dansyl-tyrosine derivatives with micromolar affinities to bacterial TSs were identified as promising leads for drug design [51]. These compounds exploit the flexibility of the folate-binding site of bacterial TSs and possess enhanced specificity because of the interactions formed with non-conserved residues outside the active site of the bacterial enzymes [43,52].

Subsequent development programs were focused only on the enhancement of the affinity and specificity of the naphthalein series. Optimization resulted in 1,2-naphthalein derivatives showing significant and dose dependent antibacterial potency against *Staphylococcus epidermidis* clinical isolate strains, without any signs of *in vitro* host toxicity and was also potent against *Enterococcus faecalis* and *Staphylococcus aureus* [53]. Importantly, the bacterial strains in this study were resistant to most of the best known antibacterial drugs,

282 including vancomycin, hence this inhibitor can be regarded as a promising future antibacterial

283 agent [53].

284 Since the observed multiple binding modes of these naphthalein derivatives significantly

285 impeded structure-based drug design, recent studies aimed to find inhibitors displaying a

286 unique binding mode [54]. Therefore, starting from phenolphthalein, and following a

287 retrosynthetic approach two compound libraries of two different scaffolds were designed [54].

288 X-ray crystallographic screening of the greatest potency hits revealed that some of those

289 present in a common unique binding mode. Further improvement of these initial hits resulted

290 in carboxamide derivatives with moderate, albeit specific potency against *Ec*TS [55].

291 Following this deconstruction approach, drug design studies starting from a complex *h*TS

292 inhibitor led to compounds, which displaced dUMP from its classical active site position in

293 the ternary complex. One of these inhibitors showed K_i=0.31μM, and more than twenty-fold

294 selectivity against *Pc*TS [56]. Initial hits for the *Enterococcus faecalis* TS enzyme are also

295 reported in the same study, which could be optimized in the future based on the recently

296 published crystal structure of *Ef*TS [57]. In addition the fact that 5-formyltetrahydrofolate co-

297 purifies with *Ef*TS from cell extract and is observed at the active site of *Ef*TS in the crystal

298 structure raises fundamental questions about how the folate and thymidylate biosynthesis are

299 coupled and regulated in these bacteria [57].

300 The thymidylate synthases of protozoan parasites like Plasmodia and Trypanosoma are fused

301 to dihydrofolate reductase forming a bifunctional enzyme. In these cases drug development

302 focuses mostly on DHFR inhibition (see section 3.2.).

303

304   **Table 3.** Recent results on the inhibition of classical thymidylate synthases (TS)

Organism	Year	Summary	Ref.
Lactobacillus casei	1999	Several crystal structures of *Lc*TS bound to dUMP and phthalimidic derivatives, which are designed to selectively inhibit TS enzymes of pathogenic species.	[48] [49]
	1999 2001	Structure-based design of dansyl-tyrosine derivatives, which specificly inhibit bacterial TSs, possibly due to interactions formed with non-conserved residues close to the active site.	[51] [52]
	2005	Molecular dynamics simulations explaining activity and species-specificity of the best active compound from Ref [51] based on the predicted binding mode of the inhibitor to *Lc*TS.	[43]
	2008	Changing the rigidity of dansyl-tyrosine derivatives oppositely alters effectivity of those against *Lc*TS and *Ec*TS.	[58]
	2011	Retrosynthetic method to design novel compounds exhibiting unique binding modes to *Lc*TS led to promising candidates having single binding orientation.	[54]
Pneumocystis carinii	2000	Based on the crystal structure the fungal specific β-sheet and the greater size of the active site of *Pc*TS can be exploited for specific antifungal drug design.	[47]
	2003	Greater flexibility of parasite TSs compared to eukaryotic enzymes allow the design of specific antiparasite agents.	[41]
	2013	Following of a deconstruction synthesis approach starting from a complex *h*TS inhibitor resulted in novel specific inhibitors of *Pc*Ts with low micromolar affinity (best compound showed K_i of 0.31μM and more than 20-fold selectivity).	[56]
Enterococcus faecalis	2006	In an extensive antibacterial TS inhibitor study a 1,2-naphthalein derivative proved to have MIC of 2.5μg/mL against *E. faecalis*.	[53]
	2011	Identification of a benzonitrile substituted dioxo-isoindol derivative as a good initial candidate for *Ef*TS inhibitor design.	[54]
	2012	The crystal structure of *E. faecalis* thymidylate synthase serving as a basis of specific inhibitor development. 5-formyltetrahydrofolate found at the active site of *Ef*TS introduces questions about the connections between the folate and thymidylate biosynthesis in *E. faecalis*.	[57]
	2013	A retrosynthetic inhibitor design starting from a complex *h*TS inhibitor resulted in only moderately potent drugs against *Ef*TS.	[56]
Staphylococcus aureus	2006	Several 1,2- and 1,8-naphthalein acting as TS inhibitors were proven to have MIC of 0.5-5 µg/mL against *S. aureus*.	[53]
Staphylococcus epidermidis	2006	During a thorough antibacterial TS inhibitor study a 1,2-naphthalein derivative showing significant and dose dependent potency against *S. epidermidis* clinical isolate strains was identified.	[53]

305

306

2.2. Inhibitors against flavin-dependent thymidylate synthase, ThyX

ThyX, a thymidylate synthase with drastically different active-site geometries and distinct enzymatic mechanisms as compared to the classical TS isoenzymes, has been discovered in 2002 [59–61]. The flavin-dependent ThyX applies a unique chemical cascade that does not follow the general scheme of biological methylation, but uses the following steps instead: i) activation of the nucleotide that involves no covalent modification but only electrostatic polarization of dUMP by the enzyme's active site ii) methylene transfer from folate mediated by N5 of FAD [39] (Figure 3). As a consequence of the marked alterations in chemical mechanism from that of the classical thymidylate synthases along with the observed structural differences, inhibitors of classical TSs have only a reduced effect on ThyX enzymes [62–65].

The exclusive existence of a distinct class of thymidylate synthase in a plethora of major pathogenic microorganisms including also *Bacillus anthracis*, *Clostridium botulinum*, *Mycobacteria* and *Treponema pallidum*, opened a new horizon for developing of antibiotic inhibitors with reduced toxicity [66] (Table 4). The emergence of mutations diminishing the effectivity of classical antifolate drugs against classical TSs of these organisms also underlines the high relevance of this novel approach [67]. Mutational studies showing that ThyX, but not the classical TS is essential in *M. tuberculosis* also supports the validity of the flavin-dependent enzyme as a drug development platform [68].

Structure based design is aided by crystal structures of ThyX enzymes from several species [62,65,69–75]. Extensive studies on numerous substituted deoxyuridine monophosphate derivatives resulted in drugs that clearly distinguished between *Mt*ThyX and *Mt*TS and showed, albeit moderate, inhibition on *Mt*ThyX, but not on *Mt*TS. However, some of these drugs also possessed dual potency being active against *Mt*TMPK as well [76–80]. High throughput screens for non-substrate analogue inhibitors of ThyX activity, using *Paramecium bursaria chlorella virus* (PCBV)-1 ThyX, a well-studied model for flavin-dependent thymidylate synthases [63,81,82], resulted in several selective inhibitors of *Mt*ThyX and *Hp*ThyX [83]. One of these, namely 2-bromo-8-hydroxy-1,4-naphthoquinone also showed cellular activity against genetically modified *E. coli* strains in which the chromosomal copy of TS was replaced by PBCV-1 ThyX. Further derivatization of this compound led to drug molecules displaying high potency against *Helicobacter pylori* ThyX and showing also modest, but significant activity in an animal infection model [84]. Since other similar

338   naphthoquinone derivatives have already passed clinical trials this scaffold seems to be very

339   promising target for ThyX inhibitor design [83].

340 **Table 4.** Recent results on the inhibition of flavin-dependent thymidylate synthase (ThyX)

Organism	Year	Summary	Ref.
Thermotoga maritima	2003	Crystal structure of *Tm*ThyX reveals an common fold of ThyX family.	[62]
	2012	X-ray crystal structures of *Tm*ThyX with several folate derivatives are serving as useful models for drug design. The previously proposed mechanism of arginine mediated methylene transfer was eliminated by study of mutant R174K *Tm*ThyX enzymes.	[73]
	2013	Conformational change is essential for substrate binding of *Tm*ThyX. Compounds locking open conformation of the substrate-binding loop might act as specific inhibitors.	[74]
Paramecium bursaria chlorella virus-1	2004	Analysis of FAD-dependent thymidylate synthase ThyX from PBCV supports that ThyX-specific inhibitors that do not affect classical TS enzymes can be designed.	[63]
	2006	Conformation of key residues at the active site of PBCV-1 ThyX differs from earlier reported ThyX structures, suggesting structural changes during catalysis. The reaction proceeds without methylene enzyme formation.	[81]
	2007	Report on benzoyl and triazole derivatives that demonstrated inhibition of the catalytic activity of PBCV1 ThyX.	[82]
		2-hydroxy-1,4-naphthoquinone derivatives (1,4-NQs) are tight binding inhibitors of PBCV-1 ThyX *in vitro* and *in vivo*.	[83]
	2014	Orchestrated fast reactions of the native substrates of ThyX, bypasses NADPH oxidase activity during the enzymatic reaction in aerobic microorganisms, enabling effective ThyX activity in oxygen rich cellular milieu.	[85]
Mycobacterium tuberculosis	2004	*Mt*TS mutations represent a pathway for development of antifolate drug resistance.	[67]
	2005	Crystal structure of *Mt*ThyX. ThyX enzymes are strongly conserved amongst evolutionarily distant organisms based on structural, functional and genomic comparisons.	[69]
	2006	Soaking of the crystal containing *Mt*ThyX-FAD-BrdUMP ternary complex into NADP+ solution results instead of a quaternary complex in a binary complex of NADP+ and Br-dUMP. For implication of data detailed mechanistic studies are required.	[70]
	2008	Extensive mutational study reveals a serine and a histidine as the key residues of *Mt*ThyX enzyme activity. It is still unclear which residues contribute to binding of methylenetetrahydrofolate (MTHFR) and NADPH.	[86]
	2008	Study of *Mt*TS and *Mt*ThyX kinetics shows that both enzymes have low catalytic activity. A folate-based inhibitor revealed high selectivity against *Mt*TS over *Mt*ThyX, entailing the possibility that reciprocal inhibitors of *Mt*ThyX may exist.	[64]
	2011	Testing substituted 2'-deoxyuridine monophosphate analogues against *Mt*ThyX.	[76]

		Best compound showed IC$_{50}$=0.91 μM and lacked activity against *Mt*TS (IC$_{50}$ > 50 μM).	
	2012	Identification of weak *Mt*ThyX inhibitors, which exhibited no activity against *Mt*TS Compounds were either substrate or inhibitor of *Mt*TMPK, this simultaneous action might be advantageous for drug design.	[77]
	2012	2-hydroxy-1,4-naphthoquinone derivatives (1,4-NQs) inhibit the activity of *Mt*ThyX and *Hp*ThyX, but not that of *h*TS. Other 1,4-NQs have passed clinical trials, which designates this scaffold a very promising target for ThyX inhibitor design.	[83]
	2013	5-alkynyl uridine analogues in which the sugar moiety has been replaced by an acyclic phosphonate were designed against *Mt*ThyX based on binding model from NMR data. Weak inhibition of ThyX is achieved (43% inhibition at 50 μM inhibitor concentration).	[78]
	2015	C-5 modified nucleosides with antimycobacterial activity were tested against thymidylate synthases of *M.tub*. These showed lack of activity against the *Mt*TS, while IC$_{50}$ of the best compound against *Mt*ThyX was 8.32 μM. Mechanism of action of these compounds could only partially be associated with the inhibition of *Mt*ThyX.	[79]
	2015	Inhibition of *Mt*ThyX by 5-FU is contributing to the mechanism of anti-mycobacterial action of this drug.	[87]
	2015	High throughput crystallization of *M. tuberculosis* proteins resulted in structures of *Mt*ThyX bound to FAD and FdUMP deposited in the PDB.	[75]
	2016	Mechanism of ThyX action fundamentally differs from that of classical TSs. The folate in this case transfers the methyl group not directly to dUMP but to the flavin cofactor. This is a hitherto unseen methylation scheme.	[39]
Helicobacter pylori	2002	Identification of the first flavin-dependent thymidylate synthase in *H. pylori*.	[59]
	2004	Based on a mutational study Ser 84 in *Hp*ThyX is responsible for dUMP activation.	[60]
	2011, 2012	Crystal structures and characterization of *Hp*ThyX enzyme aiding species specific drug design.	[71] [72]
	2015	2-hydroxy-1,4-naphthoquinone derivatives display potent inhibition of *Hp*ThyX activity. One of these has shown modest, but significant activity in an animal infection model.	[84]

341

3. Dihydrofolate reductase inhibitors

Dihydrofolate reductase is a well-validated therapeutic target of the folate pathway. There exist two major groups of these enzymes: the monofunctional DHFR and bifunctional DHFR-TS enzymes.

3.1. Monofunctional DHFR

Currently extensive studies are in progress against DHFRs of *Pneumocystis* and *Mycobacteria* since these parasites lack thymine salvage pathway and rely solely on *de novo* synthesis of this pyrimidine base [11,88] (Table 5). However, DHFRs of other pathogens also seem to be effective and highly studied targets (Table 5).

3.1.1. *Pneumocystis carinii*

The high resolution crystal structure of *Pc*DHFR initiated structure-based inhibitor design against this enzyme [89]. Starting from piritrexim by replacing the carbon linker (C9) to nitrogen and adding a methyl group to form a triamine resulted in series of pyrido-pyrimidine compounds with enhanced selectivity against *Pc*DHFR and *Tg*DHFR-TS [90]. Structure analysis confirmed that this N9-methyl group interacts more favorably with Ile123 present at the substrate binding site of *Pc*DHFR than with Val115, which resides at the same place in *h*DHFR [91]. Although optimization of the substituents of the phenyl ring attached to N9 resulted in compounds with enhanced selectivity against both *P. carinii* and *P. jirovecii* DHFRs [92], the structure activity relationship is not fully apparent based on the enzyme-inhibitor co-crystal structures [91]. Similar diaminoquinazoline derivatives in spite of showing higher effectivity were proven to be less selective against parasitic DHFRs as compared to pyrido-pyrimidines [93,94]. Studies of compounds with the arylthio-substitued furo-pyrimidine scaffold resulted in inhibitors with improved selectivity, however inadequate cellular uptake of these drugs prevented their further development [95]. This clearly indicates that studies on bacterial cell cultures and human cells assessing whole cell activity and toxicity are necessary to fully evaluate drug candidates against *Pneumocystis* species.

368   3.1.2.   *Bacillus anthracis*

369   The resistance of *B. anthracis* against trimethoprim promoted research for a potent inhibitor
370   of DHFR of this bioterrorism agent parasite [96]. Testing of numerous classes of compounds
371   resulted in some promising potent and selective hits [97,98], while some propargyl-
372   derivatives were turned out to be potent but not selective inhibitors of *Ba*DHFR [99].
373   Structural information from crystallographic, NMR and mutational studies [100–102]
374   combined with systematic analysis of substituents on the 2,4-diaminopyrimidine scaffold led
375   to compounds with improved potency and selectivity against *Ba*DHFR [103–109]. It has also
376   been shown that efficiency can be further enhanced by applying the favored enantiomer
377   instead of a racemic mixture of active compounds [106,109].

378   3.1.3.   *Enterococcus faecalis*

379   In case of *E. faecalis* the first studies about inhibitors against *Ef*DHFR were recently
380   published [110]. Investigation of 2,4-diaminopyrimidine derivatives proven to be active
381   against *Ba*DHFR [104], revealed that those are also potent inhibitors of *Ef*DHFR. Modeling
382   studies concluded that propargyl linked compounds may even be more suitable inhibitors of
383   DHFR mutant *E. faecalis* strains. Since the mutation causing steric clash in case of other
384   inhibitors does not affect these derivatives because of their better fit to the active site pocket
385   of the dihydrofolate substrate [110].

386   3.1.4.   *Staphylococcus aureus*

387   The first line therapy of community-associated methicillin-resistant *Staphylococcus aureus*
388   (CA MRSA) is a combined formulation under the brand name Bactrim targeting the folate
389   biosynthesis of the bacteria, containing trimethoprim, a dihydrofolate reductase inhibitor and
390   sulfamethoxazole, which is a dihydropteroate synthase inhibitor. However, emergence of
391   DHFR mutations leading to trimethoprim resistant strains necessitates the development of
392   novel inhibitors that effectively act against these mutant *Sa*DHFRs to prolong the
393   applicability of this class of antibiotics. Derivatization of trimethoprim resulted in a 5-benzyl-
394   2,4-diaminopyrimidine, Iclaprim, which showed favorable antibacterial activity on TMP-
395   resistant *Staphylococcus aureus* strains, and has reached phase 3 trials [111,112]. However
396   these trials for the treatment of hospital-acquired, ventilator-associated, or health-care-

397 associated pneumonia were terminated due to financial resource limitations[1]. Other
398 2,4-diaminopyrimidine compounds proven to be effective inhibitors of *B. anthracis* DHFR
399 were also tested against *S. aureus*. In spite of their favorable anti-staphylococcal potency,
400 these leads were optimized later only against *Ba*DHFR [105,113].

401 Propargyl-linked compounds with the same diaminopyrimidine scaffold, targeting DHFRs of
402 various parasites were also optimized against *Sa*DHFR [99,114,115]. These studies resulted in
403 active and selective inhibitors of both wild-type and a TMP-resistant mutant enzyme [116–
404 120]. Based on these experiences development of inhibitors of Gram-negative bacteria,
405 *Klebsiella pneumoniae* DHFR is also in the pipeline [121].

406 Derivatives containing the 2,4-diaminoquinazoline scaffold known for their high potency on
407 *Pc*DHFR and *Trypanosomal* DHFR-TSs [93,94,122], were parallelly developed against
408 *S. aureus* and *Mycobacteria*. The inhibitors designed during this project were also very
409 promising candidates for future therapies targeting *Sa*DHFR [123,124]. However high serum
410 binding of these compounds may decrease their *in vivo* efficacy. Recent *in silico* screening
411 studies resulted in completely new scaffolds with favorable *in vitro* potency, initiating a new
412 line of *Sa*DHFR inhibitor design [125].

413 Recently it has been shown that exposure of DHFR targeting drugs induced hypermutator
414 thymine auxotroph mutants [126,127], in which acquiring of antibiotic resistance was
415 significantly more prevalent [128]. These phenomena might raise debates about the use of
416 DHFR therapeutic pathway against *S. aureus*.

3.1.5. *Streptococcus mutans*

418 Trimetrexate analogues were identified as very potent inhibitors of *S. mutans* DHFR, a
419 derivative with enhanced selectivity potently impaired cell growth and formation of *S. mutans*
420 biofilms [129].

3.1.6. *Mycobacterium tuberculosis*

422 In addition to the extensive efforts against *Mycobacterial* ThyX, classical DHFR inhibition is
423 still applied against Mycobacteria. This approach is supported also by a recent verification of
424 DHFR as a target of one of the first antituberculosis agents, para-aminosalicylic acid (PAS), a
425 prodrug that after being activated by the folate pathway inhibits *Mt*DHFR [130,131].

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[1] https://clinicaltrials.gov/ct2/show/NCT00543608?term=NCT00543608&rank=1

426   In this case however, it is questionable if *Mt*DHFR inhibition acts *via* inducing thymineless

427   cell death [132], since it has been proven that the flavin-dependent ThyX can provide enough

428   dTMP for normal bacterial growth in a classical thymidylate synthase (*Mt*TS) deficient strain

429   [68], bypassing the need of *Mt*DHFR. As an alternative explanation, disruption of the

430   reactions centered around S-adenosylmethionione is suggested to be a primary cause of

431   lethality of *Mt*DHFR inhibitors [132]. This is also concordant with a model explaining

432   resistance of *M. tuberculosis* cell lines defective in *Mt*TS function to PAS, an inhibitor of

433   *Mt*DHFR [68,133]. In these mutant bacteria, more reduced folates remain available for other

434   essential one-carbon addition reactions, which results in increased bacterial survival [131].

435   However, further studies are required to fully resolve this question.

436   As PAS-resistant DHFR mutant *M. tuberculosis* strains are emerging and since PAS toxicity

437   leads to gastrointestinal ailments, it is of great importance to find alternative *Mt*DHFR

438   inhibitors [134–137].

439   First trials against *Mt*DHFR applied 1,6-dihydro-2,4-diamino-1,3,5-triazin derivative

440   (WR99210) and its analogues, which have been proven to be potent against *Mycobacterium*

441   *avium* [138]. These compounds were active in cellular assays, but unfortunately showed high

442   toxicity in host cells which prevented their further development [139]. Aiming to identify

443   inhibitors with better profiles certain studies hypothesized that filling the glycerol binding

444   pocket observed in the crystal structures of human and *M. tuberculosis* DHFR by the inhibitor

445   will enhance both potency and selectivity [140–142]. Pyrimethamine analogues with the triol-

446   mimicking trihydroxypentyl group were proven to be potent against *Mt*DHFR [141], however

447   no report is available about effects of those on *M. tuberculosis* growth. It is plausible that this

448   hydrophilic modification prevents these hits to penetrate through the waxy mycobacterial cell

449   wall, based on the fact that the inverse, lipophilic modification of methotrexate dramatically

450   increased the whole-cell activity of the original molecule [132].

451   Recently, a 2,4-diaminoquinazoline fragment was identified in a HTS search and was

452   derivatized based on trimetrexate to have enhanced antimycobacterial potency [139,143].

453   Promising hits were also found amongst the compounds with 2,4-diamino-triazin and

454   tetrahydro-1,3,5-triazin-2-amine scaffolds [144–147].

455   The vast number of recent reports about inhibitor research against *Mt*DHFR indicates that the

456   extensive work has not yet resulted in a fully adequate candidate against this target. Further

457 drug development will hopefully lead to novel therapeutic agents to fight against drug

458 resistant *Mycobacterial* species.

3.1.7. Candida species

460 While the TS enzyme of Candida species is not yet covered in literature, DHFR inhibitors

461 were tested and fine-tuned against these fungi. The first potent inhibitors against *Ca*DHFR

462 were 1,3-diaminopyrrolo-quinazolines, which although being very effective both *in vitro* and

463 *in vivo* were proven to be even more active against *h*DHFR [148]. In parallel the thorough

464 optimization of 2,4-diaminopyrimidines resulted in a family of propagyl-linker containing

465 derivatives, which are potent and selective inhibitors of *Cg*DHFR and *Ca*DHFR with

466 significant antifungal effects and low host cell toxicity [115,149–154]. Nevertheless finding

467 an adequate explanation for the observed inconsistencies between target inhibition and

468 antifungal activity of these compounds in case of *C. albicans* [154] is of utmost importance

469 for the future drug development programs. Still it is worth revisiting previously dismissed

470 *Ca*DHFR inhibitor leads with moderate *in vitro* activity, since it is possible that those have

471 sufficient antifungal potency [154]. Recent modeling studies present a validated inverse

472 docking method for compound selectivity prediction, which could also promote the

473 development of these antifungal DHFR inhibitors [155].

474 **Table 5.** Recent results on the inhibition of monofunctional dihydrofolate reductases (DHFR)

Organism	Year	Summary	Ref.
Pneumocystis carinii	2008	Methyl or ethyl substitution of the linker N9 atom of the 2,4-diaminoquinazoline inhibitors enhanced the potency of those. The original low selectivity of the compounds was unaffected.	[94]
	2010	Compounds having the arythio-substituted furo-pyrimidine scaffold resulted in *Pc*DHFR inhibitors with improved selectivity, however these classical folates can not enter to *P. carinii* cells due to lack of transfer apparatus.	[95]
	2013	2,4-diaminopyrimidine derivatives methylated at the N9 linker were highly active and selective inhibitors of *P. jirovecii* and *P. carinii* DHFRs *in vitro*.	[92]
	2015	Enhanced potency and selectivity of the best compounds from Ref [92] is due to van der Waals interactions of the N9-methyl group at the active site.These are more favorable between Ile123 (present in both *Pc*DHFR and *Pj*DHFR) than those with the corresponding Val115 in *h*DHFR. Overall structure–activity correlations of inhibitors are less evident.	[91]
B. anthracis	2006	2,4-diamino-5-deazapteridine and pyrimidine derivatives showed *in vitro* activities against *Ba*DHFR and effectively impaired growth of *B. cereus*. Selectivity of these inhibitors is to be enhanced.	[97]
	2007	2,4-diaminopyrimidine derivatives, attached to a dihydrophthalazine ring showed high potency and selectivity against *Ba*DHFR and were also active against *B. anthracis* Sterne. Best compound referred later as RAB1.	[98]
	2007	Crystal structure of *Ba*DHFR provides an accurate pharmacophore for structure based design of inhibitors.	[100]
	2008	Propargyl-linked 2,4-diaminopyrimidine derivatives were active against *B. anthracis* Sterne and potent but not selective inhibitors of *Ba*DHFR.	[99]
	2009	Structure of *Ba*DHFR in solution based on NMR measurements reveals flexible parts of the active site.	[101]
	2009	Crystal structure *Ba*DHFR with RAB1 serves as structural foundation for development of derivatives with enhanced properties.	[103]
	2010	Identification of important active site contacts of *Ba*DHFR inhibitors in a mutational and crystallographic study.	[102]
	2012 2014	Derivatives with various substituents at the dihydrophthalazine moiety of RAB1 showing no major difference in potency and selectivity.	[104] [107]
	2013	Substitutions at the C6 position of the 2,4-diaminopyrimidine scaffold of RAB1 lead to compounds with attenuated potency against *Ba*DHFR.	[105]
	2013	S-enantiomers of potent propargyl-linked 2,4-diaminopyrimidines inhibitors are more active against *Ba*DHFR. Concentration of S-stereoisomer diluted by the R-enantiomer is to be considered during activity studies.	[106]
	2015	Identification of RAB1 sites which are sensitive to modification, substitution at these positions led to reduced potency.	[108]
	2015	Studies on derivatives with various substituents at the dihydrophthalazine moiety of RAB1showed that compounds with allyl and vinyl substituents are significantly more potent than RAB1 both *in vitro* and *in vivo*.	[109]
Enterococcus	2014	RAB1 analogues are potent inhibitors of *Ef*DHFR and have promising	[110]

faecalis		whole-cell activity. Based on modeling results propargyl-linked inhibitors can be effective against *Ef*DHFR mutants because those fit tighter to the site of the folate substrate.	
Staphhylococcus aureus	2009	F98Y mutation in *Sa*DHFR may be responsible for trimethoprim resistance through making favorable a second position for cofactor binding, which impedes inhibitor binding.	[116]
	2010 2013	*B. anthracis* inhibitor 2,4-diaminopyrimidine-dihydrophthalazine derivatives were potent against *Sa*DHFR and presented anti-staphylococcal activity. No report on further optimization of these compounds against *S. aureus*	[113] [105]
	2011	Structure-based design of 2,4-diaminoquinazoline derivatives against *Sa*DHFR resulted in potent inhibitors with adequate antibacterial activity. Selectivity of the compounds is to be enhanced.	[123]
	2012	Propargyl-linked 2,4-diaminopyrimidines proved to be active against several parasites were fine-tuned against MRSA and *S. pyogenes* by the incorporation of additional pyridyl-heterocycles. The resulting potent inhibitors of *Sa*DHFR and *Sp*DHFR were also active against clinical isolates of several antibiotic resistant *Staphylococcal* strains.	[117]
	2012	Resistance mutations in *Sa*DHFR were induced by propargyl-linked 2,4-diaminopyrimidine derivatives with moderate frequency. Effectivity of these inhibitors decreased by the mutations however the resulting MICs are still acceptable (2.5 μg/ml).	[118]
	2013	Crystal structures of *h*DHFR with propargyl-linked 2,4-diaminopyrimidines of high antibacterial potency serve as a basis for selective inhibitor design.	[119]
	2014	Attaching a 7-substituted-benzimidazol-1-yl moiety to the 2,4-diaminoquinazoline scaffold led to highly selective *Sa*DHFR inhibitors with great potency against *S. aureus.* High serum binding might significantly reduce the level of *in vivo* efficacy of these compounds.	[124]
	2014	Multiple virtual screenings of large compound libraries for *Sa*DHFR inhibitors resulted in hits with promising anti-staphylococcal properties. Those were found to be active against *Sa*DHFR but not toxic against mammalian cells. Development of these novel scaffolds is in progress.	[125]
	2015	Potency of enantiomers of propargyl-linked 2,4-diaminopyrimidine derivatives against *Sa*DHFR is significantly different from each other.	[120]
	2015	Trimethoprim-sulfamethoxazole antifolate therapy triggers *Sa*TS mutations leading to thymine-dependent small colony variant formation, which is less virulent, but more persistent than the wild type bacteria.	[127]
Klebsiella pneumoniae	2014	Propargyl-linked 2,4-diaminopyrimidine derivatives inhibit *Kp*DHFR and impair *Klebsiella pneumoniae* cell growth. Crystal structures of *Kp*DHFR with these compounds will aid the design of compounds with enhanced selectivity.	[121]
Streptococcus mutans	2014	Identification of trimetrexate analogues against *Sm*DHFR resulted in compounds, which selectively (SI>100) inhibited cell growth and formation of *S. mutans* biofilms.	[129]
Mycobacterium tuberculosis	2000	Inhibitor bound three-dimensional structure of *Mt*DHFR aids structure based drug design against this target enzyme.	[140]
	2002	First proof that besides being active against *M. avium* the dihydro-	[138]

		diamino-triazin derivative WR99210 potently impairs *M. tuberculosis* cell growth *via MtDHFR* inhibition.	
	2007	Pyrimethamine analogues having triol substituents accommodating the glycerol binding site of *Mt*DHFR were proven to be potent inhibitors of the enzyme.	[141]
	2012	A 2,4-diaminoquinazoline fragment was identified in a HTS project as a potent *Mt*DHFR inhibitor and showed promising target specific effects in cellular assays.	[139]
	2013	Antimalarial drug para-aminosalicylic acid (PAS) perturbs folate metabolism in *M. tuberculosis* as a prodrug, which after bioactivation by other enzymes of the folate pathway acts as a *Mt*DHFR inhibitor.	[130] [131]
	2014	Missense mutations residing within the active site coding region of dihydrofolate synthase, one of the PAS activation enzymes, found in clinical isolates of *M. tuberculosis* were identified as the causative of PAS resistance.	[134]
	2014	Unlike methotrexate its diester derivatives with increased lipophilicity exhibited significant whole-cell potency against *M. tuberculosis* possibly because of enhanced penetration rate. It is suggested that these derivatives act through the disruption of methyl-transfer mediated by S-adenosylmethionione.	[132]
	2014 2015	Development of 2,4-diamino-triazin derivatives resulted in *Mt*DHFR inhibitors with favorable whole cell activity against *Mtb* (MIC < 2μM) along with low and moderate cytotoxicity.	[144] [145] [146]
	2015	Minireview on mechanism of PAS action and resistance.	[135]
	2015	Study of a focused library including more than 2000 compounds identified a 2,4-diaminoquinazoline derivative with promising *in vivo* antitubercular efficiency and low host cell toxicity.	[143]
	2015	Modeling study on *Mt*DHFR proposed selective and potent agents against *Mt*DHFR.	[142]
	2015	*In silico* screening for *Mt*DHFR inhibitors identified two compounds with the tetrahydro-1,3,5-triazin-2-amine scaffold, which were active against *M. bovis* BCG (MIC < 5μM).	[147]
	2016	Mutation resulting PAS resistance in *M. tuberculosis* revealed that gene *Rv2671* was misannotated. Overexpression of Rv2671 protein resulted in bacterial escape of PAS treatment, which observation led to the discovery that this protein is a DHFR.	[136]
Candida glabrata Candida albicans	1996	Development of 1,3-diaminopyrrolo-quinazolines against *C. albicans* resulted in compounds with great *in vivo* and *in vitro* potency. However these were even more potent against *h*DHFR and toxic for HCT cell lines.	[148]
	2004	Inconsistencies were found in the case of drugs targeting *C. albicans* between the effect of those on *Ca*DHFR activity and on parasite growth. Designed compounds were found to be potent but not selective.	[149]
	2008	Based on *Cg*DHFR crystal structure two drugs possessing subnanomolar potency against the enzyme and significantly impairing *C. glabrata* growth were designed. These were also highly selective *in vitro* and showed low toxicity against mammalian cells.	[115]
	2009	Structure activity relationship study of propargyl-linked 1,3-diaminopyrimidines targeting *Ca*DHFR.	[150]

		Antifungal activity is still not well correlated with *Ca*DHFR inhibition. Resulting compounds are toxic to human cells in the concentration effective against *C. albicans*.	
	2009	Derivatives of the best active 1,3-diaminopyrimidine compounds from Ref [115] were outperformed by the unmodified compounds in terms of activity against *Cg*DHFR In the crystal structures of *Cg*DHFR only R-enantiomers of the inhibitors were found in the active center.	[151]
	2011	Structure based study on improving the affinity of moderately potent compounds from Ref [150] against *Ca*DHFR. Increasing hydrophobicity at certain positions enhanced the extent of the van der Waals contacts.	[152]
	2013	Replacing a phenyl group of the best active compounds from Ref [115] with aromatic or alicyclic heterocycles resulted in derivatives with decreased efficiency of *Ca*DHFR inhibition and selectivity ratios.	[153]
	2014	Three compounds from a set of novel propargyl-linked 1,3-diaminopyrimidines proved to possess outstanding antifungal activity (MIC<1 μg/mL) and *in vivo* selectivity.	[154]
	2016	Validation of an *in silico* inverse docking method for predicting selectivity of *Ca*DHFR inhibitors.	[155]

475

3.2. Bifunctional DHFR-TS

Protozoa encode bifunctional DHFR-TSs, in which the TS domain is fused to the carboxy terminal of the DHFR domain by a junction peptide of varying size. Thus the two enzyme domains do not share a common folate binding site, unlike in the case of bifunctional dCTP deaminase – dUTPase [28]. The fusion of the two proteins facilitates dihydrofolate elimination through metabolic channeling and allows sufficient coordinate control of folate metabolizing enzymes, which might represent a biological advantage of such bifunctional DHFR-TSs [156]. The junction of the two proteins is generally not considered as the source of differences in drug actions, which is rather assigned to the sequence diversity [157,158]. Although this flexible linker does not influence the action of classical antifolates it might be a subject of specific inhibitor design, since it modulates enzyme activity in some specific cases [159,160]. Compounds binding to the dimer interface and acting as allosteric inhibitors of DHFR-TSs are also in the scope of drug design projects [161,162]. Still, the main approach targeting these bifunctional DHFR-TS enzymes is the development of specific DHFR inhibitors (Table 6).

3.2.1. Plasmodium species

Fast adaptation of *Plasmodium falciparum* DHFR-TS by specific mutations in the active site triggers continuous drug development programs against the emerging mutant enzymes.

In most cases mutations which led to drug-resistance were associated with steric exclusion of the conformationally constrained inhibitors. Based on this observation compounds with increased flexibility have been developed against *Pf*DHFR-TS to overcome fast adaptation of the parasite [163]. Combination of this principle and structure based drug design aided by crystal structures of substrates and inhibitors in complex with wild-type and quadruple mutant *Pf*DHFR–TS resulted in compounds which inhibit both the wild type and the mutant protozoan enzyme [163–165]. It has also been shown that one of the derivatives, namely P218, displays *in vivo* activity against wild-type and pyrimethamine-resistant malarias [163]. Since the pre-clinical safety studies have been completed for the drug candidate P218, it may proceed to first in-human tests[2], meanwhile a recent molecular modeling analysis might facilitate the synthesis of P218 derivatives with enhanced performance [166]. In parallel with

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[2] MMV (Medicines for Malaria Venture) Research and Development

505   these studies new scaffolds for inhibitor design against *Pf*DHFR-TS were also studied to

506   different extents [166–170].

507   Besides mutations in *Pf*DHFR–TS, it has been shown that gene amplification of GTP-

508   cyclohydrolase, the first enzyme in the folate synthesis pathway of the parasite, is strongly

509   associated with antifolate drug resistance, revealing this enzyme as a potential new target to

510   be considered for antimalarial drug design [171].

511   ### 3.2.2.   Leishmania

512   While the *Plasmodium* enzyme is the target of pyrimethamine, one of the few clinically active

513   anti-malarial agents, this drug despite the high similarity of protozoan TS-DHFRs is

514   ineffective to treat leishmaniasis. This difference in pyrimethamine inhibition is associated

515   with the observation that this drug acts on the DHFR domain which − unlike the highly

516   conserved TS domain − is more variable between species, albeit these differences are much

517   smaller, than those between the human and protozoan enzymes. Other common antimicrobial

518   DHFR inhibitors such as cycloguanil and trimethoprim were also not effective against

519   *Leishmania*.

520   In *Plasmodium* point mutations in the DHFR gene are the source of pyrimethamine resistance,

521   while *Leishmania* acts against antifolates by amplification of the gene encoding DHFR [156].

522   As such it is expected that *Leishmania* is less capable to develop resistance-inducing

523   mutations.

524   Some quantitative structure activity relationship schemes of some antifolates against *L. major*

525   have already been established in early studies, however, these were abandoned, possibly

526   because of insufficient selectivity profile of the compounds [172,173]. Later, during the

527   development of *Trypanosomal* DHFR-TS inhibitors some moderately active inhibitors of

528   *L. infantum* and *L. donovani* have been reported [122,174]. The structure of *Lm*DHFR-TS has

529   been solved, albeit the crystallographic data is not available in Protein Data Bank [158].

530   Recent studies report only *in silico* testing of limited set of compounds against homology

531   models of *L. donovani chagasi* and *L. major* [158,175,176]. However recently promising

532   preclinical development candidates against *L. donovani* exhibited moderate inhibition of

533   *Ld*DHFR-TS and inhibitory effects on promastigotes and amastigotes by triggering their

534   apoptotic cascade [177].

535   ### 3.2.3.   Trypanosomas

536   To overcome drug resistance of *T. cruzi*, *T. brucei* and *L. infantum* series of compounds were

537   tested against DHFR-TS of these parasites, which resulted in good activity and selectivity

538   leads against the protozoan enzymes [174]. Pyrimidine analogues including trimetrexate, a

539   DHFR inhibitor used in pneumocystis pneumonia therapy, were active *in vitro* but exhibited

540   only limited activity *in vivo* [178,179]. Diaminoquinazoline derivatives showed a more

541   promising *in vivo* profile [122,180] and hence these are in the focus of extensive structure

542   based drug design [181–185]. Based on these studies potent inhibitors against Trypanosomal

543   DHFR-TSs have already been developed, however overcoming selectivity problems is still a

544   challenge.

545   **3.2.4. *Toxoplasma gondii***

546   Comprehensive research has been performed to find selective inhibitors against *Toxoplasma*

547   *gondii* DHFR-TS as a validated drug target [186]. Since *Toxoplasma* cannot salvage dTTP

548   from an extracellular source, indirect inactivation of thymidylate synthase *via* DHFR

549   inhibition is lethal to the parasite [187]. The design of *Tg*DHFR-TS inhibitors has largely

550   relied on *in vitro* screening and homology modeling [114,188–190], where fine-tuning of

551   human DHFR inhibitors against the *T. gondii* enzyme resulted in some potent and selective

552   drug candidates [190]. Future studies will be directed towards improving the best active

553   tricyclic pyrimido[4,5-b]indole scaffold based on the comparison of the recently determined

554   crystal structures of *Tg*DHFR-TS with its human counterpart [190,191].

555   Exploiting this structural information research projects aiming to identify allosteric inhibitors

556   targeting the TS-TS dimer interface of the *Tg*DHFR-TS have also been launched [190,191].

557   The moderately potent compounds reported from these studies serve as proof-of-concept and

558   may lead to design and optimization of novel class of potent and selective inhibitors to treat

559   toxoplasmosis [161,162].

560   **3.2.5. *Cryptosporidium hominis***

561   The crystal structure of *Cryptosporidium hominis* DHFR-TS was determined in the early

562   2000s [192,193], enabling structure based drug design of selective inhibitors of the enzyme.

563   Derivatization of trimethoprim by applying a propargyl linker resulted in novel series of

564   classical antifolates with nanomolar inhibitory constants (K_i) against *Ch*DHFR-TS [114,194]

565   and remarkable *in vivo* activity [40,195]. However, difficulties were observed in transport of

566   the best potent compound through the vacuolar membranes of the parasite [40], which could

567 be overcome by loading the inhibitor into PLGA nanoparticles fused to *Cryptosporidium*

568 specific antibody [196]. Future development strategies will focus on improving the selectivity

569 against the human enzyme without compromising the activity against *Ch*DHFR-TS by

570 applying computer-aided design [40].

571 It has been shown that mutations in the linker region, especially inside the crossover helix of

572 the *C. hominis* DHFR, impair the catalytic rate of the enzyme, which implies that the linker is

573 necessary for optimal dihydrofolate reductase activity. Initiated by this finding, studies

574 applying virtual screening and structure based design independently resulted in mid-

575 micromolar allosteric inhibitors [159,160]. Subsequent synthetic development of these proof-

576 of-concept compounds to possess higher affinity to the identified surface cleft will

577 presumably eventuate in more potent, novel inhibitors against *Ch*DHFR-TS.

578

579 **Table 6.** Recent results on the inhibition of bifunctional dihydrofolate reductase – thymidylate
580   synthases

Organism	Year	Summary	Ref.
Plasmodium falciparum	1997	Identifying mutations responsible for drug resistance of *Pf*DHFR-TS.	[197]
	2003	Crystal structure of wild-type and mutant *Pf*DHFR-TS enzymes serves as templates for designing novel drugs against resistant-mutant parasites. Junction region might be a target for selective inhibitors interfering with interdomain interactions.	[164]
	2004	Report on low nanomolar level inhibitors targeting the mutant enzymes with good antimalarial activities against resistant *P. falciparum* parasites and low or moderate cytotoxicity candidates for novel antimalarials.	[198]
	2009	Novel inhibitors with guanidine scaffold were found to be active against wild-type and mutant *Pf*DHFR-TS enzymes. Co-crystal of novel inhibitors with a drug-resistant mutant *Pf*DHFR-TS.	[199]
	2010	Drug candidate, QN254 however showed relative *in vitro* selectivity towards the Plasmodium DHFR enzyme possesses inadequate therapeutic index tested in rats. Compound relinquished.	[200]
	2012	Identification of selective and potent inhibitors of wild-type and mutant *Pf*DHFR-TS enzymes, with good metabolic properties. Compound P218 was denominated as a pre-clinical candidate.	[163]
	2013	4,6-diaryl-2-aminopyrimidine derivatives proved to be promising leads for inhibitor design against *Pf*DHFR-TS.	[167]
	2014	Compounds from Brucea mollis Wall. ex kurz were *in silico* checked against wild type and mutant *Pf* DHFR-TS. Inhibitors with better binding affinity than pyrimethamine were identified.	[168]
	2014	Molecular dynamics simulation of interactions between rigid and flexible antifolates of wild-type and pyrimethamine-resistant mutant of *Pf*DHFR-TS. Description of key inhibitor binding residues.	[169]
	2014	Molecular dynamics analysis of inhibitor P218 binding to wild-type and mutant *Pf*DHFR-TS.	[166]
	2014	Guanylthiourea derivatives with IC_{50} value of 100 μM and 400 nM were developed against *Pf*DHFR-TS.	[170]
Plasmodium ovale	2012	Antifolate drugs showed similar kinetic and sensitivity profiles with *Po*DHFR-TS as compared to those of the *P. falciparum* and *P. vivax* enzymes.	[201]
Plasmodium vivax	2001	Transgenic Plasmodium lines expressing *Pv*DHFR-TS for screening anti-P. vivax compounds targeting this enzyme.	[202]
	2006	Testing compounds on a *Pv*DHFR-TS-dependent bacterial strain, revealed that inhibitors of this enzyme are similar to those of *Pf*DHFR-TS. Adequate correlation was found between the *in vitro* enzyme inhibition constants and the IC_{50} values.	[203]
Leishmania major	2012	*In silico* modelling study of *Lm*DHFR-TS and virtual screening for its inhibitors.	[176]
	2012	Promising preclinical development candidates with moderate inhibition of *Ld*DHFR-TS showed inhibitory effects on *L. donovani* promastigotes	[177]

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		and amastigotes by triggering of the apoptotic cascade.	
Leishmania donovani	1999	Series of compounds were tested against *T. cruzi*, *T. brucei* and *L. infantum* DHFR-TS. Leads for drug development with good activity and selectivity against the protozoan enzymes were identified.	[174]
	2010	Modeling the structure *of Leishmania donovani chagasi* DHFR-TS to aid future drug design programs.	[175]
Trypanosoma brucei, cruzi	2002	2,4-diaminopyrimidines are potent inhibitors of the *Trypanosomal* DHFR-TS enzymes *in vitro*, but show only limited activity *in vivo*.	[178] [179]
	2005	The quinazoline derivative antifolate, trimetrexate is proven to a potent but not selective inhibitor of *Tc*DHFR-TS. Outset of drug development is the improvement of the selectivity of this compound.	[180]
	2005	2,4-diaminoquinazoline-based compounds inhibited *Tc*DHFR-TS and *Tb*DHFR-TS and have *in vivo* activity in a rodent model of Chagas disease, but lack activity against the *L. donovani*.	[122]
	2008	First crystal structures of apo and inhibitor bound *Tc*DHFR. 3D-QSAR analysis of inhibitors of *Tc*DHFR activity. Identification of several highly potent inhibitors of *Tc*DHFR from libraries of antifolate compounds.	[181] [182] [183]
	2010	Introducing chemical modifications on trimetrexate did not resulted in additional favorable contacts with *Tc*DHFR nor disfavor *h*TS binding.	[184]
	2011	First Crystal structure of DHFR domain of *Tb*DHFR with NADPH and inhibitors. *Tb*DHFR is similar to pyrimethamine resistant mutant *Pf*DHFRs. During inhibition design steric hindrance of Thr86 clash should be considered	[185]
Toxoplasma gondii	2002	*De novo* pyrimidine biosynthesis is essential for virulence of *T. gondii*.	[186]
	2007	Rational lead design based on homology model of *Tg*DHFR-TS and crystal structure of *Ch*DHFR-TS. Change of the one carbon linker in trimethoprim with a longer but rigid propargyl linker to access hydrophobic pocket of DHFR active site resulted in compounds displaying high potency and selectivity against *Tg*DHFR-TS and *Ch*DHFR-TS.	[114]
	2008	Among potent compounds constructed during the development of inhibitors against *h*TS, a class of derivatives was proven to be only marginally active on the human target. These however showed high potency and selectivity against *Tg*DHFR-TS.	[188]
	2013	Comparative docking to the homology model of *Tg*DHFR-TS applying different softwares for screening the same drug library resulted in several potential inhibitors of this enzyme.	[189]
	2013	First crystal structure of *Tg*DHFR-TS aiding structure based inhibitor design.	[191]
	2013	Compounds with single-digit nanomolar K_i for *Tg*DHFR-TS, with 28- and 122-fold selectivity over human TS (*h*TS) were synthesized on the basis of the potent bicyclic *h*TS inhibitor nolatrexed.	[190]

	2013	β-strand mimicking peptides that target dimer interface of *Tg*DHFR-TS inhibit enzymatic activity in a species-specific manner.	[162]
		Non-conserved residues in the linker between TS and DHFR play a key role in domain–domain communication and in peptide interaction.	
	2013	*In silico* screening for allosteric inhibitors at the interface between the two TS domains.	[161]
		Identified compounds showed moderate inhibition of *Tg*DHFR-TS but no selectivity against *h*TS.	
Cryptosporidium hominis	2003, 2005	Crystal structures of *Ch*DHFR-TS revealing protein-ligand interactions provide template for structure-based drug design against *Ch*DHFR-TS.	[192] [193]
	2007	Highly efficient inhibitors of *Ch*DHFR-TS (cf. at *T. gondii*).	[114]
	2008	Structure-based inhibitor development resulted in enhanced affinity compounds from Ref. [114] against *Ch*DHFR-TS, without compromising selectivity.	[194]
	2009	*Ch*DHFR-TS crossover helix is indispensable for adequate enzyme activity since mutations in this region resulted in a drastic reduction of catalytic rate.	[204]
	2008	Novel non-active site inhibitors with mid-micromolar potency against of *Ch*DHFR-TS were identified by a virtual screening revealed inhibitory potential of an allosteric pocket of this enzyme.	[159]
	2013	Small molecule compound binding at the species-specific helical protein interaction surface could result in catalytic inhibition and enzyme destabilization.	[160]
	2013	A novel series of classical antifolates, were proven to be potent inhibitors of *Ch*DHFR-TS.	[195]
		Inhibitor bound crystal structure reveals key structural differences between *Ch*DHFR-TS and *h*TS and aids the design of parasite specific agents.	
	2014	Identification of a potent inhibitor of *Ch*DHFR-TS with anti-cryptosporidial activity in cell culture.	[40]
		Difficulties in delivery of the potent compounds through the vacuolar membranes of the parasite were observed.	
	2015	PLGA nanoparticles fused to *Cryptosporidium* specific antibodies loaded with a potent inhibitor of *Ch*DHFR-TS specifically targeted the parasite.	[196]
		This formulation reduced the level of parasites by 200-fold in cell culture as compared to the 4.4-fold decrease upon normal inhibitor addition.	

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583 # 4. Other promising targets

584 ## 4.1. Serine hydroxymethyltransferase inhibitors

585 Since serine hydroxymethyltransferase (SHMT) plays a key role in the dTMP synthesis
586 (Figure 1) it is a highly relevant target for antiparasite drugs. Still, inhibitor development was
587 only reported in the case of Plasmodium SHMT, which enzyme was validated as an
588 antimalarial platform [205–209]. Differences in the structure of the ligand binding pockets of
589 human and Plasmodium SHMTs have been exploited during the design of species-specific
590 inhibitors against the protozoal enzyme [209]. The excessive efforts for developing *Pf*SHMT
591 inhibitors resulted in leads with high selectivity margin relative to mammalian cell lines and
592 active also against the tested multidrug resistant *Plasmodium falciparum* strains [210,211].
593 However further studies are required to enhance the low metabolic stability of the best active
594 *Pf*SHMT inhibitors.

595 Characterization of *Mycobacterial* and *Trypanosomal* SHMTs have been performed, as a first
596 step of drug design against these enzymes [212–217]. It has been shown that unlike
597 eukaryotic SHMTs including those of other trypanosomatids, *T. cruzi* SHMT does not
598 oligomerize in solution [217]. While the genome of *M. tuberculosis* encodes two different
599 dimeric SHMTs, which also differ from the tetrameric mammalian enzymes [212,213]. In
600 most SHMTs except, among others, the *Mycobaterial* enzymes, a strictly conserved lysine
601 forms covalent bond with the cofactor pyridoxal phosphate. *Mycobacterial* SHMTs uniquely
602 display significant changes in the conserved threonine-rich octapeptide sequence near this
603 active site lysine residue [212,213], which might explain the slightly distinct catalytic
604 properties of these enzymes compared to other SHMTs.

605 These marked differences of *T. cruzi* and *Mycobacterial* SHMTs to other isoenzymes might
606 facilitate specific drug development against these targets, whereas the high similarity between
607 *Leishmanial* and human SHMTs renders the design of specific inhibitors of *Ld*SHMT
608 challenging [214,215].

609

4.2. Thymidine kinase inhibitors

It has been proven that the pyrimidine nucleotide salvage is indispensable *in vivo*, by knocking out thymidine kinase (TK) the key enzyme of the pathway in mice [218]. Thus TK is also a potential objective of antiparasite drug development [218–222]. Such as in the case of *Cryptosporidium parvum*, thymidine kinase is found to be an effective target in anti-cryptosporidial therapy [222], since the treatment of the bacteria with fluorinated pyrimidine derivatives processed by the pro-drug activator *Cp*TK resulted in inhibition of parasite growth in an *in vitro* model of infection and was found effective in a mouse model, as well.

Leishmania major TK (*Lm*TK) knockout mutants also showed lower proliferation rates, morphological defects and were found to be less infective [219]. However, a recent crystallographic study revealed that the active site of *Lm*TK is analogous to that of the human enzyme [220]. This is also demonstrated by the highly similar kinetic parameters associated with the binding of substrates and inhibitors, depicting the design of selective *Lm*TK-specific inhibitors even more elusive.

While *T. brucei* thymidine kinase (*Tb*TK), which was shown to be a pseudo-dimer of covalently linked tandem repeat of monomers, has broader substrate specificity than the human enzyme and is therefore a more feasible drug target [221].

Thymidine kinase inhibition is not applicable against several parasites including *Pseudomonas aeruginosa*, *Helicobacter pylori, Toxoplasma gondii* and *Mycobacteria* which do not encode this enzyme [11,187], although the lack of thymine nucleotide salvage sensitizes these organisms for inhibitors of the *de novo* synthesis pathway.

4.3. Thymidylate kinase inhibitors

As TMPK catalyses the formation of dTDP from dTMP, it is essentially important in the dTTP synthesis pathway for DNA synthesis, and also as an antiparasite drug target [223]. Inhibition of *Staphylococcus aureus* TMPK has resulted in *in vivo* anti-staphylococcal efficacy in mouse model and the inhibitor molecule was proven to be selective against the human enzyme [224,225]. Compounds with nanomolar inhibition potency against *Pseudomonas aeruginosa* thymidylate kinase have also been developed, however those were ineffective against the bacteria possibly due to poor penetration of the drug through the complex cell membrane [226]. Investigation of thymidine monophosphate analogs against *Mt*TMPK resulted only in very weak inhibitors [227,228], whereas compounds with thymidine core and their acyclic bioisosteres were found to be more promising lead candidates with micromolar or lower inhibitory constants (K_i) and favorable selectivity [229–231]. Recently three novel compound series were also tested against *Mt*TMPK [232]. The development of 1,6-naphthyridinone compounds has not led to high potency *Mt*TMPK inhibitors, thus the focus was shifted to cyanopyridones. These showed single digit nanomolar *in vitro* activity, but were ineffective in cellular assays. The third series containing sulfoxide or sulfone substituents showed anti-mycobacterial activity at low micromolar concentrations. The correlation analysis of *in vitro* and *in vivo* activities revealed that the observed antimycobacterial effect is not exclusively originates from *Mt*TMPK inhibition, hence further target validation is required in case of these compounds. The same holds for inhibitors developed against the *Pf*TMPK, which exhibit promising antimalarial activity and selectivity between *P. falciparum* and human enzymes, but the mechanism of *in vivo* action is yet equivocal [233]. To best of our knowledge, no report is yet available on *Leishmanial* TMPKs, also in the case of *B. anthracis* TMPK only a preliminary study has been reported to date [234].

5. New waves: proteinaceous inhibition of dUTPase and UNG

Recently a Staphylococcal protein (Stl) has been identified as a competitive inhibitor of a phage related dUTPase with nanomolar inhibitory constant [235–237]. It has also been shown that the inhibition is independent from the phage specific insert and Stl binds and inhibits *Mycobacterial* dUTPases *in vitro* and *in vivo* [238,239]. These developments shed light on the possibility of using protein inhibitors to target enzymes in the thymidylate biosynthesis pathway. Proteinaceous inhibition and the use of proteins as drugs have several advantages and disadvantages, as well. Among the positive factors, it should be mentioned first of all, that macromolecular inhibition may offer unprecedented specificity, and can also be tailored using site-specific mutations to target dUTPases from different species. However, the use of proteins as drugs may imply many technical difficulties, among which the question how we can achieve an effective concentration of the macromolecular inhibitor in the vicinity of the target protein remains to be elusive. Still, despite all technical difficulties, macromolecular drugs are up coming and it can be expected that such approaches will prove to be successful in the next few years.

Another proteinaceous inhibitor within the DNA damage and repair pathways is the uracil-DNA glycosylase inhibitor Ugi. A recent study suggests that depletion of the uracil DNA glycosylase (UNG) sensitizes tumor cells to FdUrd, because of activation more error prone DNA repair mechanisms against the incorporated 5F-uracil [236]. We propose that the potential significance of a proteinaceous inhibitor against UNG can be tested in pathogens, as well. In this regard a pioneering study on the recently discovered Ugi protein from Staphylococcus aureus attempts to modulate the inhibitor ability of this protein against human herpesvirus UDG [240,241]. Based on the promising results of these first experiments, the Ugi inhibitor may serve as a new type of drug candidate molecule.

Conclusions

We have reviewed the current state of using drugs against thymidylate biosynthesis to fight pathogenic microorganisms. Some major general aspects may be emphasized in these efforts. Since in many cases, e.g. with dUTPases and thymidylate synthases, the target pathogen enzyme has a closely-related human homologue as well, the problem of selectivity has to be addressed. This can be approached in two ways: on the one hand, the designed drug candidate

686 may possess chemical moieties that enhance selectivity [13], whereas on the other hand, the

687 targeted enzyme surface needs to include species-specific segments [15].

688 In more convenient scenarios, the target enzyme in the pathogenic microorganisms possesses

689 either somewhat altered three-dimensional structure or completely different protein fold,

690 allowing a more straightforward approach for pathogen species-specific drug design.

691 Examples in these cases include e.g the Plasmodium dUTPase where one protein segment

692 shows a distinct conformation as compared to human dUTPase, as well as the dUTPases from

693 Trypanosomes and Leishmania species, which are representatives of the all-α dimeric

694 dUTPase family possessing also an altered mechanism of action as compared to the more

695 general all-β trimeric dUTPases. In the family of thymidylate synthases, some pathogens, like

696 *Mycobacterium tuberculosis* and *Helicobacter pylori*, luckily encode this enzymatic activity

697 in a protein (nick-named as ThyX) that is fully divergent from its human counterpart.

698 Further, to overcome the mechanisms of development of resistance, the novel drug candidates

699 need to possess new mechanisms of action. It is also a promising concept to apply

700 combination therapies by simultaneous application of drug candidates targeting different

701 enzymes. Finally, during the fine-tuning of drug-like compounds, the key properties that are

702 significant for drug action (eg solubility, penetration properties, specificity, toxicity) should

703 be optimized in parallel to each other.

704

712

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