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A STUDY ON DIHYDROFOLATE REDUCTASE AND ITS INHIBITORS: A REVIEW

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ABSTRACT: Dihydrofolate reductase (DHFR) was discovered in the late 1950s by investigators searching for folate-dependent enzymes involved in 1-carbon metabolism, with its already known application as anti-cancer and as antibiotics. This study focuses on structure of Dihydrofolate, its interaction with specific amino acids, mechanism of DHFR catalysis. The reason of study was to focus on compounds which can inhibit DHFR and has applications as antifungal agents, antimalarial agents, ant tuberculosis agents, for Leishmaniasis and Trypanosomiasis treatment. Also, to find out the reason of resistance to antifolates and ways to overcome them, recent drugs under each class of agents and drugs under clinical trials.

INTRODUCTION: Sidney Futterman fractionated a chicken liver extract and obtained a preparation that catalyzed the reduction of both folate and DHF to THF in 1944. He also found that Methotrexate and Aminopterin, another anticancer drug, inhibited both of these processes. In 1988 Hitchings, Elion and Black were honoured with Nobel Prize in physiology in medicine for selective binding of Trimethoprim and other pharmaceuticals to target dihydrofolate [1].

However molecular nature of selectivity still not understood in spite of fact that DHFR is the most thoroughly and comprehensively studied enzymes most fundamental mechanism of functioning of DHFR and its nature of interactions with the inhibitors need to be understood [1]. DHFR is found ubiquitously in all dividing cells of prokaryotes and eukaryotes.

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The mammalian enzymes are all highly similar in sequence, while each bacterial form is distinct. The DHFR sequence in humans is 30% similar to that of *E. coli* and 70% similar to other mammalian DHFRs. The standard source for the enzyme is the mammalian and avian liver [2].

Structure: Enzyme Dihydrofolate reductase is a relatively soluble water soluble protein with a molecular weight of 18000-20000 Da synthesised on activation of human DHFR located on q22 region of chromosome no 5 .Enzyme consists of eight β sheets which makeup a rather rigid structure of protein molecule. All forms of enzyme consists of four α helices two of which form a substrate binding site and other two consist of coenzyme binding domain. This enzyme contains no disulphide bonds and metal ions are not necessary for activity [2]. Most structural elements are common to all enzyme forms (most commonly studied are *E. coli*, *Pneumocystis carinii* and Human DHFR) loop1 is located between α helix B and β sheet A and the *cis* peptide bonds between two lysine residues located at junction of β sheet e and α helix F.

Enzyme active site is located in hydrophobic pocket surrounded by α helix B, the central β sheets (**a, e, b**) and loop1 Refer **Fig. 1**.[2]

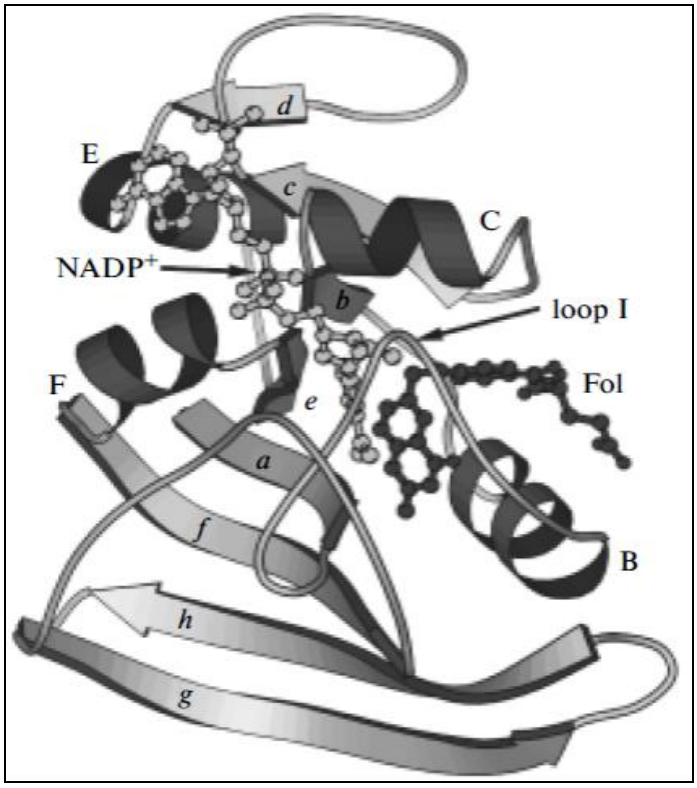
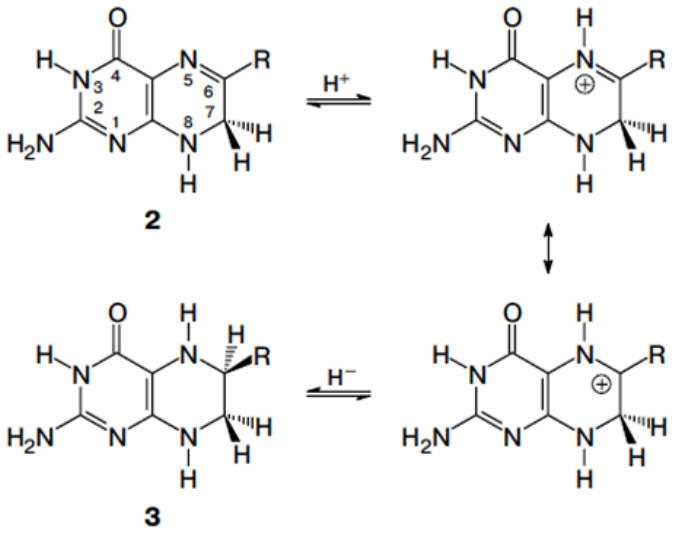


FIG. 1: TOPOLOGY OF PROTEIN AND ARRANGEMENT OF SUBSTRATE (FOL) AND COENZYME (NADP$^+$) IN THE COMPLEX OF *ESCHERICHIA COLI* DHFR. B-sheets are represented by lowercase letters (a-g) and α helices by capital letters [2]

Mechanism of DHFR Catalysis: Dihydrofolate reductase catalyses the conversion of 7, 8 Dihydrofolate to 5, 6, 7, 8-Tetrahydrofolate using coenzyme NADPH and the proton of water molecule respectively as the donor of hydride ion. The mechanism of enzyme catalysis remains a subject to be extensively studied but kinetic and structural aspects of the reaction, the role of individual amino acids in the reaction were examined. The key stage the transfer of hydride ion molecule from coenzyme molecule to substrate was also studied by quantum chemical method with aim to determine the structure of transition state and energy profile involved in the reaction [2].

1. **Mechanism of Substrate Protonation**: Key stage of the catalytic process involves two main steps protonation of substrate **2** (dihydrofolic acid) at N (5) atom and transfer of hydride ion to positively charged intermediate to form neutral reaction product **3**.



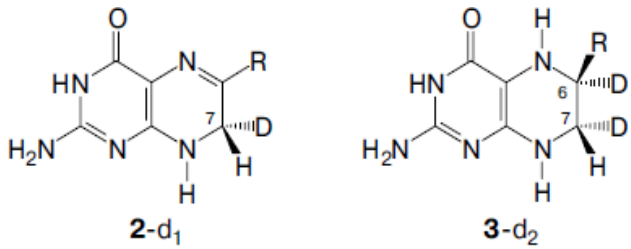
For enzymatic reaction to proceed, dihydrofolate must protonate at N(5) atom of pteridine residue of **2** or at N(8) atom for the reduction of folate.

Amino acid involved in protonation is Aspartic acid in case of *E.coli* and Glutamic acid in case of humans (Glu 30 in DHFR).

Tetrahydrofolic acid was found to exist in keto and enol forms. Analysis by NMR and Raman spectroscopy demonstrated keto forms is major tautomeric form of substrate in complex with DHFR. In the end NADPH is oxidised to NADP$^+$ [2]

2. **Transfer of Hydride Ion:** Rate of transfer of hydride ion to substrate is key stage of catalytic process. It was found that reduction of 7, 8-Dihydrofolate was accompanied by transfer of hydrogen atom located in 4-*pro* R position of NADPH to C-6 atom of dihydrofolate.

   The use of nicotinamide coenzyme which was selectively labelled with deuterium at 4-*pro* position of dihydropteridine group which was subsequently converted to tetrahydrofolate-$d_2$which possessed the deuterium atom at same side of dihydropteridine ring [2].



Kinetics of Hydride Transfer: In the DHFR reaction, early kinetic investigations showed that product release is rate limiting step and enzyme catalysed chemical transformation is exceptionally efficient. Overall reaction involves addition of proton and hydride ion to DHF. At neutral pH it has value of 450 s^{-1} at 298 K and at this pH main contribution of limiting rate comes from release of tetrahydrofolate. The rate constant for hydride transfer from NADPH to DHF is pH dependent and at pH=6.5 with a value of 960s^{-1} step becomes irreversible.

Mutation studies have shown that deletion of residues on M-20 loop has large impact on rate constant of hydride transfer step also mutation of gly-121 reduces the rate constant by a factor of 163 [3].

Fig. 2 shows it was showed that hydride transfer step might occur before the protonation step because the average time during which water was found was significantly increased within hydrogen bonding distance of N-5 position of DHFR.[3]

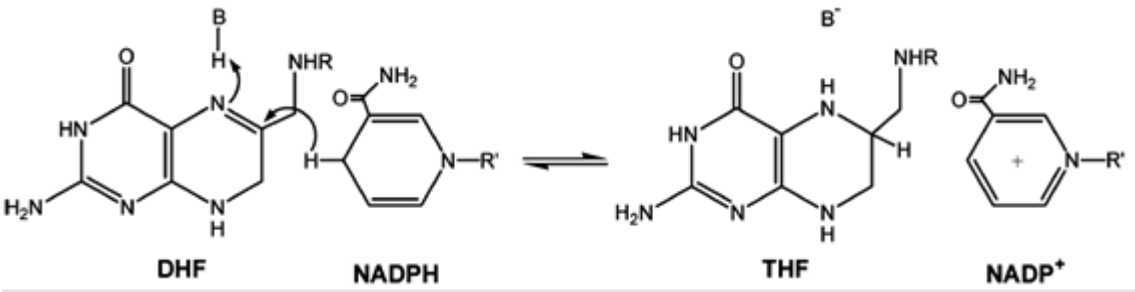


FIG. 2: REACTION CATALYSED BY DHFR. THE REACTANT SUBSTRATE IS DHF (DIHYDROFOLATE), THE PRODUCT SUBSTRATE IS THF (TETRAHYDROFOLATE) AND COFACTOR IS NADPH/$NADP^+$ (NICOTINAMIDE ADENINE DINUCLEUTIDE PHOSPHATE [4]

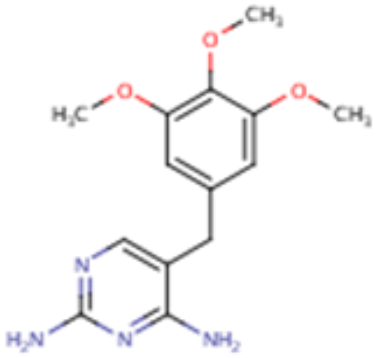
APPLICATIONS:

1. **As Antibacterial agents:** The increasing frequency of microorganisms displaying resistance to known chemotherapeutic agents has led to continued interest in the development and research on potent, broad-spectrum antibacterial agents. Among the most promising strategies for the development of new antibacterial therapeutics is the targeting of proteins, DHFR is usually an important target for treatment of variety of microbial infections [5].

Commonly used as antibiotics:

A. **Trimethoprim (TMP):** It is a pyrimidine inhibitor of dihydrofolate reductase, it is an antibacterial related to pyrimethamine. It is potentiated by sulfonamides and the trimethoprim-sulfamethoxazole combination is the form most often used for Tuberculosis patients. Trimethoprim is a successful pyrimidine antifolate due to its selective mode of action. It is five to six times more active on bacterial DHFR as compared to mammalian DHFR. This is the reason for its better tolerability and pharmacokinetic properties [6].

Structure:



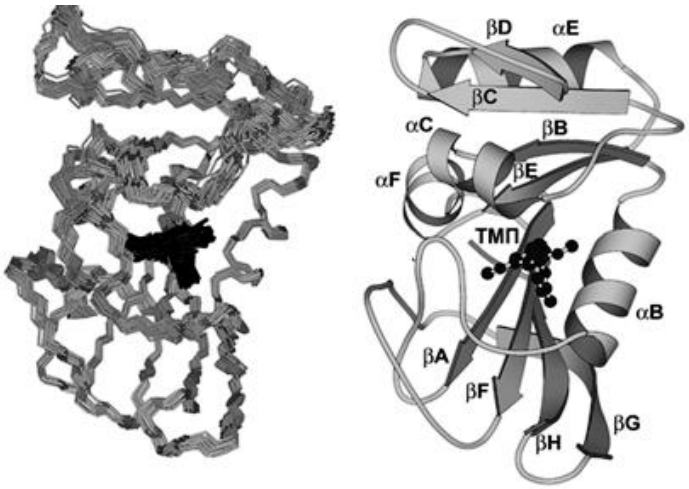
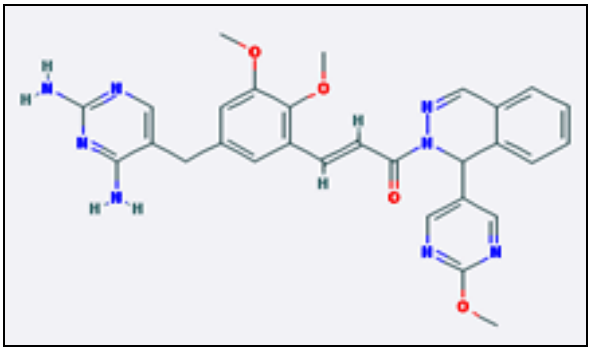


FIG. 3: INTERACTION OF TRIMETHOPRIM WITH *L. CASEI* DHFR WHERE BLACK PROTEIN CHAIN IS DRUG AND β SHEETS ARE REPRESENTED BY ARROWS PDB CODE-2HM9 [5]

Mechanism of action: Trimethoprim's (TMP) affinity for bacterial dihydrofolate reductase is several thousand times greater than its affinity for human dihydrofolate reductase. Trimethoprim binds to dihydrofolate reductase and inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF). THF is an essential precursor in the thymidine synthesis pathway and interference with this pathway inhibits bacterial DNA synthesis [7].

Pharmacodynamics: Trimethoprim is a pyrimidine analogue that disrupts folate synthesis, an essential part of the thymidine synthesis pathway. Inhibition of the enzyme starves the bacteria of nucleotides necessary for DNA replication. The drug, therefore, exhibits bactericidal activity [7].

B. **BAL0030543, BAL0030544 and BAL0030545:** They are dihydrophthalazine inhibitors with in vitro potency against gram-positive pathogens are recently discovered antibiotics and are in preclinical development stage by Basillea pharmaceuticals, Switzerland. These three dihydrophthalazine antifolates have improved potency compared to that of trimethoprim and activity against gram-positive pathogens resistant to other drug classes. They also have demonstrated *in vitro* activity against multiresistant staphylococci and *Streptococcus pneumonia*.[8]



BAL 0030543

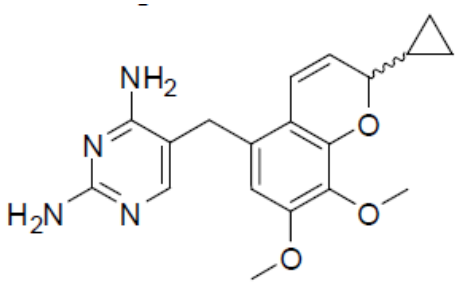
BAL0030543 and BAL0030544 had the lowest MIC (0.03g/ml) of the four DHFR inhibitors tested against methicillin sensitive *S. aureus* (MSSA) strains, and the maximum MIC for the BAL compounds was 0.25 g/ml (BAL0030544 and BAL0030545). The BAL compounds were markedly more potent against *S. pneumonia* strains than trimethoprim [8].

The antibacterial activities of BAL0030543, BAL0030544, BAL0030545, and the comparator drugs are shown [9];

TABLE 1: ANTIBACTERIAL ACTIVITY OF BAL0030543, BAL0030544, BAL0030545 AND THE COMPARATOR DRUGS AGAINST METHICILLIN SENSITIVE *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PNEUMONIAE* [8]

Organisms (No. of Strains)	Compound	MIC (µg/ml)		% susceptible
		50%	90%	
Methicillin sensitive *Staphylococcus aureus* (25) (MSSA)	BAL 0030543	0.03	0.03	100
	BAL 0030544	0.03	0.03	100
	BAL 0030545	0.12	0.25	100
	Trimethoprim	1	2	92
	Daptomycin	0.25	0.25	100
	Linzeolid	2	2	100
	Minocycline	0.25	0.25	92
	Vancomycin	1	1	100
Streptococcus pneumonia	BAL 0030543	0.06	0.12	100
	BAL 0030544	0.12	0.25	100
	BAL 0030545	0.12	0.12	100
	Trimethoprim	1	4	NA
	Daptomycin	0.25	0.12	NA
	Linzeolid	0.25	2	93
	Minocycline	0.12	0.25	100
	Vancomycin	**0.12**	**0.25**	**10**

C. **Iclaprim:** Iclaprim, a new selective dihydrofolate inhibitor, is based on rational drug design. It specifically and selectively inhibits purified wild type and mutant DHFR enzymes at low concentrations, being more potent than Trimethoprim.



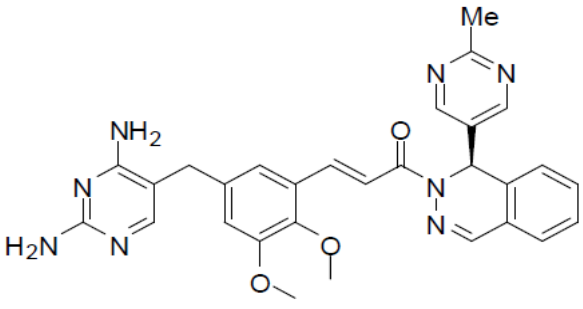
IUPAC name: 5[2-cyclopropyl-7,8-dimethoxy-2H-chromen-5-yl) methyl] pyrimidine- 2,4- diamine.

The compound has shown potent and expanded-spectrum activity against Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate, vancomycin resistant *S. aureus* (VISA, VRSA) and TMP-resistant strains [9].

Iclaprim exhibits a potent, bactericidal activity against Gram-positive pathogens (including multidrug-resistant pathogens such as MRSA) as well as show low resistance development. Provides an alternative treatment option to clinicians for the treatment of complicated skin and skin structure infections (cSSSI).

Phase 1 studies have shown that Iclaprim was well-tolerated in human subjects and has a low potential for drug-drug interactions. Phase 2 studies found that Iclaprim was well-tolerated in cSSSI patients and exhibited high clinical and microbiological cure rates that compared favourably with Vancomycin. Phase 3 studies demonstrated that Iclaprim exhibited a good safety profile and exhibits high clinical cure rates that were non-inferior to Linezolid.

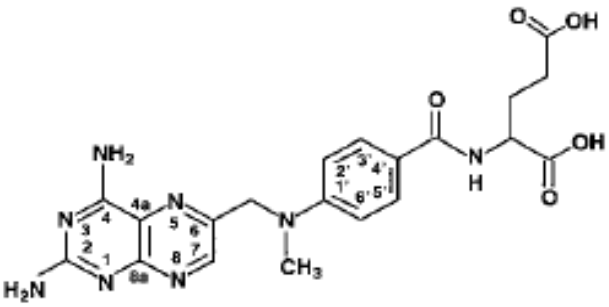
Novel 2,4-diaminopyrimidines bearing N,N-disubstituted amino methyl residues.eg., RO-64-5781 showed inhibitory activity that was up to 190000 times greater than that of TMP, allowing the coverage of TMP-resistant strains, and selectivity was at least as good as that of TMP [10].



RO-64-5781

2. **As Anticancer agents**:

a. **Methotrexate (MTX):** Methotrexate is a folic acid antagonist structurally designed to competes successfully with 7, 8 DHF for the DHFR enzyme. The direct inhibitors of DHFR causes cellular levels of 7, 8 DHF to build which in turn results in feedback (indirect) inhibition of thymidylate synthase. Methotrexate also is effectively involved in inhibiting glycine amide ribonucleotide (GAR) transformylase, a key enzyme in the synthesis of purine nucleotides [11].



IUPAC Name: (2S)-2-[(4-{[(2, 4-diamino pteridin-6-yl)methyl](methyl)amino}phenyl)formamido] pentanedioic acid

Modification of trimethoprim, an antibacterial drug with no tumor growth inhibition, led to the development of compounds 2 and 3 having appreciable anticancer activity that seem to be due to inhibition of DHFR [12].

Interaction with DHFR enzyme:

1. Binding of Methotrexate with DHFR is exclusively strong this can be attributed to number of specific interactions and amino acid sequence of protein (K_d value for eukaryotic enzymes=10^{-11}).

2. Interaction with enzyme involves protonation at N-1 atom which is responsible for sufficient coulomb interaction with a carboxy group of asparatic acid residue.

3. Coulomb interactions of α and γ carboxy group of p-methylaminobenzoylglutamate residue of the inhibitor with positively charged residues of His28 and Arg57 also play an important role of binding of MTX with enzyme.

4. The N5 position of DHF is protonated by Glu30 of DHFR and a cationic form binds to DHFR Asp27 through a cationic bend

5. The pKa of imidazole ring of residue His28 in complex with methotrexate is 1 unit higher than those in apo-enzyme or in complex with ligands devoid of γ carboxy group

6. Hydrophobic interactions of pteridine and benzoyl rings of the inhibitor with lipophillic amino acid residues involved in the enzyme binding site contribute significantly to total energy of binding of Methotrexate to DHFR.

7. Synchronisation of bond residues obtained on going from free arginine to complex in which residues interact with carboxy groups.[2,13]

Pharmacodynamics [15]:

1. Methotrexate prevents cancerous substances from being incorporated in to DNA during the "S" phase (of the cell cycle), stopping normal development and division.

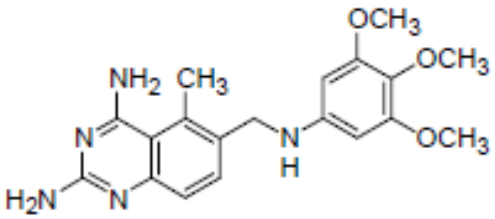
2. Tetrahydrofolic acid itself is synthesized in the cell from folic acid with the help of an enzyme, folic acid reductase. Methotrexate looks a lot like folic acid to the enzyme, so it binds to it quite strongly and inhibits the enzyme.

3. Methotrexate selectively affects the most rapidly dividing cells (neoplastic cells). It is also indicated in the management of severe, active, classical, or definite rheumatoid arthritis [13].

    a. **Trimethrexate:** Trimethrexate has been approved recently by the FDA for the use in the treatment of *Pneumocystis carinii* pneumonia (PCP) The administration of TMQ, in combination with leucovorin, is regarded as an good alternative in the treatment of PCP in AIDS patients.

TMQ therapy is currently recommended for patients with PCP who are either unable to tolerate or are resistant to first-line therapy with trimethoprim-sulfamethoxazole (co-trimoxazole). Trimethrexate is a non-classical folic acid inhibitor through its inhibition of the enzyme dihydrofolate reductase. It is being tested for efficacy as an antineoplastic agent and as an antiparasitic agent against pneumocystis pneumonia in AIDS patients 13.

Structure:



TRIMETHREXATE

IUPAC name: 5-methyl-6-{[(3, 4, 5-trimethoxy phenyl)amino]methyl}quinazoline-2,4-diamine

Mechanism of action: Trimetrexate is a competitive inhibitor of dihydrofolate reductase (DHFR) from bacterial, protozoan, and mammalian sources. DHFR catalyzes the reduction of intracellular dihydrofolate to the active coenzyme tetrahydrofolate. Inhibition of DHFR results in the depletion of this coenzyme, leading directly to interference with thymidylate biosynthesis, as well as inhibition of folate-dependent formyltransferases .The end result is disruption of DNA, RNA, and protein synthesis, with consequent cell death [13].

Pharmacodynamics: By interfering with the reduction of folic acid, trimetrexate interferes with tissue cell reproduction. Generally, the most sensitive cells to the antimetabolite effect of trimetrexate are those cells which are most actively proliferating such as malignant cells, dermal epithelium, buccal and intestinal mucosa, bone marrow and cells of the urinary bladder, because the proliferation of cells in malignant tissues is greater than in most normal tissues [13].

Quinazolinone Derivatives [14]: In order to produce potent new leads for anticancer drugs, a new series of quinazoline analogs was designed to resemble methotrexate structure and fitted with functional groups was believed to enhance inhibition of mammalian DHFR activity.

It is well known that quinazoline derivatives are potent inhibitors of epidermal growth factor receptor (EGFR) .Combining the inherent DHFR inhibition activity of the quinazolines and those functional groups in one structure was expected to produce more active compounds.

As antimalarial agents: Number of effective drugs available that interact in different ways with the biochemical life cycle of the parasites are quinine, chloroquinine, primaquine, halofantrine, pyrimethamine and proguanil. But these paprasites have soon developed resistance against these drugs [15].

Different basic approaches to drug discovery for malaria are used and among these approaches, target base approach is one of the leading approaches in malaria chemotherapy. In this connection, DHFR is a clinically validated target in anti-malarial treatment.

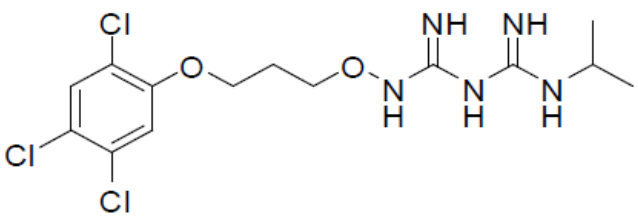
Classification: Antifolate agents used in the treatment of malarial infection are subdivided into two classes: inhibitors of dihydropteroatesynthase (DHPS), known as class I antifolates, and inhibitors of dihydrofolate reductase (DHFR), also known as class II antifolates.

Class I antifolates are Dapsone, Sulphadoxene and Sulpahanene and Class II antifolates include Proguanil, Pyrimethamine.

In search for novel inhibitors of *Pf* DHFR, molecular docking approach was carried out. *N*-hydroxyamidines, pyrimidines, triazines, urea, and thiourea-derivatives were identified as inhibitors of the wild-type and resistant mutants PfDHFR harbouring the widespread single, double, triples, and quadruple mutations of this enzyme.

Phenoxypropoxybiguanides are recent class of antimalarial prodrugs analogous of proguanil and its active metabolite Cycloguanil. WR99210, the active metabolite of PS15 has retained in vitro potency against newly emerging antifolate resistant malaria parasites [16].

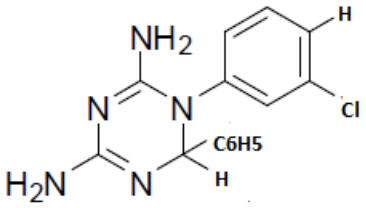
Dihydrotriazine metabolites another class exhibited potent antimalarial activity with in vitro IC_{50} values less than 0.04 mg/mL.



PS-15

Recently a new series of 4-diamino-1, 6-dihydro-1, 3, 5-triazine derivatives (cycloguanils) were reported as potent inhibitors. These inhibitors showed hydrogen bonding interactions with folate binding siteTrp48, Asp54, Arg122, Ile164, Tyr170 and Thr185.

A lead compound has been found with inhibitory activity similar to that of cycloguanil against the wild type DHFR and about 120-times more effective than Cycloguanil against the mutant enzyme. Analogues **10** and **11** were over 120-fold more effective than Cyc against the mutant pfDHFR



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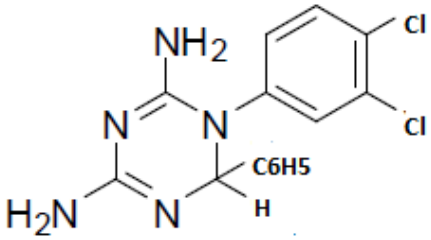

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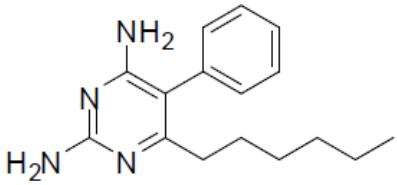
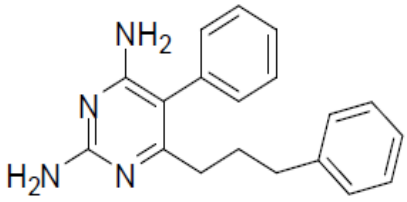
Several 2, 4-diaminopyrimidine compound are efficacious against single, double, triple and quadruple mutant varieties of the *P.falciparum* enzyme. Two compounds A10 and A11in particular displayed greater *P. falciparum* DHFR inhibition [17].



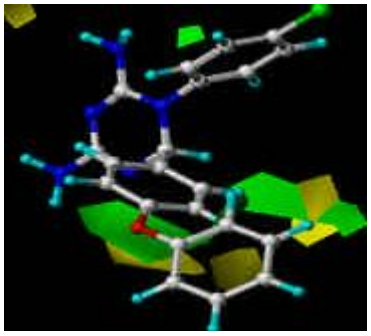
A10



A11

To visualize the content of the derived 3D QSAR models for identification of cycloguanils derivatives as DHFR inhibitors, CoMFA (Comparative molecular field analysis) and CoMSIA (comparative molecular similarity indices analysis)contour maps were generated.

The contour maps of CoMFA and CoMSIA steric field are shown below. Green and yellow represent favorable and disfavorable contour for steric site respectively. R1, R phenyl ring substituents are in steric favorable and co region is in the disfavored regions according to the contour maps. Hence substitution at R1 with simple methyl group showed increase in activity.



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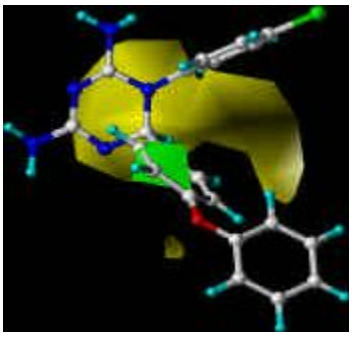


2

FIG. 4: 1 is for CoMFA (comparative molecular field analysis) and 2 is for CoMISA (comparative molecular similarity indices analysis) in which green is sterically favoured and yellow for steric disfavoured [18]

4. **Resistance to Antifolates:** Resistance of *Plasmodium falciparum* to antifolates is an important problem in antimalarial chemotherapy and has been shown to be associated with mutations in the Dihydrofolate reductase (DHFR) domain. Chloroquine, Mefloquine and other frontline drugs for the treatment and prevention of malaria are becoming increasingly ineffective.

Artemisinin analogues such as Artesunate and Arteether were later introduced and found to be quite effective, particularly against drug resistant *P. falciparum* but these causes neurotoxicity in animals [8-10]. Therefore, there is an eminent need for new and safe antimalarial drugs to combat this disease in areas of malaria endemicity [19].

Such mutation-based resistance raises serious questions on the possibility of developing new antifolates with prolonged therapeutic life, since new mutations could arise to compromise on the new antifolates. But it may be possible to develop inhibitors against which mutations would not be possible, since they would also lead to non-functional enzymes.

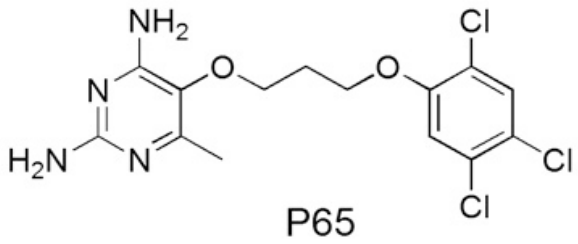
Mutation to Asn108 leads to resistance to Pyrimethamine and a moderate loss of response to cycloguanil (CYC),A mutation of Threoinine 108 along with Ala16 to Val 16 mutation provides resistance to CYC.[20]. These findings strongly suggest that the threonine-108 side chain in the *P. falciparum* enzyme makes an important interaction with the chlorophenyl group of pyrimethamine and that disruption of this interaction leads to drug resistance.[20]

Highly efficacious, and orally available antimalarial drug candidate that potently inhibits both wild-type and clinically relevant mutated forms of Plasmodium falciparum (Pf) DHFR that has recently developed includes

P65, P218 and WR 99210:

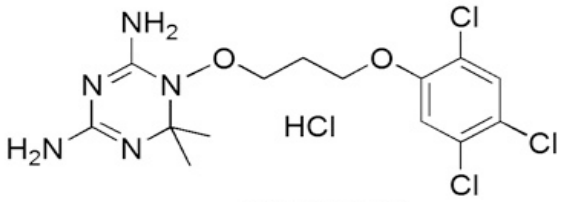
P65 [21]: Due to low bioavailability of WR99210, synthesis of P65 was done which is 2, 4 diaminopyrimidine analogue of WR99210. Triazines such as WR99210 and Cycloguanil are much more basic (pKa 10–11) than pyrimidines. Thus, at the slightly acidic pH of the gastrointestinal track, the triazines are fully protonated whereas 2, 4-diaminopyrimidines exist as a mixture of protonated and unprotonated forms.

Hence, the oral bioavailability of P65 in rats was found to be 83%, compared to less than 1% for WR99210.



P65

WR 99210 [21]: A 4, 6-diamino-1,2-dihydro-1, 3, 5-triazine WR99210 was advanced as an antifolate-based antimalarial with high activity against both wild-type and Pyrimethamine resistance associated Pf DHFR .



WR99210

WR99210 could avoid steric clash with the side chain of Asn-108 in Pyrimethamine-resistant quadruple mutant PfDHFR because of its flexible (2, 4, 5-trichlorophenoxy) propoxy side chain. Further development of WR99210 as an antimalarial was terminated because of its low bioavailability and severe gastrointestinal toxicity.

P218: P218 is an inhibitor of both wild-type PfDHFR and the highly PYR-resistant quadruple mutant enzyme. The carboxylate of P218 binds to quadruple mutant PfDHFR makes two charged mediated hydrogen bonds with Arg122, but in human DHFR the P218 side chain has no direct interaction with Arg70 that is P218 binds differently to *Plasmodium falciparum* and Human DHFR [21].

5. **As Antituberculosis agent**: As one of the oldest and most destructive human diseases, TB continues to be a massive burden on public health around the world. The rise of AIDS (acquired immune deficiency syndrome), and with it a population of people who are more susceptible to opportunistic infections, has escalated the prevalence of TB. Today, roughly 1.4 million people die every year from TB, despite the availability of chemotherapies to combat the bacterium that causes this disease. The current standard treatment for tuberculosis is a four-drug combination therapy that lasts a minimum of 6 months. In addition, several factors have led to new strains of *Mycobacterium tuberculosis(Mtb)*that are resistant to many or sometimes all of the first and second line drugs.

There have been increased efforts to develop new and improved therapeutics for tuberculosis, and this has led to a few promising drug candidates. Although DHFR has been extensively studied as an effective drug target in several pathogens and cancer cells, it has not been successfully targeted in *Mtb*

NC00094240 demonstrated a dose-dependent inhibition on the purified recombinant *Mtb* DHFR (IC_{50} = 22.4 nM), as well as DHFR-specific growth suppression of live *Mtb*, displaying an MIC_{99} of 207µM. NC00094221 belongs to the diaminoquinazoline family of compounds.[22]

A potent inhibitor containing a quinazoline ring was identified. This compound was active against the wild-type laboratory strain H37Rv (MIC (99)=207 µM). In addition, an Mtb strain with artificially lowered DHFR levels showed increased sensitivity to this compound the inhibition was target-specific.[23]

6. **Leishmaniasis and Trypanosomiasis treatment:** It occurs in 88 countries in the Africa, South Asia, and Latin America where they form a serious health problem. Visceral leishmaniasis (VL*a*) is a disease caused by infection with human protozoan parasites belonging to the *Leishmania donovani* complex. While *Trypanosoma cruzi* (Tr .c) is responsible for Chagas disease in south America and *Trypanosoma brucei gambiense* is accountable for sleeping sickness in Africa.

   WHO has selected these disease as "neglected and emerging disease", and the novel therapies to combat leishmaniasis and trypanosomiasis are urgently needed [24]. DHFR inhibitors recently studied for Leishmaniasis and Trypanosomiasis belong to 2 classes – 5 benzyl 2, 4 diamino pyrimidines and 2, 4 diaminoquinazolines [24, 25].

5 benzyl 2, 4 diaminopyrimidines: 5-benzyl-2, 4-diaminopyrimidines were prepared and assayed against recombinant DHFR form *L. Major*, *T. Cruzi*,

T. Brucei, and human. There is a major interaction between the 2, 4-diaminopyrimidine moiety and an aspartic acid residue found in the active site. The glutamate moiety of the substrate then lies in the cleft along the surface of the protein.

Straight chain and branched chain analogues were synthesized. For the 3-substituted compounds, maximum activity against all the parasitic DHFRs occurs for chain length of 2-6. As the chain length increases further the activity decrease.

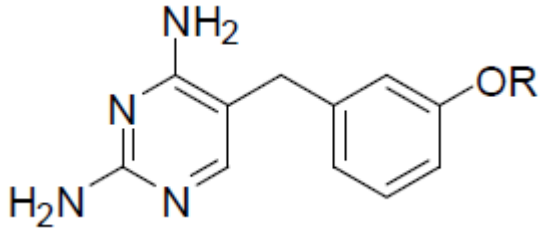
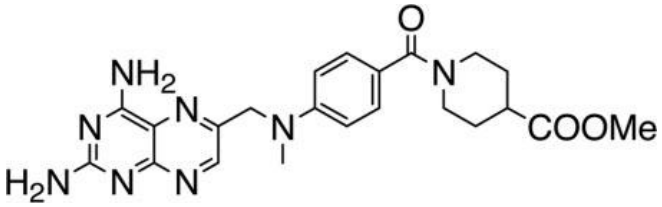


FIG. 5: 3-ALKOXY SUBSTITUTED COMPOUNDS WHERE R CAN BE C_nH_{2n+1} n=1-10 [24]

New series of 2, 4diaminopyrimidines were synthesised with the aim of maximizing activity and selectivity for the enzyme, even as getting better *in vitro* activity.

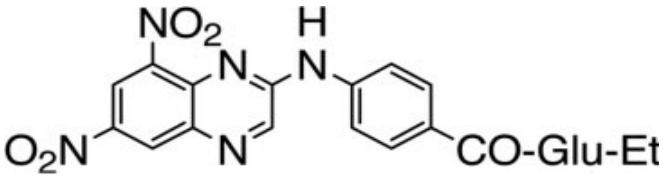
5 benzyl 2, 4 diaminoquonazolines: Notably, compound 1a, with a inhibition constant of 7 M against *Tc*PTR1 and of 4 M against *Leishmania* DHFR, it is the best inhibitor of *Leishmania*PTR1 and one of the most selective compounds After identification of 1a it was re-evaluated to identify similar compounds for further testing Compounds **1b**, **1c**, **1d** were tested against *Lm*PTR1 and the best inhibitors also against *h*DHFR. **1b** displayed *K*i values of 37 and 820 nM against *Lm*PTR1 and *Human* DHFR, respectively [25].

1a



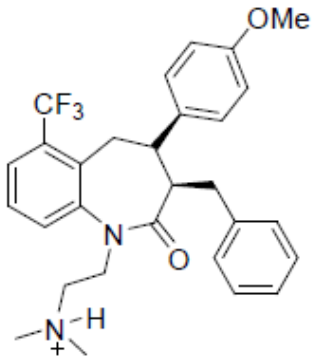
1b

1c



1d

Also in an attempt to identified the new non-2,4-diaminopyrimidine lead structures, showing activity against the *T. Cruzi* DHFR were successfully accomplished by computer-aided screening techniques. Three molecules (compounds **7**, **8** and **9**) that showed moderate enzyme inhibition activity (IC50<100 μM)were identified.



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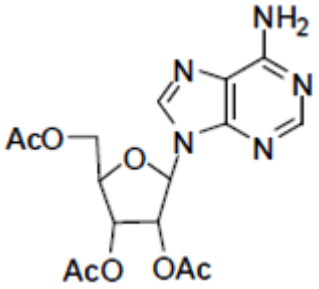

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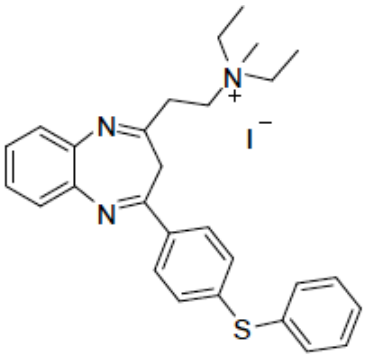


9

7. **Antifungal agents:** There have been very few studies focusing on DHFR as an antifungal target *Candida glabrata* is an emerging fungal pathogen that currently account for at least 20% of all candidemia infections. Unfortunately, C. Glabrata is resistant to the majority of clinically approved antifungal agents primarily, also causes a significant (20%) number of bloodstream infections. This can be attributed to the lower susceptibility of *C. glabrata* toward the azole compounds, especially the commonly used agent fluconazole. The therapeutic window to treat *C. Glabrata* is even narrower because *C. Glabrata* strains are also often resistant to amphotericin B [26].

Crystal structure of *C. glabrata*: The overall structure of the CgDHFR protein consists of a ten-strand central β sheet and five flanking α helices containing 217 residues. The additional two strands in the central sheet, relative to the structures of DHFR that typically have an eight-stranded β sheet, are formed from a 25 residue insert (177–202 in Cg DHFR) in the sequence. CgDHFR and *C. albicans* DHFR share 85% sequence homology. But there are also two structural differences at the active site: CgDHFR has a methionine (Met33) interacting with the pyrimidine ring of the inhibitor; CaDHFR has an isoleucine in this position (Ile33) [26].

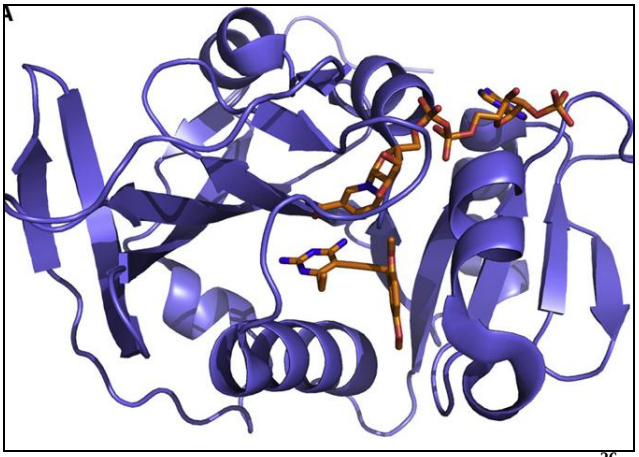
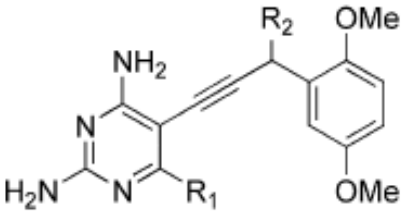


FIG. 6: STRUCTURE OF *CANDIDA GLABRATA* [26]

These propargyl linked inhibitors may also serve as potential antifungal lead compounds. These inhibitors bind the *C. glabrata* DHFR enzyme with subnanomolar potency, display greater than 2000-fold levels of selectivity over the human enzyme, and inhibit the growth of *C. glabrata*

First generation compounds:



If R_1 and R_2 is Et and H, then it was found that it has IC_{50} value for *C. glabra* is 8.1nM and Human DHFR is 1,280 nM and antifungal activity of 78 µg/ml which is most potent compound found for *C. glabra.*

Interaction with *Candida glabra* DHFR: The protonatedN1 atom and the 2-amino group of the pyrimidine ring form hydrogen bonds with Glu32 and an ordered water molecule. Additional hydrogen bonds are formed between the 4-amino group and the backbone carbonyl oxygen atoms of Ile9 and Ile121. The dimethoxyphenyl ring fits in a hydrophobic pocket composed of Met33, Ile62, Leu69, Phe66, Pro63 and Thr58 [26].

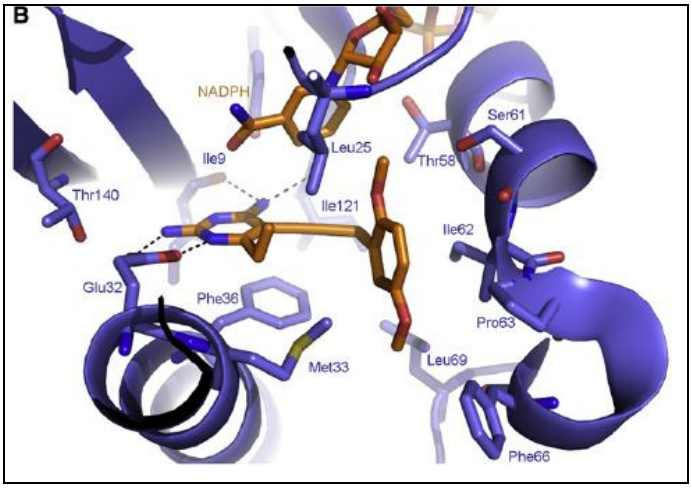


FIG. 6: INTERACTION OF FIRST GENERATION COMPOUNDS WITH CgDHFR WITH HYDROGEN BONDS SHOWN AS DASHED LINES [26]

These are compounds with a trimethoxyphenylring that also showed potent inhibition while exhibiting greater antifungal activity. It displays the best combination of potency, selectivity, and antifungal activity. The docked complex of First generation compounds in CgDHFR revealed potential interactions of the propargyl methyl group with a pocket composed of Ile121, Thr58, and Ile62. 3 methoxy group interacts with Ser61and Leu25, a 4 methoxy group has very few interactions with the protein, and a 5 methoxy group that interacts withIle62, Pro63, Met33 [26].

Second generation compounds: It seemed that larger lipophilic moieties at the 5 position on the aryl ring, relative to the simple methoxy group of compounds 11 and 6, could take better advantage of the hydrophobic interactions available from Ile62, Pro63, Met33 and Phe66.

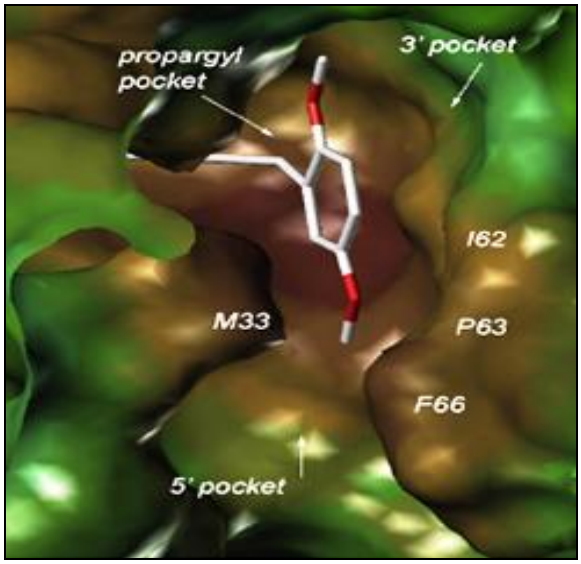
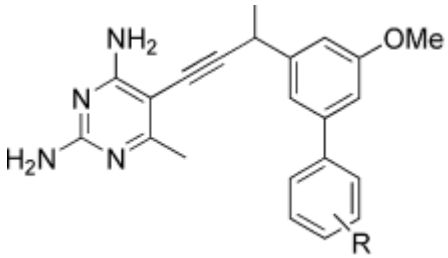


FIG. 7: INTERACTION OF SECOND GENERATION INHIBITORS WITH *C. GLABRA* DHF[26]

High-resolution structure of CgDHFR and second-generation inhibitors were designed to be significantly more potent and selective. The series of second-generation derivatives maintained methyl group at the C6 position of the pyrimidine, a propargyl methyl group, and the original 3 methoxy-substituted phenyl ring. However, the new compounds also include a second aryl ring at the meta position of the first phenyl ring.



Compound A and compound B positions on the second ring were designed with R at 3, 5 position to be CH_3 and at para position CH_3 respectively.

Not only are these compounds extremely potent, with IC_{50} values in the nanomolar (nM) range against CgDHFR, they also exhibit very strong selectivity for the fungal enzyme. It also displayed superior antifungal activity [26].

Presence of thiazolidinone ring in the compounds enhances the antifungal activity to a considerable level Compounds with phenyl ring having hydroxy group or methoxy group at ortho or para position exhibit prominent antifungal activity. Ortho derivatives possess the better antifungal activities [27].

CONCLUSION: Dihydrofolate reductase is involved in conversion of 7, 8 dihydrofolate to 5, 6, 7, 8 tetrahydrofolate. Inhibition results in the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF). THF is an essential precursor in thymidine synthesis pathway and interference of this pathway inhibits bacterial DNA synthesis. Dihydrofolate reductase inhibitors are important class of compounds have wide use in areas of anticancer, antifungals, antibiotics and Leishmaniasis and trypanosomiasis.

Iclaprim a new dihydrofolate reductase inhibitor is currently under Stage III clinical trials for complicated skin and skin structure infections. BAL 003054 and other analogues are currently under stage I for *S. pneumonia* and MRSA. Due to growing concern for resistance against malarial antifolates WR 99210 and P 65 have been developed which have high activity against pyremethamine resistant Pf-DHFR. DHFR have proven to be emerging target for treatment of wide range of cancers, fungi, bacteria.

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