Review Article

DIHYDROFOLATE REDUCTASE AS A VERSATILE DRUG TARGET IN HEALTHCARE

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Abstract: Dihydrofolate reductase is one of the important enzymes for thymidylate and purine synthesis. It has been used as a drug target for treatment of various diseases. A large number of pharmaceutical drugs have been designed to inhibit the activity of dihydrofolate reductase. However, over the period of time some organisms have developed resistance against some of these drugs. There is also a chance of cross reactivity for these drugs, as they may target the dihydrofolate reductase enzyme of other organisms. Although using NMR spectroscopy, phylogenetic sequence analysis, comparative sequence analysis between dihydrofolate enzymes of various organisms and molecular modeling studies, a lot has been unraveled about the difference in the structure of this enzyme in various organisms, yet there is a need for deeper understanding of these differences so as to design drugs that are specific to their targets and reduce the chance for cross reactivity. The dihydrofolate enzyme can also be explored for treatment of various other diseases that are associated with the folate cycle.

Keywords: Dihydrofolate Reductase; Drug target; Inhibitor; Folate cycle; NADPH

Note : Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Dihydrofolate reductase (EC 1.5.1.3) (5, 6, 7, 8 tetrahydrofolate: nicotinamide adenine dinucleotide phosphate oxidoreductase) is a pervasively present enzyme in all the organisms (Askari *et al.* 2010; Schnell *et al.* 2004). It exhibits a significant function in the synthesis of purine, thymidylate and several other amino acids like glycine, methionine and serine (Hong *et al.* 2015). It catalyzes the reduction of dihydrofolate [(H2F) (7, 8 dihydrofolate)] which leads to regeneration of tetrahydrofolate [(H4F) (5, 6, 7, 8 tetrahydrofolate)] (Schweitzer *et al.* 1990; Frey *et al.* 2012; Hong *et al.* 2015). This reaction involves the use of a cofactor which is the reduced form of nicotinamide adenine dinucleotide phosphate i.e. NADPH. Inhibiting the activity of dihydrofolate reductase leads to imbalance in the pathways involved in the synthesis of purine and thymidylate as it causes reduction in the quantum of folates present in the cell. Additionally, inhibition of dihydrofolate reductase also disrupts DNA replication (Neradil *et al.* 2015). All this disruption eventually leads to cell death. Owing to its essential role, dihydrofolate reductase has been studied as a lucrative target for designing of inhibitors that could help in treatment of several fatal diseases e.g. cancer (Askari *et al.* 2010). It has also been used as a target for various antimicrobial and antineoplastic drugs (Shakya *et al.* 2010).

In hock of its crucial role in the treatment of diseases, dihydrofolate reductase has been very well evaluated. A number of studies have been carried out in order to understand its structure, mechanism of action, enzymatic activity, the mechanism for its control at the level of transcription and transregulation, functional analysis, drug designing (*in silico*), transcription in absence of promoters (TATA) , auto regulation of translation, and regulating the process of transcription through the cell cycle.

Dihydrofolate reductase is piloted by a TATA-less promoter. This promoter is further under the control of multifarious transcription factors such as Sp1 and E2F. These transcription factors are significant for the modulation of the dihydrofolate reductase protein through the whole of cell cycle (Askari *et al.* 2010). At the G1/S phase, the concentration of these enzymes reaches its zenith.

Many reports have been put forward that suggest the autoregulation of the levels of dihydrofolate reductase is achieved by interactions between the dihydrofolate reductase and RNA interactions (Blume *et al.* 2003; Martianov *et al.* 2007). There is an involvement of dihydrofolate reductase in the conversion of synthetic folic acid (which is present in food used as supplements) into the tetrahydrofolate. This conversion takes place intracellularly. The tetrahydrofolate forms produced due to this conversion are able to enter into the metabolism of folate/homocysteine. If there is decrease in the activity of the enzyme dihydrofolate reductase then the quantum of tetrahydrofolate molecules present in the cell declines which alters the quantity of folate coenzymes and eventually perturbs the synthesis of purines and pyrimidines (Chen at al. 1994). Perhaps, this alteration can also affect the quantity of homocysteine and the process of methylation. The methyl tetrahydrofolate helps in remethylation of homocysteine in so as to produce methionine and by that establishing the procurement of S-adenosylmethionine imperative for various reactions that involve methylation (Sohn *et al.* 2009).

The family of dihydrofolate reductase gene comprises of the functional dihydrofolate reductase gene and more pseudogenes which are intronless (dihydrofolate reductaseP1–4) (Anagnou *et al.* 1994). The chromosome 5q11.2-13.2 is occupied by the functional gene. It is expressed in three isoforms of messenger RNA which are spliced alternatively at the 3' untranslated region (Morandi *et al.* 1992). The functional polymorphisms of the dihydrofolate reductase gene could be responsible for the difference in the expression or activity of dihydrofolate reductase to some extent, and consequently meddling with the peril for the diseases associated with the folate metabolism. The variations in genes may also result in impinging the therapeutic feedback to antifolate which leads to deteriorated efficacy of treatment or elevated inimical drug event prevalence.

Diversity in the structure of dihydrofolate reductase in various species

There is a vast diversity in gene families and their corresponding proteins. This is achieved by various phenomena such as random genetic drifts, mutations and natural selection. This heterogeneity has lead to a broad range of enzymes, scaffolds in cells, signal transducers and several other molecular machines present in all the kingdoms of life. The dihydrofolate reductase enzymes in various species have evolved over the years. The enzyme has accustomed to various environments and specialized functions. Its evolution is significant as it helps not only to understand the adaptation of the enzyme to different environments but also helps in establishing a deeper understanding of the evolution of drug resistant pathogens.

Dihydrofolate reductase enzyme particularly possesses a characteristic motif consisting of a dinucleotide binding domain (Rossmann fold) which is extremely conserved in the enzymes that are associated with the cofactor NAD (H) or NADP (H). It typically comprises of parallel β sheets that are flanked on each side by α helices. The α helices are intercalating between the parallel and anti parallel β sheets. Human dihydrofolate reductase has a molecular weight of 21,544 Da and consists of 186 amino acids (Blakley, 1995). In *E.coli*, it has been observed that dihydrofolate reductase posses a globular

structure and possesses 159 amino acids. It comprises of eight β sheets that are from A–H, four β helices which are labeled as B, C, E and F, and other amino acids which make loops within the elements of the secondary structure of dihydrofolate reductase. Two helices are present flanking on the either side of the sheet.

Though the general secondary structure, mechanism of action and the amino acids required for catalysis are conserved throughout the evolution of the dihydrofolate reductase enzyme yet there are certain small differences that are present in the human dihydrofolate reductase and the *E.coli* dihydrofolate reductase. The human dihydrofolate reductase is rigid in its structure as compared to the *E.coli* dihydrofolate reductase. This difference is due to the presence of loop like structures in the *E.coli* dihydrofolate reductase. The main loop regions that are responsible for imparting flexibility to dihydrofolate reductase in *E. coli* are the M20 loop (residues 14–24), the FG loop (residues 116–125) and the GH loop (residues 64–71).On comparing the crystal structures of dihydrofolate reductase, it was revealed that dihydrofolate reductase present in vertebrate lacks loop 1 motion and rotation in its sub domain (Met20 loop in *E.coli*) structures (Sawaya and Kraut, 1997). There are three major differences in structure of the vertebrate/human and *E. coli* dihydrofolate reductase which could be considered responsible for giving rise to occurrence of rigidity in human/ vertebrate dihydrofolate reductases. The first difference is the presence of a left-handed polyproline-type helix which is inserted in the loop 1 of *E.coli* dihydrofolate reductase. The second main difference is the replacement of asparagine with Gly20 in the loop 1 of dihydrofolate reductase in vertebrates which is responsible for creating a β-hairpin. Thirdly, the G-H loop in vertebrate dihydrofolate reductase is truncated which prevents the hydrogen bond formation with loop 1.

A study was carried out by Bhabha *et al.* 2013 to examine different dihydrofolate reductase enzymes from different species, both prokaryotic and eukaryotic. They used diverse techniques such as nuclear magnetic resonance, X ray crystallography, assessment of enzyme's function in cells, evaluation of dihydrofolate reductase amino acid sequences and *in vitro* under various conditions. To evaluate the role that dynamics plays in the functioning of dihydrofolate reductase, they studied the crystal structure of dihydrofolate reductase from various species. They observed that the loops in active site possess closed conformation in all the crystal structures of vertebrate dihydrofolate reductases. Also, using the ^{15}N HSQC spectra they found that the *E. coli* dihydrofolate reductase undergoes backbone conformational change in three product complexes. It is in closed conformation when it forms complex with NADPH or NADP+–substrate but in three product complexes, it is present in occluded state which allows for the influx of ligands. In contrast to *E. coli* dihydrofolate reductase, human dihydrofolate reductase always stays in closed conformation. They further carried out a detailed comparative evaluation of about 1800 dihydrofolate reductase sequences including eukaryotic dihydrofolate reductases. The main focus was given to three sites that are mainly responsible for mechanistic difference between the dihydrofolate reductases of different species. These three sites were, region A located at the end of the Met20 loop and regions B and C, which contained the hinges involved in rotation of sub domain. Most of the dihydrofolate reductase enzymes of prokaryotic organisms possessed 7 residues in region A. In dihydrofolate reductases present in the eukaryotic organisms, it was observed that the region A is more heterogeneous, with some sequences comprising 7 residues, 8 residues, and >8 residues. The dihydrofolate reductases present in the *Bacillus anthracis* (*ba*dihydrofolate reductase), *Staphylococcus aureus* (*sa*dihydrofolate reductase), *Streptococcus pneumonia*e (*sp*Dihydrofolate reductase) and *Vibrio cholerae* (*vc*Dihydrofolate reductase) possessed 7 residues in their region A. These four dihydrofolate reductases which contain 7 residues in Region A, showed about 34% to 51% identity with the *E.coli* dihydrofolate reductase. The region B contains hinge 1. The dihydrofolate reductase sequences of the bacteria consisted mainly of short sequences of region B as in *E.coli* dihydrofolate reductase which comprises of 12 residues. On the other hand, in case of eukaryotic dihydrofolate reductase, region

B usually comprise of about 18 or even more residues. However, very few exceptions are seen in certain unicellular species of eukaryotic organisms e.g. some amoebozoans and stramenopiles. The hinge 2 present in the region C was observed to comprise of His127-Leu131 in case of human dihydrofolate reductase. In *E.coli* dihydrofolate reductase it was seen to comprise of Pro105-Ala107. On observing the pattern of sequences in the regions A, B and C, it was seen that some dihydrofolate reductases possess flexible loops possessing 7 residues in region A as well as long hinges. On the other hand a few archaeal dihydrofolate reductase enzymes comprise of a rigid active site possessing 8 residues in region A and short hinges. Such dihydrofolate reductase enzymes are considered to be inefficient in their ability to exchange ligands. It was put forth that, despite the fact that human and bacterial dihydrofolate reductases possess identical three dimensional structures, their intrinsic properties vary greatly. Dihydrofolate reductase enzymes possessing 7 residues in their region A and long hinges have been found in many fungi, members of the unicellular flagellate eukaryotes (euglenozoa) and invertebrate animals.

In the study carried out by Bhabha *et al.* 2013 it was also observed that the dihydrofolate reductase enzyme in the vertebrates functions very less cellular concentrations of NADP$^+$ (~20 μM vs ~2 mM) and THF (~0.3 μM vs ~13 μM) as compared to the dihydrofolate reductase of the *E. coli* and the proportion of NADPH to NADP$^+$ varies largely between mammalian which is about 100:1 and in *E. coli* cells which is about 1:1. As the dihydrofolate reductase enzyme present in vertebrates function at low levels of NADP$^+$ and THF in the cell, they have over the time adapted to bind the cofactor firmly and release it more moderately (Bhabha *et al.* 2013).

Another study was carried out by Tony Liu *et al.*, 2013 in which they devised stringent evolutionary criteria for evaluation of amino acid sequences of the dihydrofolate reductase enzyme from different species. Presence of small mutational substitutions results in the divergence observed within an orthologous protein family. They studied the dihydrofolate reductase enzyme of about 233 species that ranged from human to bacteria where three important evolutionarily sequence divergent sites [phylogenetically coherent events] were found. They also performed various experiments to investigate catalytic consequences of various phylogenetically coherent events and observed that on introducing these three phylogenetically coherent events into the framework of *E.coli* dihydrofolate reductase enzyme, its *in vitro* properties were transformed as that of the dihydrofolate reductase enzyme present in humans (Tony *et al.*, 2013).

Structure based variation in the specificity of inhibitors of dihydrofolate reductase

Dihydrofolate reductase is widely targeted for various pathogenic and malignant diseases (Bhosle *et al.* 2016). Antifolates are the most competitive inhibitors of this enzyme. The inhibitors that are used clinically are usually specific to their target dihydrofolate reductase. At times there is a certain degree of cross reactivity with dihydrofolate reductases enzymes belonging to other species. To understand the affinity and specificity of these inhibitors towards their target dihydrofolate reductase enzymes belonging to various species, a study was carried out by Bhosle *et al.*, 2016. They recognized the possible druggable space of a dihydrofolate reductase enzyme in a substructure. This space was termed the 'supersite'. They further categorized supersites of dihydrofolate reductase enzymes into 16 site types from 56 species. This was done on the basis of pairwise structural similarity. A deeper evaluation of these supersites showed that dihydrofolate reductase enzymes display differing extents of non uniformity at positions that are structurally equivalent, in the vicinity of the binding site. They achieved an antifolate and dihydrofolate reductase network by mapping the commonly used high specificity antifolates with their respective supersites. This map was then used to describe antifolates that could be repurposed on the basis of similarity between supersites or antifolates. They were able to determine a variety of antifolates out of which 177 were specific for humans and 458 were specific for pathogens. These maps can be used efficiently for designing of inhibitors that are species specific to the dihydrofolate enzyme (Bhosle *et al.*2016).

Inhibitors of dihydrofolate reductase

Dihydrofolate reductase performs a significant role in folate biosynthesis pathway and is a well known chemotherapeutic target (Lele *et al.*, 2016). It was primarily analyzed for its anti cancer drug discovery. The two most commonly used anti cancer drugs that are inhibitors of dihydrofolate reductase are methotrexate and aminopterin. Both these drugs belong to the class diaminopteridines. Both these drugs are analogues of folic acid. Nevertheless, because of their toxic nature these drugs were specifically used for the treatment of cancer and not for treating any other infectious disease. With the development of new derivatives of diaminopteridine, the use of these drugs for other therapeutic uses has been repositioned. The new analogues have been found to possess anti parasitic, immuno suppressants, anti bacterial etc. Similarly, new compounds such as diaminopyrimidine, diaminoquinazoline and diaminodihydrotriazines are being evaluated for modifications in their structure (Lele *et al*, 2016). These modifications can help in utilizing these compounds for other medical applications and can be used in various other infectious disease conditions.

Dihydrofolate reductase as a target for antimicrobial inhibitors

Dihydrofolate reductase as a target for infection caused due to Mycobacterium tuberculosis

Tuberculosis one of the most deadly infections is caused by *Mycobacterium tuberculosis.* It has a higher occurrence in South East Asian and African countries. Although tuberculosis is a prevent-able disease yet due to robust elevation in the resistance against multiple and extensive drugs, there has been rise in the dire need for development of new drug targets for *M. tuberculosis* (Hong *et al.* 2015)*.*

Methotrexate is one of the most well known inhibitors of dihydrofolate reductase which binds with the human dihydrofolate reductase and the *Mycobacterium* dihydrofolate reductase with same selectivity. Methotrexate contains 2, 4-diaminopteridin at its core. However, the entire pteridinediamine structure is not needed and a number of non classical inhibitors have been designed. These non classical inhibitors possess pyrimidine-2, 4-diamines or its analogues at the central core [e.g. trimethoprim (TMP)] (Hong *et al.* 2015). The crystal structures of methotrexate, binding to *Mycobacterium* dihydrofolate reductase and human dihydrofolate reductase, were individually analyzed (Hong *et al.*, 2015). It was observed that the selectivity of the *Mycobacterium* dihydrofolate reductase inhibitors was based on the glycerol binding site of the *Mycobacterium* dihydrofolate reductase. The glycerol binding site is lacking in the human dihydrofolate reductase (Hong *et al.* 2015). The *Mycobacterium* dihydrofolate reductase active site comprises of two parts which are the dihydrofolate binding site and the glycerol binding site. Usually the dihydrofolate reductase inhibitors target the dihydrofolate binding site. The binding site for glycerol is close to the dihydrofolate binding site and can be considered as the extension of the dihydrofolate binding site. It has been reported that glycerol binding site can be essential for the development of inhibitors that are specific to *Mycobacterium* dihydrofolate reductase (Hong *et al.* 2015). Using *in silico* methods a group of compounds based on the effect of glycerol binding site have been obtained that are able to inhibit the activity of *Mycobacterium* dihydrofolate reductase.

While the glycerol binding site is not essential for the activity of *Mycobacterium* dihydrofolate reductase, it was reported that hydrophilic side groups may be capable of occupying this site (Hong *et al.* 2015). Based on this finding new inhibitors can be designed that are able to specifically bind to *Mycobacterium* dihydrofolate reductase.

A number of derivatives of dihydropyrimidines have been developed that bear imidazole nucleus at C-4 position (Desai *et al.*, 2016). These compounds were characterized using H-NMR, C-NMR and mass spectroscopy. These compounds showed their potency as inhibitors of *M. tuberculosis* dihydrofolate reductase. Such compounds act as leads for development of new inhibitors specific against the *M. tuberculosis* dihydrofolate reductase.

Another series of compounds were developed by EL Messery *et al.*, 2016 for inhibiting the

activity of dihydrofolate reductase *in vitro*. The series comprised of 2, 3, 6-substituted-quinazolin-4-ones. These compounds were evaluated for inhibition of dihydrofolate reductase, anti microbial and anti tumour activities .Their studies suggested that the recognition with Phe 34 is essential for binding of these compounds so that the activity of dihydrofolate reductase gets inhibited (EL Messery *et al.*, 2016).

Dihydrofolate reductase as a target for infection due to methicillin resistant Staphylococcus aureus strains

Methicillin resistant *S. aureus* are gram positive bacteria and are extremely pathogenic. They are widely present in the surroundings of hospitals (Frey *et al.* 2012). In contemporary times, the upheaval from the infections acquired in the hospital to the infections acquired in the community (Boucher and Courey, 2008; Najmi *et al.* 2003; Nikaido, 2009; Frey *et al.* 2012) and the development of resistance against multiple drugs designed for methicillin resistant *S. aureus,* has brought forth a grave hindrance in treating such infections. Various strains have come to surface and constitute about 60% of clinical *S. aureus strains* that have been secluded from intensive care units (ICU) in USA (Sakoulas and Moellering, 2008; Frey *et al.* 2012). Besides being resistant to methicillin these *S. aureus* strains could also possess resistance to aminoglycosides, tetracycline, macrolides, or various other disinfectants (Nikaido, 2009; Frey *et al.* 2012). Vancomycin is chiefly utilized to treat hospital acquired infections but there are many Methicillin resistant *S. aureus* strains that have been reported to be resistant to vancomycin (Frey *et al.* 2012). Trimethoprim is another important drug that targets dihydrofolate reductase present in *S. aureus.* However, over a period of time, the bacteria have developed resistance against this drug. Also, a number of point mutations such as Phe 98 to Tyr (F98Y) mutation of *S. aureus* dihydrofolate reductase and mutations occurring in F98Y have further helped in development of resistance against trimoptherin (Bourne *et al.* 2010).

In order to extend the lifetime of antifolates used for inhibition of dihydrofolate reductase enzyme, a new group of antifolates have been developed which are linked with propargyl and are known as the propargyl linked antifolates. They possess the potency to inhibit both the dihydrofolate enzyme and the bacterial strains. A study was carried out by Keshipeddy *et al.* 2015 to analyze the role of the single propargylic stereocenter in such inhibitors (Keshipeddy *et al.* 2015). This led them to develop a new path based on the non-racemic 3-aryl-1-butyne building blocks. This was achieved by the help of pairwise addition of asymmetric conjugate and dehydration of aldehyde. Keshipeddy *et al.*, 2015 developed a series of non racemic propargyl linked antifolate inhibitors. It was observed that these compounds possessed potent inhibition of enzyme inhibition. They also possessed antibacterial effects and were capable of forming stable ternary complexes with the wild type as well as resistant mutants.This progressive series of inhibitors displayed potent activity to counter a range of methicillin-resistant strains of *S. aureus* strains and various other Gram-positive pathogens.

RAB1 is another active molecule that is being investigated to see its role in prevention of anthrax. Its structure is based on trimethoprim but additionally it possesses an acryloyl linker and phthalazine moiety. It also includes a propyl group at a stereogenic carbon. This increases the surface area of the RAB1 by 40% as compared to trimethprim enabling more access to the important contact regions in order to interact with the binding site (Bourne *et al.* 2010). The binding of RAB1 to dihydrofolate reductase is similar to the binding of dihydrofolate which is the naturally present substrate of the enzyme dihydrofolate reductase. The binding site of *S. aureus* dihydrofolate reductase has a unique property. It has a shallow cavity on the surface which is due to the lack of stabilizing interactions which are present in other dihydrofolate reductase enzymes. Also, another characteristic property of *S. aureus* dihydrofolate reductase is its capability to accommodate bigger ligands. RAB 1 has been found to have broad spectrum applicability against a number of bacteria (especially Gram positive) that affect the human health. RAB1 was seen to be extremely effective against *S. aureus* strains (methicillin resistant and vancomycin resistant strains) (Bourne *et al.* 2010).

Dihydrofolate reductase as a target for infection due to Streptococcus mutans

Streptococcus mutans is a bacterial strain responsible for causing dental caries. A group of three inhibitors that were analogues of trimetrexate have been identified that are able to inhibit the activity of dihydrofolate reductase in *S. mutans.* These inhibitors have high selectivity towards the dihydrofolate reductase in *S. mutans* as compared to the human dihydrofolate reductase. The primary sequence of *S. mutans* dihydrofolate reductase has a similarity of only 28% with that of the human dihydrofolate reductase sequence. Their effect was further analysed using molecular modelling and docking studies which further showed that these analogues of trimetrexate were efficient compounds to develop new treatment for the dental caries (Zhang *et al.*, 2015).

Dihydrofolate reductase as a target for various other antimicrobials

A group of compounds that have shown to inhibit the activity of dihydrofolate reductase in various bacteria are 2, 4-diaminoquinazolines (Li *et al.* 2011). With the help of structure based drug design a number of 7-aryl-2, 4-diaminoquinazolines were discovered that were found to have efficient potency for inhibiting the bacterial dihydrofolate reductase. Inhibition of the bacterial dihydrofolate reductase has also been achieved with a group of (±)-6-alkyl-2, 4-diaminopyrimidine-based inhibitors. Also, a series comprising of 2 -heteroarylthio-6-substituted-quinazolin-4-one analogues has been developed, synthesized and evaluated to see their effect on inhibition of bacterial dihydrofolate reductase (Omary *et al.* 2013). A number of compounds in this series proved to have significant effect on inhibition of bacterial dihydrofolate reductase.

A new group of inhibitors was designed against the bacterial dihydrofolate reductase by Lam *et al.* 2014. These inhibitors are the 7-(benzimidazol-1-yl)-2, 4-diaminoquinazolines. The most efficient inhibitors of this group include a five-membered heterocycle at the 2-position of the benzimidazole. These inhibitors are extremely potent and selective compounds. They exploit the difference in the size of a binding pocket in vicinity of the cofactor NADPH between the bacterial and human dihydrofolate reductase enzymes. 7-((2-thiazol-2-yl)benzimidazol-1-yl)-2,4 diaminoquinazoline which is a typical enzyme of this group possesses 46700 times more selectivity towards bacterial dihydrofolate reductase as compared to the human dihydrofolate reductase (Lam *et al.* 2014).

Dihydrofolate reductase as a target in the treatment of various malignancies

Human dihydrofolate reductase and thymidylate synthase complex as a drug target for treatment of cancer

Thymidylate synthase, dihydrofolate reductase and other enzymes involved in the synthesis of thymidylate are used as targets in the chemotherapy against cancer. It has been demonstrated that human thymidylate synthase and dihydrofolate reductase form a strong complex in vitro and together they confine in the cytoplasm and nucleus of normal and cancerous human colon cell. It was observed that on treating these cancerous cells with methotrexate or 5-fluorouracil there was no effect on the distribution of any of these enzymes inside the cells. However, 5-fluorouracil was able to bring a 2.5 times decrease in the presence of complex formed together by the dihydrofolate reductase and thymidylate synthase complex in the nucleus. This outcome inferred that if thymidylate synthase was sequestered by FdUMP within the cytoplasm, it affected the translocation of dihydrofolate reductase-thymidylate synthase complex to the nucleus. Taking into consideration the strong inclination of complex formation by these two enzymes they could be a suitable contemporary target of drugs utilized in cancer therapy. In hock of these facts, some initial work has been carried out in this tangent where three dimensional structures of human thymidylate synthase and human dihydrofolate reductase, and few bifunctional dihydrofolate reductase-thymidylate synthase structures obtained from protozoans have been used as templates, to construct an in silico model for structure of human dihydrofolate reductase and thymidylate synthase complex. It comprises of one dimer of

thymidylate synthase and two monomers of dihydrofolate reductase. This intricate structure could serve as an elementary three dimensional drug target model for the inhibitors produced in future that would target the interfaces between these enzymes (Anto-siewicz *et al.* 2016).

Dihydrofolate reductase as a target for treatment of basal like breast cancer

Basal like breast cancer (BLBC) which is a molecular sub type of breast cancer characterized by a cluster of genes that is expressed by epithelial cells present in the basal or outer most layer of the mammary glands in adults. It is a major challenge because it is common in young females and has a high chance of relapse. Kalgoris *et al.*, 2014 studied the effect of a naturally present benzophenanthridine alkaloid called sanguinarine against these BLBC cells. They observed that sanguinarine was a potent inhibitor of dihydrofolate reductase. They reported that it was able to inhibit the activity of dihydrofolate reductase enzyme even in methotrextae resistant MDA-MB-231 cells (Kalogris *et al.*, 2014).

Dihydrofolate reductase as target for multi targeting drugs for anti proliferative activity

Dihydrofolate reductase has also been used by multi targeting drugs in the treament of complex disease such as cancer. Dihydrofolate reductase and thioredoxin reductase are two enzymes that are part of two totally unrelated pathways. Both these pathways are capable of contributing towards the growth and survival of cancer cells. A study was carried out by Ng *et al.* 2016, to observe the effect of simultaneous inhibition of dihydrofolate reductase and thioredoxin reductase. The compounds were designed by combining the dihydrtriazines which are the inhibitors of dihydrofolate reductase and chalcones which are the inhibitors of thioredoxin reductase. These two different inhibitors were combined at different concentrations and were used against the breast and colorectal carcinoma cells. These compounds showed inhibitory effect on growth of cancer cells. This acts as a good lead for the development of dual inhibitors of dihydrofolate reductase and thioredoxin reductase. (Ng *et al.* 2016).

5. Conclusion and future directions

Dihydrofolate reductase is a crucial enzyme participating in the folate cycle. It has been used as a target by antifolate medication utilized in treating cancer and various infections caused by bacteria. A number of drugs have been designed to inhibit the activity of dihydrofolate reductase in order to treat cancer. However, resistance has developed for some of these drugs. Also, some of these drugs are toxic and are therefore only utilized for the treatment of cancer. A number of new drugs have been designed for not only treatment of cancer but also for microbial infections. A procedure that helps in determination for the specificity of target dihydrofolate reductase from various organisms is yet to be worked on. An in depth understanding of the affinity and specificity of target dihydrolate reductase can help to design better antifolates. The inhibitors for dihydrofolate reductase can further be designed for treatment of infections caused due to fungi, parasites, protozoa, anthrax
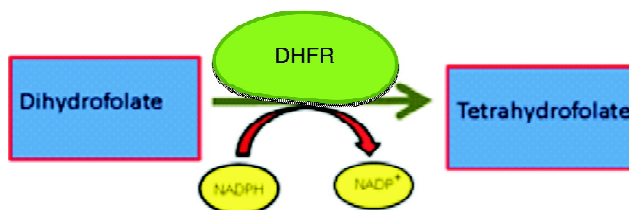

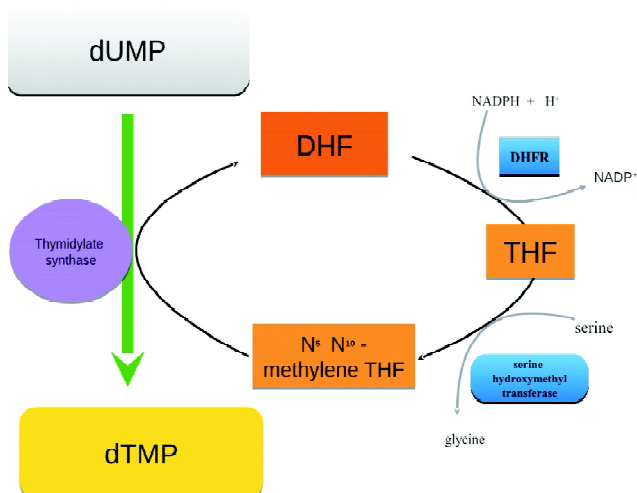*Figure 1:* **Reaction catalyzed by Dihydrofolate reductase (DHFR)**

Figure 2: **Role of DHFR in the synthesis of purine and thymidylate. DHFR: Dihydrofolate reductase, DHF: dihydrofolate, THF:tetrahydrofolate, dUMP: deoxyuridine monophosphate, dTMP: deoxythymidine monophosphate**
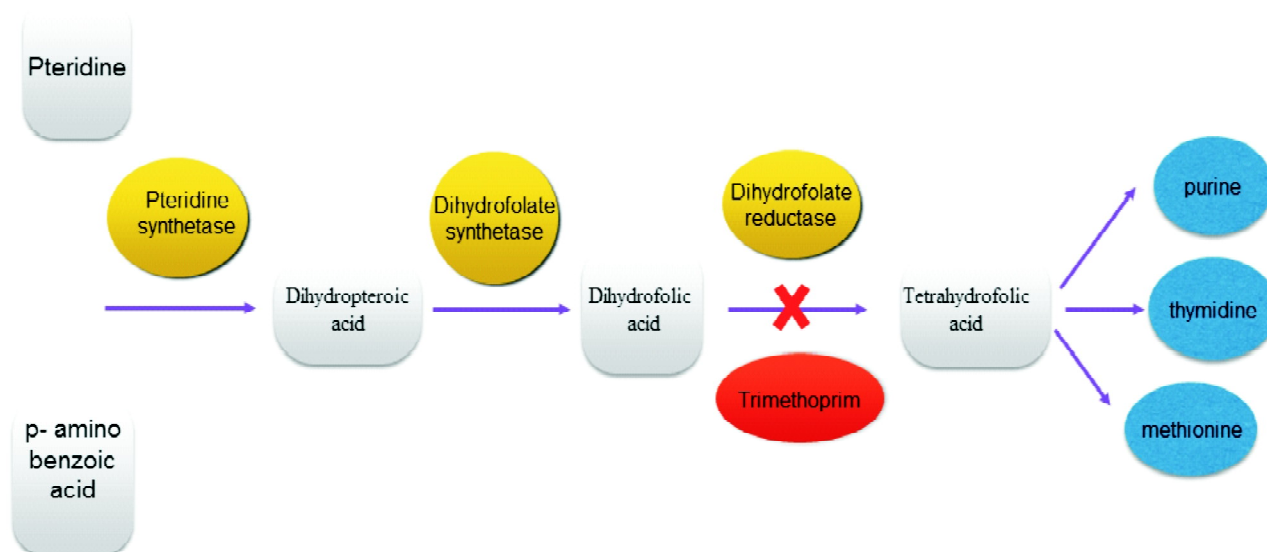
Figure 3: **Mechanism of action of inhibitors of DHFR**

Table 1
Commonly used inhibitors of Dihydrofolate reductase

Inhibitors of Dihydrofolate reductase

Class of compounds	Diaminopteridine	Diaminoquinazoline	Diaminopyrimidine	Diamino- dihydrotriazine
Structure				
Function	1. anti cancer/ anti proliferative	1. anti proliferative/ anti cancer, 2. anti cancer	1. anti bacterial	1. anti malarial

causing bacteria etc. Inhibitors can also be designed for dihydrofolate reductase enzymes of plant pathogens which can help in overcoming diseases caused in plants due to such pathogens.

Conflict of Interest

The authors do not have any conflict of interest with the contents of this manuscript.

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