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Inhibitors of *de novo* folate enzymes in *Plasmodium falciparum*

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Antifolates, inhibitors of folate synthesis or folate conversion, are used for malaria treatment. They are developed as synergistic combinations of inhibitors of dihydrofolate reductase (DHFR) and of dihydropteroate synthase (DHPS). DHPS inhibitors are sulfur-based drugs, analogs of sulfanilamide. These compounds compete with para-aminobenzoic acid in the active site of DHPS. The discovery of new antifolates is based on the identification of DHFR inhibitors; little work has been done on sulfur-based drugs because of their toxicity. As a result, only a few sulfur-based drugs are available. In this review, the hypothesis that compounds that compete with pteridine derivatives in active sites of *de novo* folate enzymes can be used as synergizers of DHFR inhibitors is discussed. If correct, this could lead to the identification of a new family of synergizers of DHFR inhibitors.

Introduction: chemotherapy in malaria

Chemotherapy remains the most important means of controlling malaria. In Africa, the quinoline chloroquine has been the most widely used antimalarial agent since the 1950s. However, the spread of resistance to this drug has prompted the use of alternative antimalarials.

The inhibition of synthesis and conversion of folate derivatives has proved a good strategy for drug development against malaria. Drugs that target folate synthesis or conversion are known as antifolates. Such drugs are: Fansidar™, the combination of pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR), and sulfadoxine, an inhibitor of dihydropteroate synthase (DHPS), see Figure 1; and Metakelfin®, the combination of pyrimethamine and sulfalene (DHPS inhibitor). Although the efficacy of Fansidar™ and Metakelfin® is comparable, Fansidar™ has been more widely used than Metakelfin® [1]. However, resistance to Fansidar™ is spreading in Africa [2], and as an alternative drug the antifolate combination of chlorproguanil (active metabolite is chlorcycloguanil, an inhibitor of DHFR) and dapsone (an inhibitor of DHPS) has been developed. This drug, known as Lapdap®, is now available in many African countries [3,4]. Proguanil (Paludrine®) is another antifolate drug that is commonly used for prophylaxis against falciparum malaria, and is metabolized to its active form of cycloguanil. Proguanil, together with atovaquone, is also used in a prophylactic combination – known as Malarone® [5,6].

Plasmodium falciparum has an intrinsic ability to select for resistance to new antimalarial drugs quickly. To slow the pace of selection of resistance, the World Health Organization (WHO) has recommended that antimalarial therapies are developed as artemisinin-based combinations. In this respect, the combination of lumefantrine–artemether (Co-artem®) [7,8] has been adapted as the first line of treatment in many African countries. However, this drug is not widely available at an affordable price in Africa. As a result, the quinoline amodiaquine (AQ) is now increasingly being used alone or in combination with Fansidar™ or artemesinin derivatives as an interim alternative, until Co-artem® is made affordable through WHO subsidies [9–12]. Two promising combinations, piperaquine–dihydroartemisinin (Eurartekin™) and pyronaridine–artesunate (Panda), are being evaluated in Africa (http://www.mmv.org). Although the antifolate Lapdap® is available in most African countries, it is not used for mass treatment in Africa because it is a monotherapy. As a result, Lapdap® has been combined with artesunate, and this combination is known as

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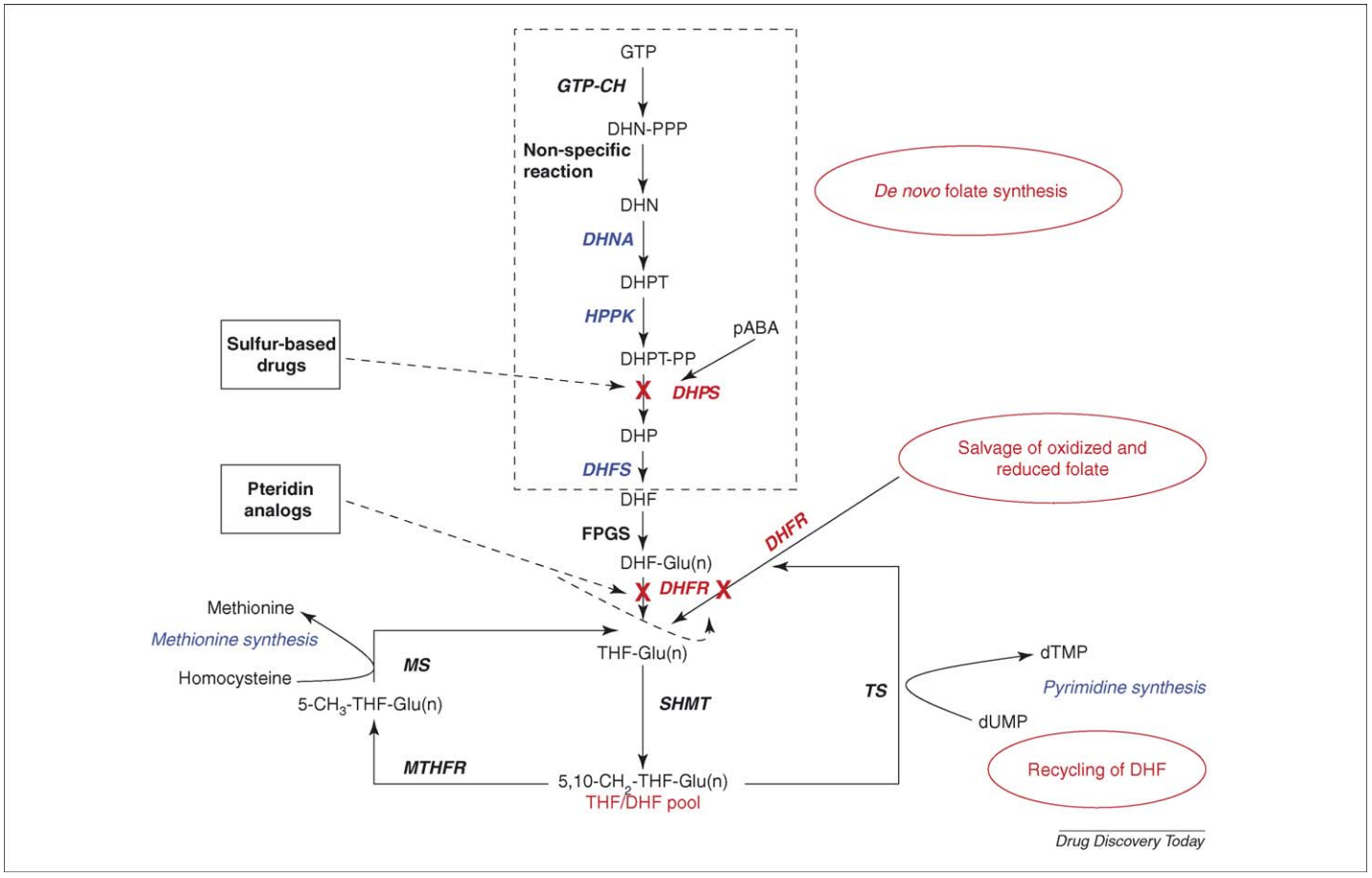

FIGURE 1

Folate biochemistry in *Plasmodium falciparum*. Abbreviations used: GTP, guanosine triphosphate; GTP-CH, GTP-cyclohydrolase I; DHN-PPP, 7,8-dihydroneopterin triphosphate; DHN, 7,8-dihydroneopterin; DHNA, dihydroneopterin aldolase; DHPT, 7,8-dihydro-6-hydroxymethylpterin; HPPK, 6-hydroxymethyl-dihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; FPGS, folylpoly-gamma-glutamate synthetase; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; TS, thymidylate synthase; DHPT-PP, 7,8-dihydro-6-hydroxymethylpterin pyrophosphate; DHP, 7,8 dihydropteroate; pABA, para-amino benzoic acid; dUMP, 2′-deoxyuridine-5′-monophosphate; dTMP, 2′-deoxythymidine-5′-monophosphate; DHF, 7,8-dihydrofolate; Glu, glutamate; THF, tetrahydrofolate.

CDA. CDA has reached Phase II clinical development [13], and Phase III is about to start in Africa. Table 1 summarizes antimalarials that are either in clinical use or in clinical development as combination therapy. Information on single active molecules that are in clinical development can be found on the Medicines for Malaria Venture website (http://www.mmv.org).

Search for new antifolates

As discussed earlier, *P. falciparum* has the ability to select for resistance against antimalarial drugs quickly. There is no single antimalarial in clinical use against which the parasite has not yet developed resistance (Table 1). In many cases resistance occurs relatively quickly, only a few years after the widespread introduction of the antimalarials. This is a matter of concern because chemotherapy is currently the only available option to control malaria. Thus, to counterbalance the pace of resistance, the identification and development of new antimalarials is urgently needed.

Rapidly dividing cells such as cancer, bacterial and malarial cells are reliant upon the availability of folate derivatives, making the inhibition of the synthesis or conversion of these vitamins an attractive target for drug development. The development of antifolates against malaria and bacteria is based on the combination of DHFR inhibitors and sulfur-based drugs as DHPS inhibitors. DHFR and DHPS inhibitors are synergistic, and this is why they are used in combination (see next section). In this combination, the activity of DHFR inhibitors is in the nanomolar range (<100 nM), whereas that of DHPS inhibitors, sulfur-based drugs, is in the micromolar range (<1 μM) [14,15]. Although sulfur-based drugs are weak antimicrobial agents on their own, they substantially increase the activity of the DHFR inhibitor, and are therefore used as a component of the antifolate combination. This use of DHFR inhibitors with sulfur-based drugs has been the dogma in antifolate development in malaria (pyrimethamine–sulfadoxine, pyrimethamine–sulfalene, and chlorproguanil–dapsone), and also in bacterial infections (trimethoprim–sulfamethoxazole). In all these combinations, sulfur-based drugs (sulfadoxine, sulfalene, dapsone and sulfamethoxazole) are active in the micromolar range, whereas DHFR inhibitors [pyrimethamine, chlorcycoguanil (active metabolites of chlorproguanil]) and trimethoprim] are active in the nanomolar range.

Because of the rapid selection and spread of antimalarial drug resistance, a lot of work has been devoted to the discovery of new potent antifolates [16]. For instance, a new family of antifolates, diaminotriazine-based compounds, is being investigated [17]. These compounds are potent inhibitors of *Plasmodium* DHFR and

TABLE 1

Summary of commonly used antimalarials and those that are in clinical development as combination therapy

	Antimalarials	Drug family[a]	Use in human	Resistance	Refs[b]
In clinical use	Chloroquine	4-Amino-quinoline	Yes	Widespread	[1,60]
	Amodiaquine	4-Amino-quinoline	Yes	Reported	[10]
	Pyrimethamine–sulfadoxine (Fansidar™)	Antifolate	Yes	Widespread	[2,61]
	Proguanil–atovaquine (Malarone®)	Triazine and Hydroxynaphtho-quinone	Yes	Reported for atovaquone	[62–64]
Recently introduced In clinic	Chlorproguanil–dapsone (Lapdap®)	Antifolate	Yes	Reported in South East Asia	[65]
	Lumefantrine–artemether[c] (Co-artem®)	Aryl aminoalcohol	Yes	Decrease sensitivity to lumefantrine?	[66]
In clinical development	Pyronaridine–artesunate[c] (Panda)	Aryl aminoalcohol	Yes for pyronaridine as monotherapy	Likely to be present in China.	[67]
	Piperaquine–dihydroartemisinin[c] (Eurartekin™)	Aryl aminoalcohol	Yes for piperaquine as monotherapy	Common in South East Asia and China	[68,69]
	Chlorproguanil–dapsone–artesunate[c] (CDA)	Antifolate	Yes for Lapdap®	Reported for Lapdap®	[65]

[a] Except for artemisinin derivatives.
[b] Relevant references.
[c] Artemether, artesunate and dihydroartemisinin are endeperoxide derivatives of the artemisinin. Resistance to artemether has recently been reported [70].

yield promising *in vivo* efficacy. Several groups, including ours, are carrying out *in vitro* screening studies to identify potent antimalarial antifolates [18–24]. With the crystallographic structure of *P. falciparum* DHFR-thymidylate synthase protein now resolved, more screening programs are expected to be initiated [25,26].

One major limitation of these studies on the discovery of new antifolates is that they target DHFR alone. Yet, every active DHFR inhibitor should be developed in combination with a DHPS inhibitor [see Box 1]. Currently, dapsone, sulfadoxine and its close congener sulfalene are the only available DHPS inhibitors used as antimalarials – although resistance to the latter two agents is increasing. To the best of my knowledge, no studies devoted to the search for DHPS inhibitors have been undertaken since the 1960s [1]. During that time dapsone, sulfadoxine and sulfalene were identified as potent synergizers of DHFR inhibitors. Since then, no other DHPS inhibitor has been found. Several reasons could account for the lack of interest in searching for new DHPS inhibitors. One could be the host toxicity associated with use of this class of antifolate [27]. The question, therefore, is: are there any alternatives to sulfur-based drugs that could be developed as potentiators of DHFR inhibitors?

BOX 1

The history of antifolate (alone or in combination) in the treatment of malaria

Proguanil was the first reported antimalarial antifolate. It metabolizes to cycloguanil, an inhibitor of dihydrofolate reductase (DHFR) [54]. The search for analogs of proguanil led to the discovery of chlorproguanil and pyrimethamine [16]. As monotherapy, these drugs were used in the past for prophylaxis and treatment with limited efficacy [1].

Sulfur-based drugs are dihydropteroate synthase (DHPS) inhibitors. They were discovered when studying prontosil, an azo dye. Prontosil is converted *in vivo* to sulfanilamide, the active compound and an inhibitor of DHPS [55,56]. From there on, analogs of sulfanilamide, which formed the class of antifolate sulfur-based drugs, were synthesized. In the past, attempts were made to use these drugs alone as antimalarials [57]. Because of their limited activity, high doses of these agents were needed, making them highly toxic in humans. They were, therefore, abandoned as antimalarials [1].

However, interest in these compounds was fostered when it was established that they were potent synergizers of DHFR inhibitors, leading to the concept of developing antifolate drugs as combinations of sulfur-based drugs (used at lower doses than those needed in monotherapy) and DHFR inhibitors [58,59]. Thus, it is the fortuitous discovery of sulfanilamide that led to the development of sulfur-based drugs.

Mechanism of antifolate synergy

DHPS mediates the synthesis of dihydropteroate, whereas DHFR controls the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) (Figure 1). The inhibition of DHPS activity decreases the synthesis of dihydropteroate, which leads to reduced DHF synthesis. Because the amount of DHF, the substrate of DHFR, is decreased, the activity of DHFR inhibitor increases. The concomitant blockage of DHF synthesis and the inhibition of DHFR make the combination of DHPS and DHFR inhibitors synergistic – one inhibitor potentiates the activity of the other.

Because the role of DHPS inhibitors is to block the *de novo* synthesis of DHF, the inhibition of any enzyme of the *de novo* folate pathway could bring about synergy with DHFR inhibitors. In addition to DHPS, known enzymes of this pathway are: dihydroneopterin aldolase (DHNA), although this enzyme has yet to be identified in *P. falciparum* [28]; hydroxymethyl-dihydropterin pyrophosphokinase (HPPK); and dihydrofolate synthase (DHFS) (see next section). The inhibition of any of these enzymes will lead to a reduction of DHF synthesis. As discussed earlier, the reduction of DHF potentiates the activity of DHFR inhibitors. Because these *de novo* folate enzymes (including DHPS) use pteridine derivatives as substrates, compounds that will compete with these pteridine derivatives (in the active site of folate enzymes) could potentiate the activity of DHFR inhibitors by blocking the *de novo* folate enzymes.

DHPS is the most interesting of all *de novo* folate enzymes. DHPS has two substrates, para-aminobenzoic acid (pABA)

and the pteridine derivative 7,8-dihydro-6-hydroxymethylpterin pyrophosphate (DHPT-PP) [28,29]. Thus, to inhibit DHPS enzyme, one could use compounds that will compete with pABA (sulfur-based drugs) or DHPT-PP. However, only sulfur-based drugs have been developed as inhibitors of this enzyme. Thus, the question is, why should this be?

Dihydrofolate reductase plays a central role in the *Plasmodium* folate pathway

The answer to the question (raised in the previous paragraph) lies in the role that DHFR plays in the malarial parasite. As shown in Figure 1, DHFR plays three main roles in the folate pathway. First, it controls *de novo* folate synthesis by blocking the synthesis of THF. Second, DHFR mediates the salvage of exogenous folate derivatives, DHF and the fully oxidized folate, by reducing them to THF. Third, DHFR recycles DHF from 2′-deoxythymidine-5′-monophosphate (dTMP) synthesis, by reducing it to THF so that it can re-enter the folate pool (Figure 1).

Inhibition of *de novo* folate synthesis cannot completely block growth of *P. falciparum* because the parasite can salvage folate from the exogenous medium, or can re-use the DHF that is derived from dTMP synthesis, bypassing *de novo* synthesis. However, the inhibition of this pathway greatly enhances the activity of DHFR inhibitors.

Change of dogma

Because screening programs are based on the identification of potent inhibitors of parasite growth, inhibitors of *de novo* folate synthesis were abandoned because, on their own, they are weaker antimalarial agents. For instance, sulfur-based drugs would never have been identified as antimalarial agents because their *in vitro* activities are in the micromolar range.

Because the role of sulfur-based drugs is to block *de novo* synthesis and any compound that exerts such activity would potentiate with DHFR inhibitors, we propose that compounds that will compete with pteridine derivatives in active sites of *de novo* folate enzymes would potentiate the activity of the DHFR inhibitors, like sulfur-based drugs. Therefore, the dogma that DHFR inhibitors can only be used in combination with sulfur-based drugs does not take into account the potential of the folate pathway. Thus, antifolates can be developed as a combination of compounds that compete with DHF in DHFR, and with any of the pteridine derivatives that are used as substrates by *de novo* folate enzymes. In the next section we present some biochemical information on these potential folate enzymes.

Folate enzymes

Most of the information on *de novo* folate enzymes was derived from studies carried out in bacteria. The potential of these enzymes as drug targets (thus as synergizers of DHFR inhibitors) has not been explored yet in *P. falciparum*. As discussed earlier, all these enzymes do not exist in mammals (humans), thus their inhibition will not affect the host folate pathway.

Dihydroneopterin aldolase

7,8-Dihydroneopterin triphosphate (DHN-PPP), the product of the GTP-cyclohydrolase I (GTP-CH) reaction, the first reaction in the *de novo* folate pathway, is hydrolyzed to the free hydroxyl form; this reaction has been postulated to involve a nonenzymatic loss of pyrophosphate, followed by nonspecific phosphatase activity that removes the third phosphate group [30] (Figure 1). The resulting substrate is converted to 7,8-dihydro-6-hydroxymethylpterin (DHPT) in the presence of DHNA (Figure 1). DHNA has not been identified yet in *Plasmodium* ([28]; however, as radiolabeled GTP or guanosine precursors are ultimately converted to folate in *P. falciparum* [31], the existence of this enzyme to mediate a key step along this pathway appears to be mandatory in the *de novo* folate pathway.

The crystal structure of this enzyme has been resolved in bacteria (*Staphylococcus aureus*) [32]. Several hydrophobic residues are located in the active site area, providing a suitable environment for pteridine [33] and probably analogs of pteridine binding. In bacteria, work has been carried out to identify pteridine derivatives as DHNA inhibitors [34], and recently the use of CrystaLEAD high-throughput X-ray crystallographic screening has identified interesting lead compounds that can be used to design potent DHNA inhibitors [35]. Thus, the *de novo* folate pathway can be blocked by inhibiting DHNA in bacteria, a possibility that can be extended to *P. falciparum*.

Hydroxymethyl-dihydropterin pyrophosphokinase and dihydropteroate synthase

HPPK catalyzes the diphosphorylation of 2-amino-4-hydroxy-6-hydroxymethyl-dihydropterin. The resulting compound then condenses with pABA to generate dihydropteroate, a reaction mediated by DHPS. HPPK and DHPS occur as a bifunctional protein in *Plasmodium* [36,37]. In bacteria, depending on the species, HPPK occurs monofunctionally (*Eschericha coli*) or bifunctionally with other folate enzymes such as DHNA (*Streptococcus pneumoniae*) [38].

Hydroxymethyldihydropterin pyrophosphokinase

Crystal structures of this enzyme have been resolved in many bacterial species [39–41], and work has been reported in the development of analogs of pteridine as HPPK inhibitors. Most tested compounds exhibited limited efficacy, probably arising from their poor penetration into bacterial cells [42]. Interestingly, the inhibition of HPPK has been achieved in bacteria with the use of bisubstrate molecules, compounds formed of pterin and an adenosine moiety [43], pointing to a possibility of their use in *P. falciparum*.

Dihydropteroate synthase

The crystallographic structure of DHPS has been resolved in many bacterial species, including *E. coli*, *S. aureus* and *Mycobacterium tuberculosis* [44–46]. It is the only enzyme of the *de novo* folate pathway that is used as a drug target in the clinic. As discussed earlier, this enzyme has two substrates: a pteridine derivative and pABA. Currently, only analogs of pABA, sulfur-based drugs, are used as anti-DHPS inhibitors.

In bacteria, attempts have been made to explore the potential of the inhibition of this enzyme with pteridine derivatives, and bisubstrate compounds consisted of pteridine analogs coupled with pABA. DHPS inhibitors were identified; however, their potencies were limited by poor compound transport through bacterial cell membranes [33,47,48]. Thus, the proof of principle that pteridine analogs can block DHPS has already been demonstrated, and we hypothesize that such compounds could synergize with

DHFR inhibitors. This enzyme is a validated target against *P. falciparum*, thus it merits further consideration.

Dihydrofolate synthase

DHFS catalyzes the conversion of dihydropteroate to DHF by the addition of a single L-glutamate moiety; this reaction is the final step in *de novo* folate synthesis. The gene encoding *P. falciparum* DHFS has been characterized [49,50] and is expressed as a bifunctional protein that also exhibits folylpolyglutamate synthase (FPGS) activity, which adds further glutamate residues to the molecule [29]. In bacteria, DHFS and FPGS activities can be found on the same or different proteins, depending on the species [33]. The potential of this enzyme as a drug target has already been demonstrated with the use of analogs of pteridine in bacteria [51,52]. However, no work has been in carried out in *P. falciparum* as yet.

How to identify these compounds

Based on the previous explanation, an inhibitor of the *de novo* folate pathway would have an *in vitro* activity in the micromolar range. Because dapsone is the most active inhibitor of the *de novo* folate pathway, it is proposed that a promising inhibitor of the *de novo* pathway should be at least as good as dapsone. The IC_{50} of dapsone (the inhibitory concentration that kills 50% of the parasite) against multidrug-resistant isolates is ~50 μM [53]. Thus, a potential inhibitor of the *de novo* folate pathway (and synergizer of DHFR inhibitors) should have an IC_{50} *in vitro* no greater than 50 μM. Once such compounds are identified, analysis of their synergistic interaction with DHFR inhibitors would then be carried out.

Conclusion

The development of antifolate in the treatment of malarial and bacterial infection is based on the use of a combination of DHFR and DHPS inhibitors. DHFR inhibitors are compounds that compete with the pteridine derivative DHF in the active site of DHFR, whereas DHPS inhibitors are sulfur-based drugs, competitors of pABA. In this combination, the role of the DHFR inhibitors is to block the *de novo* folate pathway, explaining the existence of synergy between DHFR inhibitors and sulfur-based drugs. The observation of folate biochemistry indicates that compounds that can block *de novo* synthesis, by competing with pteridine derivatives in the active site of *de novo* folate enzymes, can also synergize with DHFR inhibitors. Thus, such compounds could be used in combination with DHFR inhibitors, a change to the dogma that DHFR inhibitors can only be used with sulfur-based drugs. The proof of principle that compounds that compete with pteridine derivatives in the active site of *de novo* folate enzymes could function as inhibitors of *de novo* folate enzymes has already been established in bacteria, although none of them have yet been studied as synergizers of antibacterial DHFR. Thus, the possibility exists to identify a new class of potentiators of DHFR inhibitors – both in bacteria and malaria.

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