Inhibition of Hypusine Biosynthesis in Plasmodium: A Possible, New Strategy in Prevention and Therapy of Malaria

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Abstract: The increasing drug resistance of malaria parasites against chemotherapeutics enforces new strategies in finding new drugs. Here, we describe a new class of compounds the piperidone 3-carboxylates which show an antiplasmodial effect *in vitro* and *in vivo.* This effect might be caused by inhibition of eukaryotic initiation factor (eIF-5A).

Key Words: Hypusine, dipyridyl-piperidone carbonic esters, antiplasmodial, initiation factor eIF-5A, deoxyhypusine synthase (DHS).

INTRODUCTION

Recent results published from the roll back malaria campaign show climbing malaria rates with estimated 500 million cases [1] a year. The goal for treatment of 60% of infected patients 8 hours after the onset of symptoms has not been fulfilled yet. It also has not been achieved that more than half of at-risk pregnant women and children younger than five years receive preventive drug treatment. Prevention and treatment of malaria could be greatly improved with existing methods if increased financial and labor resources were available.

Resistance mechanisms to conventionally used drugs have been elucidated. The antifolate drug combination sulfadoxine-pyrimethamine is linked to three to four mutations in the dihydrofolate reductase (*dhfr)* [2] gene and to one or two mutations in the dihydropteroate synthase (dhps) gene [2]. Surprisingly, these mutations have arisen infrequently and their current widespread distribution is due to gene flow [3]. Resistance of *P. falciparum* to chloroquine is largely due to mutations affecting the *pfcrt* and *pfmdr I* genes that have also arisen infrequently but are now widely distributed [4]. Both drugs, i.e. chloroquine and sulfadoxine-pyrimethamine, have long half-lives; therefore, drug-resistant parasites are at a selective advantage in communities where these drugs are widely used [5]. However, in the absence of drug use, resistant parasites seem not to be as well transmitted as wild-type parasites.

Combination therapy with drugs having different modes of action is now the referred strategy in malaria treatment to prevent the spread of parasites against one component of the fixed dose combination. The most widely spread coformulated ACT, i. e. artemether-lumefantrine, is already available in Asia [6], and with promising effectiveness in Africa [7]. The high efficacy of the fixed dose combination of dihydroartemisinin-piperaquine has already been shown in Southeast Asia while a fixed dose combination of artesunate and chlorproguanil-dapsone is designed for the African market where costs play a big role. An important progress is also made using intermittent preventive treatment in pregnancy (IPTp). IPTp protected against maternal anemia and low birth weight [8]. Unfortunately, the efficacy of IPTp is reduced in HIV-positive women [9]. Subsequent studies in Tanzania [10] have shown that the use of sulfadoxine-pyrimethamine applied to children at specific times during the first year reduced the incidence of severe malaria and anemia.

Enzymes of the Polyamine Pathway in Plasmodium Might be Potential New Drug Targets

The increasing resistance of the malaria parasites has enforced new strategies of finding new drug targets. During the last years the polyamine pathway (Scheme **1**) has been exploited for antiparasitic chemotherapy. Targeting of enzymes of the polyamine pathway like the ornithine decarboxylase (ODC) [EC 4.1.1.17] [11] adenosylmethionine decarboxylase (AdoMetDC) [4.1.1.50] [11] and the spermidine synthase (SPDS)[EC 2.5.1.16] [12] turned out to be valuable.

ODC has been recently cloned, sequenced and overexpressed in *Escherichia coli* and was found to be a bifunctional enzyme [13] which exhibits both S-adenosylmethioninedecarboxylase and ODC activity with a total molecular mass of 330 kDa separated through a hinge region. This bifunctional nature has been found in three different malaria species. The hinge region consists of four parasite- specific regions which are essential for protein interactions. The hinge region is indirectly involved in the specific activity of this bifunctional protein. Thus, the parasitic specific elements within the hinge region might be a valuable target for the design of new polyamine inhibitors.

ODC and AdoMetDC have been proven to be valuable targets for the development of inhibitors: One of the first enzyme inhibitors are methylamino-5-deoxyadenosine, an analogue of methyl adenosine reducing the AdoMetDC activity, and α–difluoromethylornithine (DFMO) being a competitive, irreversible inhibitor of ODC which alkylates the active site of this enzyme. Meanwhile a next generation of ODC and AdoMetDC inhibitors has been synthesized since

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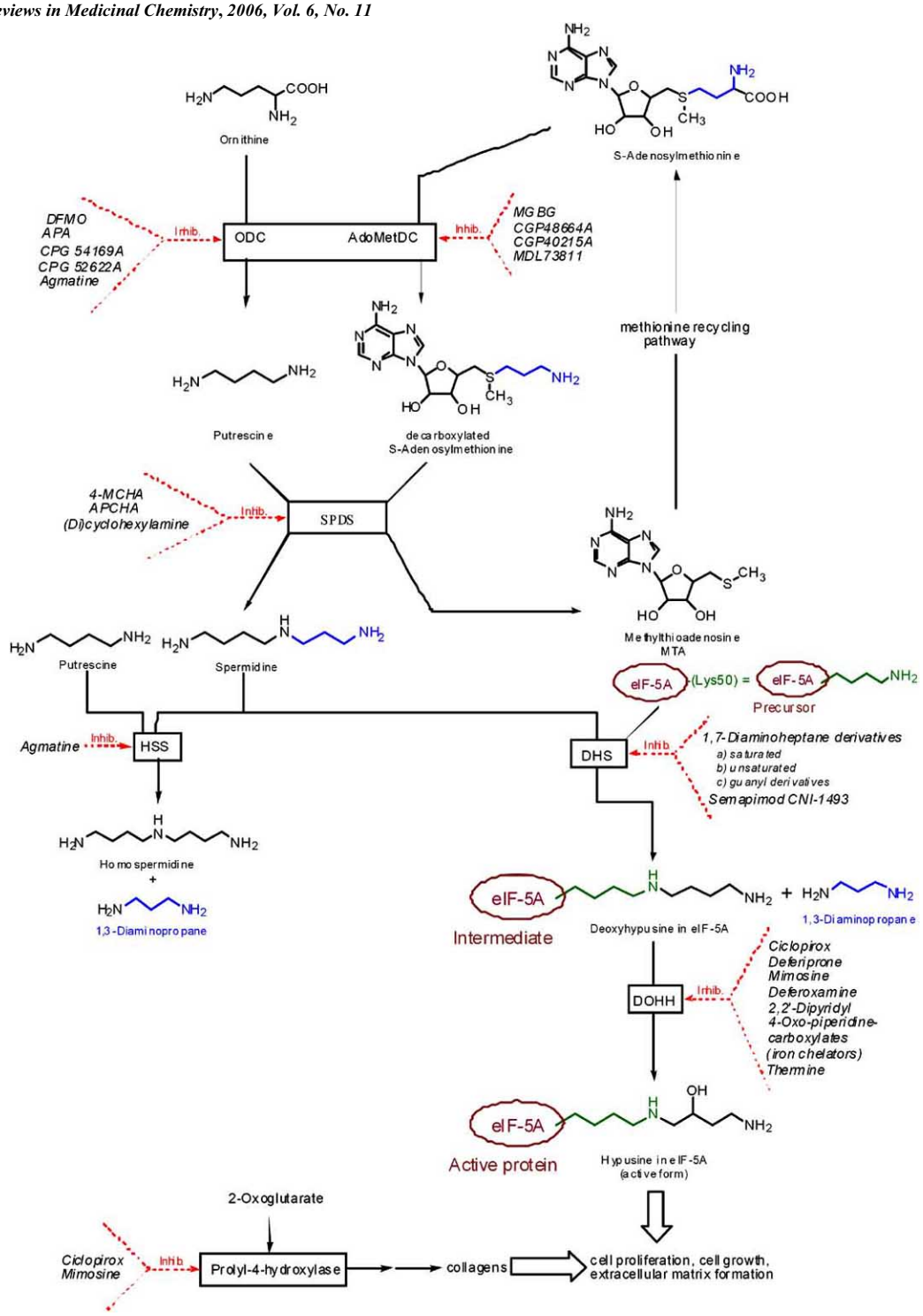


Scheme (1). Polyamine biosynthetic enzymes are important drug targets in Plasmodium: Only one pathway for the biosynthesis of putrescine catalyzed by ornithine decarboxylase (ODC) exists in Plasmodium. Spermidine synthase (SPDS) catalyzes the transfer of the aminopropyl-moiety from decarboxylated S-adenosylmethionine to spermidine. Decarboxylated S-adenosylmethionine which derives from the methionine recycling pathway is formed under catalysis of S-adenosylmethionine decarboxylase (AdoMetDC). The aminobutyl moiety of spermidine can either be transfered to putrescine by homospermidine synthase (HSS) or to eukaryotic initiation factor (eIF-5A) to form hypusine. In the first step of hypusine biosynthesis deoxyhypusine synthase (DHS) catalyzes the transfer of the 4-aminobutyl moiety of spermidine to the ε-amino group of one specific lysine residue in eIF-5A to form deoxyhypusine. In the second step this intermediate is subsequently hydroxylated by deoxyhypusine hydroxylase (DOHH) to complete hypusine biosynthesis and thus eIF-5A maturation. DHS can accomodate putrescine as an alternative butylamine acceptor instead of eIF-5A(Lys) to form homospermidine. Prolyl-4-hydroxylase (bottom of the scheme) is a 2-oxo-glutarate-dependent dioxygenase which is a key enzyme in the biosynthesis of collagens and differs from DOHH in its superhelical structure.

DFMO hardly affected the erythrocytic stages. The new inhibitors of ODC are related to 3-aminooxy-1-propanamine (APA) while the new AdoMetDC inhibitors are derivatives of bis-(guanylhydrazone) (see Fig. (**1**)) [14]. All APA derivatives and bis- (guanylhydrazone) derivatives with one exception had an antiproliferative effect on cultured *P. falciparum* by decreasing the intracellular polyamine concentration in a dose dependent manner after separating the compartments of the red blood cells (RBCs) and the parasites. In the case of APA-like ODC inhibitors which are structural analogues of putrescine the antiplasmodial effect could be blocked by supplementation with 500 μM putrescine but not when spermidine was added to the parasite cultures. The APA-like inhibitors showed K_i values in the low nanomolar range and are of similar potency. In contrast, the K_i values of the bisguanylhydrazone-type AdoMetDC inhibitors were in the μmolar range. The inhibitory effect of APA-like inhibitors on ODC protein is rather specific since the aminooxy-group, i.e. a hydroxylamine ether, can react with a carbonyl function forming an irreversible oxime with the PLP cofactor in the active site of the ODC enzyme [14]. These results were based on previous findings which showed that the drug inhibited ODC, AdoMetDC and SPDS from mice irreversibly with K_i values ranging from 2,3 μM to 50 μM [15]. *In vitro* experiments performed with a concentration of 0.5 mM of the drug in promyelocytic leukemia cells inhibited growth which could only be restored by spermidine.

The spermidine synthase has also been shown to be an important target in Plasmodium. It is expressed in trophozoites in the early stage of parasite development. The protein is a cytoplasmatic polypeptide of 321 amino acids with a molecular mass of 36,6 kDa with an N-terminal extension of unknown function which can only be found in plants. Besides the function of the transfer of an aminopropyl group from S-adenosyl methionine to putrescine, spermidine synthase (see scheme (**1**)) from the parasite can also catalyze the synthesis of spermine [12]. Among the spermidine synthase inhibitors tested against *P. falciparum* spermidine synthase, trans-4-methylcyclohexylamine (4MCHA) was found to be most potent with a K_i value of 0.18 μM. In contrast to the situation in mammals, where inhibition of spermidine synthase has no or only little effect on cell proliferation, 4MCHA was an efficient inhibitor of *P. falciparum* cell growth *in vitro* with an IC_{50} of 35 μMol, indicating that *P. falciparum* spermidine synthase represents a putative drug target. Although SPDS has been characterized from many sources only the first crystal structure of a SPDS from the prokaryote *Thermotoga maritima* [16] has been characterized in a ligand-free and as a substrate-analogue complexed state with the analogue S-adenosyl-1,8-diamino-3-thiooctane (AdoDATO).

Recently the first crystal structure from SPDS of the model nematode *C. elegans* has been obtained [17]. Biochemical characterisation of the recombinantly expressed protein revealed a high degree of similarity i.e. in the molecular mass (35 kDa) to other eukaryotic SPDS. The amino acid identity between the nematode and the human protein is 57%, 48% to the plant *Nicotiana sylvestris* and 43% to *T. maritima.* Although the *C. elegans* SPDS resembles its mammalian counterpart the nematode enzyme has some specificities: i) the protein from the nematode has a low affinity towards the substrate decarboxylated S-adenosylmethionine and ii) the less pronounced feed back inhibition by 5' thiomethyladenosine. The structure of the putrescine binding hydrophobic cavity in *T. maritima* SPDS resembles the topology of the nematode protein. In particular Asp170 is responsible for deprotonating the attacking group in *T. maritima* whereas Tyr76 and Ser171 are thought to be involved in binding and proper orientation of the diamine. The corresponding amino residues are Tyr94, Asp188 and Ser189 in the nematode protein. In total 20 amino residues are conserved in inhibitor binding. Of these 16 amino acids are conserved whereas four amino acids have been exchanged in the SPDS of the nematode (Met67 to Gln85, His77 to Gln95, Glu178 to Pro195 and Trp244 to Met261).

Hence it remains remarkable [14] that although *P. falciparum* lacks a spermine synthase gene (determined by a BLAST search in the databases) parasitized RBCs (red blood cells) accumulate spermine with an average concentration of 34 nmol per 10^{10} cells when the intracellular putrescine concentration decreases due to ODC inhibition. These results can be interpreted by the fact that spermidine synthase can catalyze the reaction to spermine [14].

The polyamine biosynthesis in Plasmodium was previously understood as the pathway from ornithine to spermidine. However, recent findings indicate that the biosynthesis of the novel amino acid hypusine is present [18] in Plasmodia. This process occurs in a two step mechanism. Deoxyhypusine synthase (DHS) [EC 1.1.1.249] transfers an aminobutyl moiety from the triamine spermidine to a specific lysine residue in the eIF-5A precursor protein to form deoxyhypusine (see scheme **1**). In a second step deoxyhypusine hydroxylase (DOHH) [EC 1.14.9929] completes hypusine biosynthesis through hydroxylation [19]. DHS is a ubiquitous protein in eukaryotes which resembles homospermidine synthase (HSS) [EC 2.5.1.44] [20, 21] in its reaction mechanism, an enzyme involved in the production of pyrrolizidine alkaloids (Pas) in plants. While HSS, in comparison to DHS has lost the ability to bind the eIF-5A(lys) to its surface it can only catalyze the transfer of the aminobutyl moiety from spermidine to putrescine to form symmetric homospermidine [22]. In contrast to HSS, DHS can catalyze the synthesis of symmetric homospermidine because of its lower substrate affinity.

Both enzymes share common mechanistic properties, i.e. the formation of an enzyme-imine intermediate, NAD^+ as cofactor and the transfer of the aminobutyl moiety from spermidine. It was shown that HSS, the first specific enzyme in the biosynthesis of the necine base moiety of PAs in Angiosperms, was originally recruited from DHS [20,21]. However, phylogenetic analysis of 23 cDNA sequences coding for HSS and DHS of various angiosperm species revealed at least four independent recruitments of HSS from DHS: one within the *Boraginaceae,* one within the monocots, and two within the Asteraceae family [23].

Since the essential function of DHS in eukaryotic cell proliferation has been demonstrated by inhibitor experiments [24] with branched-chain saturated and unsaturated 1,7-diaminoheptane derivatives because of their structural similarity to the substrate spermidine, we used this first approach

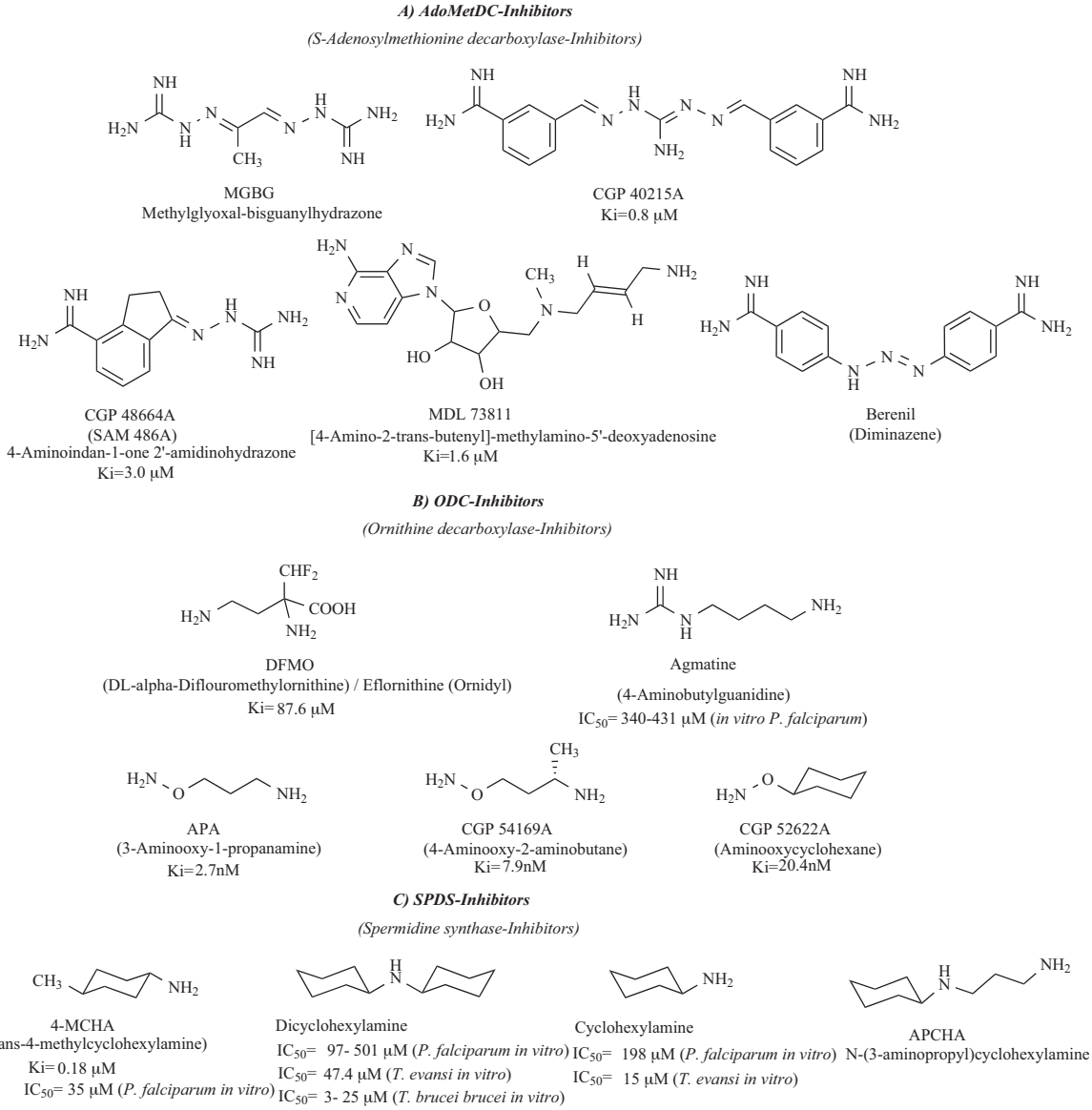


Fig. (1). A: The new generation of S-adenosylmethionine decarboxylase inhibitors (AdoMetDC) are derivatives of bis-(guanylhydrazone). Methylglyoxal-bisguanylhydrazone (MGBG) was the first inhibitor to be tested in Plasmodium with different efficacy on the developmental stages of the parasite. In contrast to the K_i values of ODC inhibitors the K_i values of AdoMetDC inhibitors are in the μMol range. CGP 40215A showed effective *in vitro* inhibition of *P. falciparum* and *in vivo* inhibition of *P. berghei.* Most notably it had potent acivity against early and late stages of African trypanosomiasis. 4-Aminoindan-1,2'-amidinohydrazone (Methylglyoxal-bisguanylhydrazone) has already been tested in phase II trials against tumors and was shown to block mammalian cell growth. 4-[Amino-2-trans-butenyl]-methylamino-5'-deoxyadenosine (MDL 73811), an adenosine derivative, prevented progression of *P. falciparum*-infected erythrocytes into schizonts. However, it was ineffective in a *P. berghei* mouse model. Diminazene (Berenil) which is an antitrypanosomal agent preferentially used in animal trypanosomiasis (surra) caused by *Trypanosoma evansi* binds to DNA duplexes, in particular to AT rich domains in the minor groove of double DNA helices.

B: Inhibition of ODC in Plasmodium. The first ODC inhibitor was DL-alpha-difluoromethylornithine (DFMO). The inhibitor irreversibly inhibits ODC by alkylation of the active site of the enzyme. The efficacy of ODC is limited to the liver stages rather than the erythrocytic stages of Plasmodium. ODC is also effective in treating the cerebral state of African sleeping sickness caused by *Trypanosoma brucei rhodesiense*. Meanwhile a new generation of ODC inhibitors related to 3-aminoxy-1-propanamine (APA) have been synthesized with K_i values in the nM range and tested in Plasmodium. These drugs form an irreversibe oxime with the PLP cofactor of the enzyme. Agmatine, an analogue of putrescine leads to stimulation of antizyme which downregulates ODC activiy. The inhibitory effect of the drug has only been shown in *P. falciparum in vitro* cultures.

C: The generation of spermidine synthase (SPDS) inhibitors in Plasmodium. From the spermidine synthase inhibitors tested trans-4-methylcyclohexylamine (4-MCA) was the most potent inhibitor. Dicyclohexylamine (DCA) was not only efficient in Plasmodium, but turned out to be effective in different Trypanosome species *in vitro*. However, the drug was ineffective in a Trypanosoma mouse model.

to study the antiplasmodial effect in *Plasmodium falciparum* strains with different chloroquine susceptibility. These experiments showed moderate inhibition on chloroquine susceptible and resistant *P. falciparum in vitro* cultures with IC_{50} values ranging between 319 μM and 466 μM after 48 hours of drug treatment [18]. To investigate whether a separate *hss* locus exists in Plasmodium we used the aminobutylguanidine compound agmatine (see Fig. (**2**)) as a competitive analogue of putrescine, which is the acceptor for the aminobutyl moiety in the reaction catalyzed by HSS. The antiplasmodial effect of this inhibitor was comparable to inhibition of DHS with IC_{50} values in the range of 340 μM and 431 μM after 48 hours. Recently the antiplasmodial effect of agmatine was even more confirmed in a rodent malaria model of *P. berghe*i K173 strain [25]. Spermidine antagonized the antimalarial effect of agmatine for *P. berghei* K173 strain. Finally the occurrence of homospermidine in Plasmodium proven by GC/MS analysis suggested at least one of the spermidine metabolizing enzymes to be present in the parasite.

Molecular and Functional Analysis of Genes Involved in Hypusine Biosynthesis in Plasmodium

Molecular cloning and functional analysis has been performed for *odc, s-adometdc and spds* genes so far in Plasmodium while molecular analysis of genes involved in hypusine biosynthesis is currently underway. During the last years we have isolated two genes involved in hypusine biosynhesis [26], i.e. nucleic acids encoding deoxyhypusine synthase (*dhs)* and eukaryotic initiation factor 5A *(eIF-5A*) in the malaria parasites *P. falciparum* and *P. vivax.* We have recently functionally analyzed the 162 amino acid sequence of eIF-5A from *P. vivax* causing tertiary malaria. EIF-5A from *P. vivax* causing benign malaria is 97% identical to its homologue from *P. falciparum* [27] and expression in *E. coli* resulted in a protein of 20 kDa which was purified in one step by Nickel chelate chromatography. EIF-5A is transcribed equally in different developmental stages of the parasite's life cycle (trophozoites and schizonts) and recruites from homologues of higher plants [manuscript in revision]. A recently performed phylogenetic analysis with eIF-5A proteins from *Plasmodium vivax* [26] and from *Plasmodium falciparum* [27] with that of other eukaryotic homologues shows that both apicomplexan eIF-5A proteins are to a higher degree more similar to their homologues from plants, i. e. *Arabidopsis thaliana* (61%) and *Nicotiana plumbaginifolia* (59 %). The modification of *P. vivax* eIF-5A precursor protein by *P. falciparum* deoxyhypusine synthase and human deoxyhypusine synthase has been successfully demonstrated [manuscript in revision] although to a lower extent in case of human DHS enzyme because of its reduced substrate affinity. The function of eIF-5A in the life cycle of the parasite remains to be elucidated by mutational analysis. The function of *eIF-5A* is also still unexplained for its eukaryotic homologues. Previous findings [28] indicate that eIF-5A functions as a nucleocytoplasmatic shuttle protein of a subset of mRNAs related to the G1/S cell cycle transition. These data suggested that *eIF-5A* may be operational in posttranscriptional processing of a specific subset of mRNAs. These transcripts may encode factors that are required for cell viability and efficient proliferation [29]. This notion was even more supported by the finding that *eIF-5A* is acting as a cofactor of the human immunodeficiency virus type 1 (HIV-1) Rev mRNA transport factor [30]. A comparable function of eIF-5A in the proliferation of malaria parasites would increase its role as an important target in malaria therapy.

We have recently cloned a 1491 bp nucleic acid sequence from *P. falciparum* strain NF 54 encoding deoxyhypusine synthase [26]. The alignment of the DHS amino acid sequence from Plasmodia shows 57% amino acid identity to DHS from the plants *Nicotiana tabacum* [31] and *Lycopersicon esculentum* [32]. The parasite DHS enzyme has a homology of 53% to the HSS protein from *Senecio vulgaris* (Asteraceae) [20] and 57% homology to that of *Senecio vernali*s [21], respectively. The spermidine and NAD^+ binding sites of Plasmodium DHS are highly conserved in different species, i.e. human, plant HSS and the protozoan *T. brucei rhodesiense.* However, in the putative DHS protein from *T. brucei rhodesiense* three amino acid exchanges appear within the spermidine binding site, i.e. lysine 329 is replaced with leucine (*T. brucei* DHS numbering), aspartic acid 316 with glutamic acid and alanine 309 with glycine [26].

While the human DHS enzyme has been purified to chemical homogenity [33] and its x-ray structure has been elucidated, neither kinetic studies have been performed for DHS from the parasite nor a crystal structure of the protein is existing. The first x-ray structure of human DHS [33] revealed four active sites of the homotetrameric enzyme located within deep tunnels. Each active-site entrance was blocked by a ball-and-chain motif composed of a region of extended structure capped by a two-turn alpha-helix under conditions of high ionic strength and acidic pH. Recently under physiological conditions the enzyme was crystallized with a free swinging ball-and-chain motif which did not block the enzyme at its active site [34]. The DHS competitive inhibitor N-guanyl-1,7-diaminoheptane (GC7) is observed to be bound within the putative active site of the enzyme in the new crystal form (Form II) after exposure to the inhibitor. Mutations affecting the NAD^+ binding site severely reduced the spermidine and inhibitor binding. The spermidine binding was mostly affected when mutations at amino acid positions E137A, D238A, and D342A occurred.

DHS from *P. falciparum* strain Dd2 has a fasta score of 48 to human DHS encoded by transcript variant 1 which is the only human transcript encoding a functional DHS. Most of the amino acid identities are within the spermidine and NAD binding sites so that the binding and structure for appropriate inhibitors should be predictable for the parasite enzyme.

For a phylogenetic comparison between the human pathogenic malaria parasite *P. falciparum* and the more benign malaria parasite *P. vivax* we isolated a 1368 bp nucleic acid sequence (unpublished results) encoding a putative *dh*s gene from *P. vivax* Salvador PEST strain I by means of expressed sequence tags. The amino acid sequence identity between the *dhs* genes from the human malaria parasites is 63%. The amino acid identities are 66% to *P. yoelii* and 65% to *P. berghei*, respectively.

While molecular cloning of the *dhs* gene has been performed in a variety of eukaryotes now the first *dohh* genes

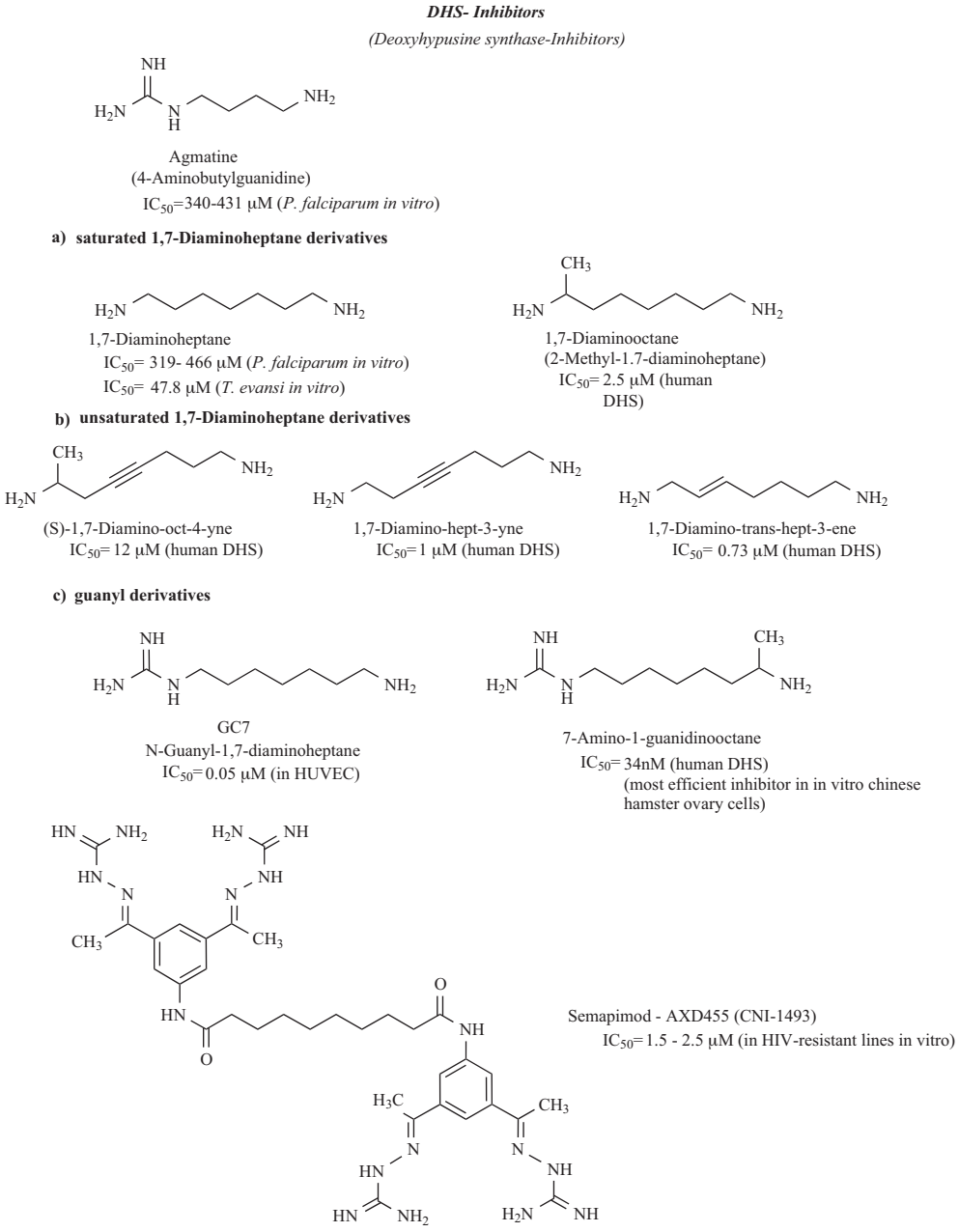


Fig. (2). Inhibitors of the spermidine binding pocket of DHS which are derivatives of 1,7-diaminoheptane (a [18, 24], b [24], c [44,33,24,42]). The structure revealed two basic moieties separated by 7-8 methylenes either branched-chain saturated or unsaturated which fit the active site of the enzyme. The most potent inhibitor from this series of compounds is N-guanyl-1,7-diaminoheptane [44,33] which shows inhibition *in vitro* and in cultured cells. Meanwhile the low molecular compound CNI-1493 [42], a tetravalent guanylhydrazone, showed a comparable inhibitory effect as N-guanyl-1,7-diaminoheptane. The inhibitory mechanism of CNI-1493 on human DHS is still unexplained.

from yeast and human have been obtained [35]. Computational analysis of the deoxyhypusine hydroxylase structure (DOHH) revealed that it is a HEAT-repeat protein (human huntingtin elongation factor 3, a subunit of protein phosphatase 2A and the target of rapamycin) containing eight helical hairpins (HEAT motif) and two potential metal coordination sites which are composed of four strictly conserved HE sequences. DOHH appears to be a unique protein hydroxylase distinct from the family of Fe(II)- and 2-oxoacid-dependent hydroxylases. In contrast to DHS no DOHH proteins have been found in *archaea* [35]. A *Saccharomyces cerevisiae* DOHH-null strain grew only slightly slower than its parental strain while DOHH seems to be functionally more related in fission yeast [35].

Even after the first partial purification of the DOHH enzyme which was obtained from rat testis [19] the catalytic action of this enzyme was suggested to be different from that of prolyl- and lysyl hydroxylases. The catalytic site of DOHH turned out to be a metal ion. *In vitro* inhibition studies on the enzyme revealed spermine and its analogue thermine [36] to be competitive inhibitors. Since there is a lack of specific inhibitors to evaluate DOHH as a potential drug target, the molecular cloning of DOHH is the first critical step toward characterization of structure and mechanism and toward development of specific inhibitors of this important eukaryotic enzyme.

Strategies in Prevention of eIF-5A Formation

In principle two different strategies of inhibition of *eIF-5A* formation exist: One strategy is inhibition of *eIF-5A* modification by means of DHS protein and a second one focuses on DOHH inhibition. The most elegant proof has been recently demonstrated by RNA interference of human DHS [38] in HIV-1 virus replication. HIV-1 replication depends on the action of the viral regulatory protein Rev [39]. *EIF-5A* has been previously reported to be a cellular cofactor of the Rev pathway [40, 41]. In summary the data show that HIV-1 replication could be blocked by inhibiting modification of eIF-5A protein by means of the DHS enzyme. Besides the approach on the molecular level the new inhibitor CNI-1493, formerly semapimod (Fig. (**2**)) which has already been applied for treatment of Morbus Crohn disease [42], has been shown to inhibit DHS in a dose dependent manner. CNI-1493 is a tetravalent guanylhydrazone, *N*,*N*9-bis[3,5-bis[1(aminoiminomethyl)hydrazonoethyl]phenyl]decanediamide tetrahydrochloride. In case of Morbus Crohn it suppresses formation of antiinflammatory cytokines like TNF-α by MAP kinase inhibition.

Blocking the biological activity of *eIF-5A* by means of its first modification step has recently been demonstrated to be an alternative in HIV-1 treatment since *eIF-5A* is an important cofactor in HIV-1 replication [43]. CNI-1493 had an IC_{50} value between 1.5 to 2.5 μM *in vitro* in HIV-resistant lines. Therefore the identification of new DHS inhibitors could be one step in the development of new HIV-therapies.

The most commonly used competitive inhibitor of DHS is N1-guanyl-1,7-diaminoheptane GC7. The inhibitory effect of GC7 on DHS was previously observed in human umbicilal vein endothelial cells (HUVEC) [44]. GC7 at 10 μM caused almost complete inhibition of hypusine biosynthesis and led to cytostasis of HUVEC. It has also been shown that GC7 suppressed the growth of N2a-mouse neuroblastoma cells and DS19- murine erythroleukemia cells at micromolar concentrations [45].

In a separate study the antiproliferative effect of GC7 on several tumorigenic cell lines under various growth conditions was studied [46]. GC7 seems to interfere with different steps in progression of the cell cycle and inhibits DHS in a competitive way by binding to its active site [33] which can be observed in crystallized form II being recently obtained for DHS enzyme and the inhibitor GC7. This structural context supports the results of previous biochemical investigations of the deoxyhypusine synthase reaction mechanism.

Branched-chain saturated and unsaturated 1, n-diaminoalkane, alkene and alkine derivatives containing 7 or 8 methylene groups inhibit the human DHS in the micromolar range of concentration [24]. In this series, the 1,7-diamino-trans-hept-3-ene exhibits the highest inhibitory potency, which can be easily enhanced by replacing one of the amino groups with a guanidine moiety, for comparison see 7-amino-1-guanidinooctane and 1,7-diaminooctane (see Fig. **2**).

In contrast to the variety of DHS inhibitors, only a few inhibitors of DOHH exist since a partially purified enzyme preparation has only be obtained from rat testis [19]. Inhibition of DOHH, the metalloenzyme catalyzing the final step in hypusine biosynthesis was shown in HUVEC cells by the antifungal drug ciclopiroxolamine, a small chelating molecule which targets the essential metal atom of the enzyme. For 4 additional chelating compounds deferiprone, deferoxamine, mimosine and 2,2-dipyridyl the following order of IC_{50} values was determined: deferoxamine > 2,2-dipyridyl > deferiprone >mimosine (IC_{50} 5-200 μM, see Fig. **3**) [47]. Even though the structures seem to be very different, they have one moiety in common, the ß-hdroxyketone group which is able to complex the iron atom of the enzyme. However, dipyridyl does not show this group, but is able to complex the metal ion by means of nitrogens.

Ciclopirox showed the strongest antiangiogenic activity and inhibitory effect on cell proliferation suggesting a new potential in treatment of solid tumors.

Antiretroviral effects have also been achieved by means of DOHH inhibition with α-hydroxypyridones specifically L-mimosine, a plant amino acid, and deferiprone, an experimental drug, which inhibited deoxyhypusyl hydroxylase in T-lymphocytic and promonocytic cell lines [48]. The HIV-1 protein Rev, critical for translation of incompletely spliced retroviral mRNAs encoding capsid elements requires eIF-5A as a host cell protein. By interfering with the translation of incompletely spliced retroviral mRNAs these compounds restrict HIV-1 to the early, nongenerative phase of its reproductive cycle. Selective suppression of retroviral protein biosynthesis and preferential apoptosis of retrovirally infected cells by α-hydroxypyridones point to a novel mode of antiretroviral action.

Recently during a screen for early respondents to L-mimosine treatment [49] two proteins, i.e. eIF-5A and its precursor form were identified in a 2D gel electrophoresis and shown to be inhibited by the drug while there was only mimosine-induced expression observable in case of differentiation related gene 1 (*drg1*).

The mimosine-induced expression of Drg-1 was mainly attributable to increased transcription likely by the c-Jun/AP-1 transcription factor. Since induction of Drg-1 is an early event after mimosine treatment and is observed before a notable reduction in the steady-state level of mature *eIF-5A*, *eIF-5A* does not appear to be involved in the modulation of Drg-1 expression.

These findings prompted us to test whether mimosine would affect *P. falciparum in vitro* cultures with different choroquine susceptibility in a similar way. The determined IC_{50} value was 32 μM for the chloroquine susceptible (CQS)

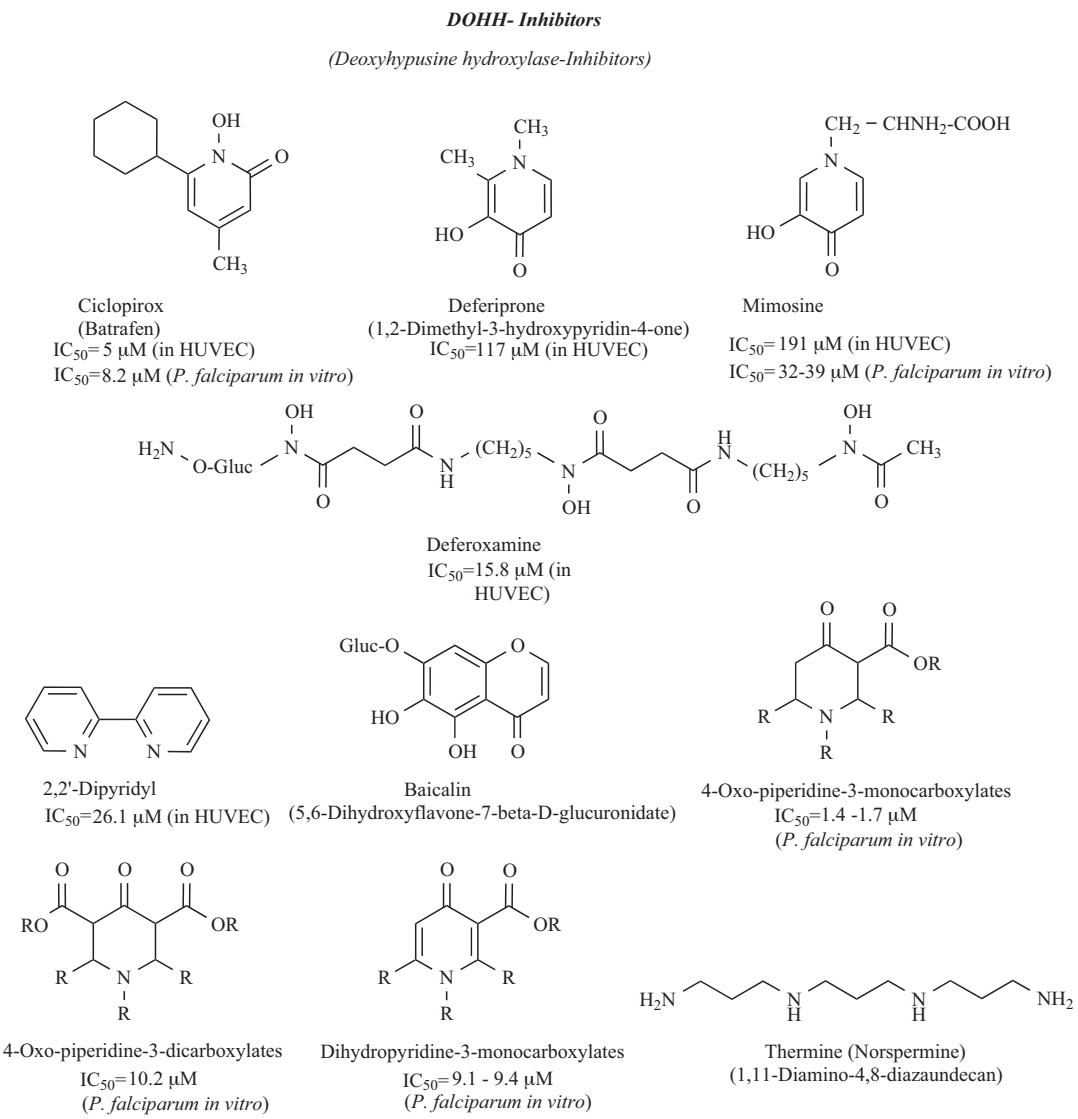


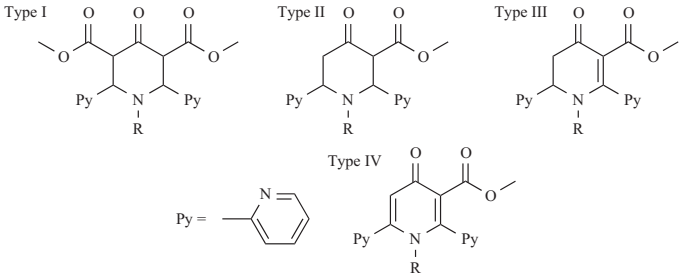
Fig. (3). Inhibition was mainly performed on purified DOHH enzyme from rat testis. Since the crystal structure of yeast and human DOHH protein has not been analyzed and only a predicted structure has been described, the reaction mechanism of these compounds can be interpreted as chelating iron which is part of the four strictly organized histidine glutamate coordination sites. The following compounds are part of the following references: Ciclopirox [53], deferiprone [47], mimosine [47], deferoxamine [47], 2,2– dipyridyl [47], 4-Oxo-piperidine-3-monocarboxylates [53], 4-Oxo-piperidine-3-dicarboxylates [53], dihydropyridine-3-monocarboxylates [53], thermine [36].

strain and 39 μM for the chloroquine resistant (CQR) strain, respectively. However, mimosine was toxic in a rodent malaria model.

The 4-pyridone skeleton in mimosine, especially the ß-hydroxyketone moiety, provided the basis for the development of a new lead structure for an initially small series of different saturated and non saturated mono and dialkyl 4-piperidone mono- and dicarboxylates which were previously synthesized [50,51,52] (Table **1**). These compounds should be able to complex the iron atom of the enzyme either by means of the ß-ketocarboxylate which can tautomerize to an enolcarboxylate, or by means of the pyridine rings. Table **1** shows the *in vitro* biological activity of seven different saturated and non-saturated dipyridine substituted mono- and diesters tested in CQS *P. falciparum* strains NF54 and CQR R [53].

In summary the data indicate that saturated 4-piperidone monoesters show the most prominent inhibitory effect (DOHHI-3, DOHHI-7) while a 4-piperidone diester was less efficient in growth inhibition *in vitro* [53]. The average IC_{50} values which we obtained for the 4-oxo-piperidine monoesters in *P. falciparum* strain NF54 were determined to be 1.7 μM for an N-p-chlorobenzyl substitution (Table **1**, type II, DOHHI-3) and 1.4 μM for an N-allyl substituted derivative (Table **1**, type II, DOHHI-7), respectively, suggesting that the N-substitution is of minor importance. The 4-oxo-

Table 1. Structural Formulae of the Compounds Studied and Their Determined IC_{50} Values in *P. falciparum* Chloroquine Susceptible (CQS) and Chloroquine Resistant (CQR) Strains NF54 and R Respectively



DOHHInhi-bitors	Type	R	H	*P. falciparum* strain	Average IC_{50} [μM]
1	I	benzyl	24	NF54	10.2± 5.9
2	III	benzyl	24	NF54	4.7± 2.7
3	II	4-Cl-benzyl	48	NF54	1.7± 0.9
4	III	4-Cl-benzyl	24	R	18.0 ±10.4
5	IV	4-Cl-benzyl	36	R	9.4 ±5.4
6	IV	4-CH_3-benzyl	24	R	9.1±5.2
7	II	allyl	24	NF54	1.4±0.8

piperidine diester DOHHI-1 shows a higher IC_{50} value of 10.2 μM (DOHHI-1) *in vitro* in comparison to the monoester. The oxidation products, the tetrahydropyridinemonoester (DOHHI-2, DOHHI-4) and dihydropyridine monoesters (DOHHI-5, DOHHI-6) although both being structurally rather similar to the lead compound mimosine were less efficient in growth inhibition compared to saturated 4-piperidone monoesters. Most notably the highest average IC_{50} value was determined to be 18.0 μM for CQS NF54 strain with the DOHHI-4 inhibitor, a tetrahydropyridine monoester.

Due to the double bonds in DOHHI-2, 4, 5, and 6, the carbonyl function in position 4 cannot form an enol which is favourable to a metal chelation. Thus, the high IC_{50} values of the dihydro- and tetrahydropyridines point to the importance of the metal ion complexation of the enzyme for an efficient inhibition. Obviously, the two pyridine rings cannot serve as complexation partner for the metal as the β-hydroxycarbonyl function does.

However, the 4-oxo-piperidine diester DOHHI-1 although less active *in vitro* applied in a daily dose of 300 mg / kg body weight seems to be a promising compound *in vivo* since it extends significantly the survival time of 20% of the BALB/c mice to 19 days post infection in comparison to the untreated control. Despite a prolonged survival time a decrease in parasitemia in DOHHI-1 treated mice compared to the control was not detectable. This observation might be caused by the fact that inhibition of *eIF-5A* might lead to a reduced synthesis of tumor-necrosis factor (TNF-α) in mice [54] which is a key factor of death in mice. Previous data have shown that high concentrations of TNF are involved in pathological processes of Plasmodia while low concentrations are important for the control of *Plasmodium* infection [55].

The high daily dosage of DOHHI-1 drug which is 300 mg/kg body weight resembles its application in DFMO treatment of *Trypanosoma brucei gambiense* causing African sleeping sickness commonly used in the range of 100 mg kg^{-1} body weight for adults and 150 mg kg^{-1} for children [56] given at intervals of 6-14 days as short infusions.

Perspectives

Having identified a new lead structure, the 4-oxo-piperidine-3-carboxylate, the future work will concentrate on the enhancement of the inhibitory potency as well as the optimization of pharmacokinetic parameters, i.e. ADMET (= absorption, distribution, metabolism, elimination and toxicity). Therefore a wide variation of the substitution pattern will be necessary. However, the increase of the water solubility of the inhibitors has to be kept in mind, because this is an important prerequisite for high *in vivo* efficacy. This goal can likely be achieved by means of introduction of more polar substituents in various positions, especially the pyridines will be replaced with more "hydrophilically" substituted aromatic rings. Another aim for the future has to be the cloning of DOHH from *P. falciparum* in order to test the biological activity of this 2,6-dipyridine substituted 4-oxo-piperidine ester on the expressed and purified enzyme.

ABBREVIATIONS

ACT = Artemether combination therapy

IPTp = Intermittent treatment in pregnancy

ODC = Ornithine decarboxylase

AdoMetDC = Adenosyl methionine decarboxylase

SPDS = Spermidine synthase

DFMO	=	α–difluoromethylornithine
APA	=	3-aminooxy-1-propanamine
4MCHA	=	Trans-4-methylcyclohexylamine
RBCs	=	Red blood cells
DHS	=	Deoxyhypusine synthase
DOHH	=	Deoxyhypusine hydroxylase
DOHHI	=	Deoxyhypusine hydroxylase inhibitor
EIF-5A	=	Eukaryotic initiation factor 5A
HSS	=	Homospermidine synthase
Pas	=	Pyrrolizidine alkaloids
Odc	=	Ornithine decarboxylase gene
s-adometdc	=	s-adenosyl-methionine decarboxylase gene
spds	=	Spermidine synthase gene
dhs	=	Deoxyhypusine synthase gene
eIF-5A	=	Eukaryotic initiation factor 5A gene
GC7	=	N-guanyl-1,7-diaminoheptane
NAD	=	Nicotinamide adeninedinucleotide
Dohh	=	Deoxyhypusine hydroxylase gene
HEAT-repeat protein	=	Human huntingtin elongation factor 3, a subunit of protein phosphatase 2A and the target of rapamycin
Rev protein	=	Regulatory viral protein
CNI-1493	=	*N*,*N*9-bis[3,5-bis[1(aminoiminomethyl)hydrazonoethyl]phenyl]decanediamidetetra-Hydrochloride
drg-1	=	Differentiation related gene 1
CQS	=	Chloroquine susceptible
CQR	=	Chloroquine resistant
HUVEC	=	Human umbicilal vein endothelial cells
DOHHI	=	Deoxyhypusine Hydroxylase Inhibitor
TNF-α	=	Tumor necrosis factor α
DCA	=	Dicyclohexylamine
MGBG	=	Methylglyoxal-bisguanylhydrazone
MDL 73811	=	4-[Amino-2-trans-butenyl]-methylamino-5‘-deoxyadenosine

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