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REVIEW ARTICLE

Assessment of deoxyhypusine hydroxylase as a putative, novel drug target

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Abstract Antimalarial drug resistance has nowadays reached each drug class on the market for longer than 10 years. The focus on validated, classical targets has severe drawbacks. If resistance is arising or already present in the field, a target-based High-Throughput-Screening (HTS) with the respective target involves the risk of identifying compounds to which field populations are also resistant. Thus, it appears that a rewarding albeit demanding challenge for target-based drug discovery is to identify novel drug targets. In the search for new targets for antimalarials, we have investigated the biosynthesis of hypusine, present in eukaryotic initiation factor 5A (eIF5A). Deoxyhypusine hydroxylase (DOHH), which has recently been cloned and expressed from *P. falciparum*, completes the modification of eIF5A through hydroxylation. Here, we assess the present druggable data on *Plasmodium* DOHH and its human counterpart. *Plasmodium* DOHH arose from a cyanobacterial phycobilin lyase by loss of function. It has a low FASTA score of 27 to its human counterpart. The HEAT-like repeats present in the parasite DOHH differ in number and amino acid identity from its human ortholog and might be of considerable interest for inhibitor design.

Keywords Deoxyhypusine hydroxylase · *Plasmodium* · Malaria · Drug target

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Abbreviations

DHS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
eIF5A	Eukaryotic initiation factor 5A
HUVEC	Human vascular endothelial cells
OPA	Ortho-phtaldialdehyde
SDS–PAGE	Sodium dodecylsulfate
DEAE FF	Diethylaminoethyl cellulose fast flow
GC/MS	Gas chromatography/mass spectrometry
SCRATCH	A protein structure and structural feature prediction server
HEAT	Huntingtin protein, elongation factor 3, the protein phosphatase subunit 2A and the target of rapamycin

Introduction

Hypusination in eukaryotic initiation factor 5A (eIF5A) is a unique posttranslational modification which so far has only been discovered in this protein (Park 2006). Hypusine was first isolated by Shiba et al. (1971) from brain cells and identified as an [N(epsilon)-(4-amino-2-hydroxybutyl)lysine].

The biosynthesis of hypusine occurs in two consecutive steps. In the first step, deoxyhypusine synthase (DHS) transfers the 4-aminobutyl moiety to a specific lysine residue in eIF5A (Umland et al. 2004), while in the second step of hypusine biosynthesis, deoxyhypusine hydroxylase (DOHH) completes this posttranslational modification by hydroxylation (Park et al. 2006) (Fig. 1). Over recent years, DHS proteins have been characterized from human (Joe et al. 1995; Liao et al. 1998), plants (Duguay et al. 2007; Wang et al. 2005; Ober and Hartmann 1999), fungi (Tao and Chen 1995) and parasites (Kaiser et al. 2003, 2007).

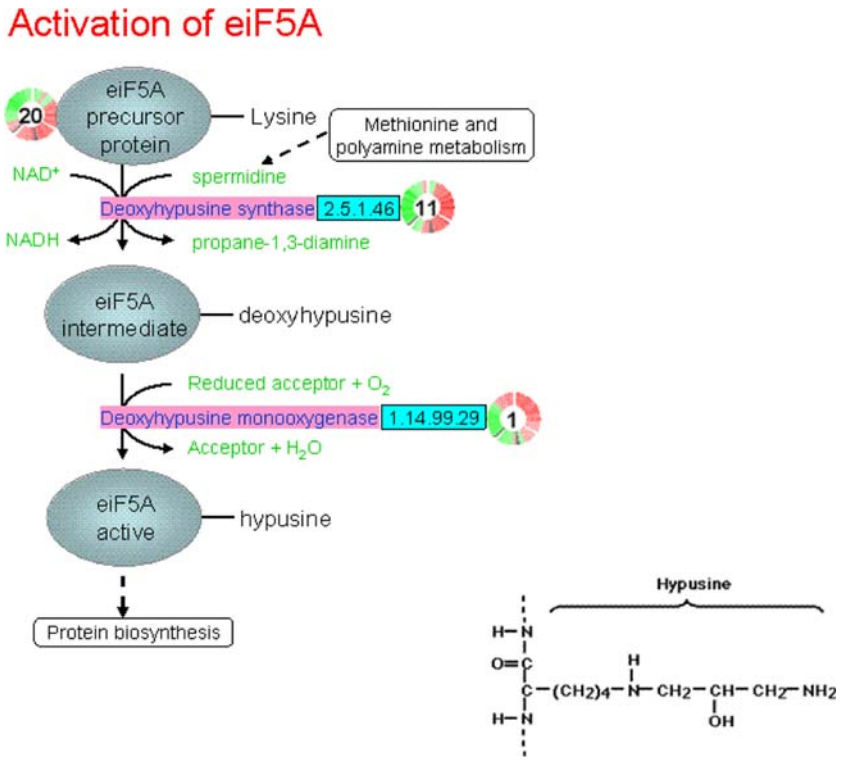

Fig. 1 Eukaryotic initiation factor 5A is activated in two steps. The first enzyme, deoxyhypusine synthase (DHS), catalyzes the NAD-dependent cleavage of spermidine and transfer of its 4-aminobutyl moiety to the ε-amino group of a specific lysine residue to form the deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] residue. The second enzyme, deoxyhypusine hydroxylase (DOHH), hydroxylates this intermediate to form the hypusine residue and mature eIF5A. Recently it was shown that hypusine-containing eIF5A promotes translation elongation (Saini et al. 2009; Gregio et al. 2009). The substrate spermidine of deoxyhypusine synthase is formed by an aminopropyl-moiety transfer from decarboxylated *S*-adenosylmethionine to putrescine which is catalyzed by spermidine synthase. Numbers 1, 11 and 20 provide a link to the *dohh* gene locus in different *Plasmodium* strains

However, information about DOHH proteins from different species is still limited.

Deoxyhypusine hydroxylase (DOHH) was first partially purified and characterized from rat testis in 1985 by Abbruzzese et al. It was shown that the enzyme required addition of sulfhydryl groups for catalytic activity, while the catalytic mechanism was different from prolyl and lysyl hydroxylases. Fe^{2+} was supposed to be a cofactor of DOHH. Gene expression was observed in different developmental stages of the brain (Abbruzzese 1988). The highest specific activities of DOHH were determined in the parietal cortex during the first 5 days of life. After this period, DOHH activity declined to less than 50% of the level in the newborn within 15 days. The active site of the enzyme from rat, which contained Fe^{2+} as a cofactor, was blocked by two catecholpeptides containing a 3,4-dihydroxybenzoyl- and a 2,3-dihydroxybenzoyl moiety. The 3,4-dihydroxybenzoyl-containing compound was more potent with a K_i value of 32 mM.

The next decade of research focused intensively on DOHH inhibitors in different cell systems to investigate the biological function of the enzyme in cell proliferation. Within a panel of compounds, the lead structure mimosine inhibited progression of cells from the G1 to S-phase by DOHH inhibition (Hanauske-Abel et al. 1994). DOHH reactivation occurred rapidly after inhibitor withdrawal and correlated with synchronized entry into the S-phase.

Andrus et al. (1998) demonstrated antiretroviral effects of alpha-hydroxypyridones (i.e. mimosine and deferiprone) on HIV-1 multiplication in T-lymphocytic and promonocytic cell lines. This effect was attributed to DOHH inhibition and subsequent inhibition of eIF5A modification by hypusination. Selective suppression of retroviral biosynthesis was attributable to inhibition of Rev protein which is critical for translation of incompletely spliced mRNAs requiring eIF5A as a cellular cofactor.

Hitherto molecular cloning of DOHH has only been successful for genes from human (Park et al. 2006), *Saccharomyces cerevisiae* (Park et al. 2006), bovine (Huang et al. 2007) and the parasite *Plasmodium falciparum* (Frommholz et al. 2009). This might be attributed to the difficulties in the analytical procedures with respect to the activity assay of this protein.

The following review will critically discuss the recently obtained genomic data from different *dohh* genes and address structural predictions of the encoded proteins with respect to a druggable target.

What is druggable in *dohh* genes?

The first *dohh* gene was cloned from a *Saccharomyces cerevisiae* GST-ORF library in a biochemical genomics approach (Park et al. 2006). The isolated *dohh* gene complemented a yeast deficient mutant YJR070C. The *dohh* gene from *Saccharomyces cerevisiae* was already identified in a former two-hybrid DNA screen as Lia1, a ligand of eIF5A (Thompson and Cano 2003). The nucleic acid data from the yeast *dohh* gene provided the basis for the identification of the human and bovine (Huang et al. 2007) single copy orthologs. Meanwhile the first *dohh* gene from the human malaria parasite *P. falciparum* has been isolated and expressed (Frommholz et al. 2009). Structural analyses of the four *dohh* genes identified so far revealed that they belong to a family of HEAT-like repeat proteins which comprise the Huntingtin protein, elongation factor 3, the protein phosphatase subunit 2A and the target of rapamycin (Park et al. 2006). Two distinct HEAT-like repeat motifs are present in all DOHH proteins. However, significant differences can be observed with respect to the similarity and number of the HEAT-like repeats. These differences in the HEAT repeat structure might be of considerable interest for the design of new DOHH inhibitors.

Plasmodium (*P.*) *falciparum* and *P. vivax* belong to the order *Apicocomplexa* which is characterized by the presence of an apicoplast, essential for the parasite to invade its human host. The apicoplast is thought to be the relic of a chloroplast derived from an ingested red alga. Such chloroplasts, in turn, are thought to be of cyanobacterial (prokaryotic) origin. Although the apicoplast has lost all the photosynthetic capacity (Maréchal and Cesbron-Delauw 2001), it retains some metabolic pathways of the chloroplast which are therefore potential targets for anti-malarial drugs.

In *Plasmodium*, five HEAT-like repeats are exhibited, in contrast to four in the human ortholog (Frommholz et al. 2009) (Fig. 2). The function of the fifth HEAT-like repeat in the *dohh* gene from *Plasmodium* is unclear and needs further analysis with respect to the activity of the enzyme. Interestingly, the first two repeats from *Plasmodium* show significant identities to E-Z type HEAT-like repeat proteins (IPR004155 (InterPro), PF03130 (Pfam), SM00567 (SMART) present in phycocyanin lyase subunits of cyanobacteria (Scheer and Zhao 2008).

However, the purified DOHH protein displayed no phycocyanin lyase activity, suggesting that plasmodial DOHH arose originally from an EF/type cyanobacterial phycobilin lyase that gained a new role in hydroxylation.

In conclusion, the low FASTA score of 27 of *Plasmodium* DOHH to its human ortholog and the significant amino acid identity of the two-first HEAT-like repeats in the DOHH protein between *Plasmodium* and phycocyanin lyases from cyanobacteria are important features for inhibitor design in the future.

Structural predictions of different DOHH proteins will contribute to inhibitor development in the future

At present, a predicted X-ray structure only exists for the human protein. Eight tandem HEAT-repeats are organized in a symmetrical dyad in an α-helical structure. Each of the four domains in human DOHH consists of a highly characteristic histidine-glutamate motif to chelate Fe^{2+}. Although parasitic DOHH consists of an additional HEAT-hairpin, it shares the four common His-Glu coordination sites for Fe^{2+} with the identified DOHH proteins from yeast, human and bovine. The purified human recombinant DOHH is a mixture of active holoenzyme containing 2 mol of iron/mol of DOHH and inactive metal-free apoenzyme (Kim et al. 2006). The two molecular species could be distinguished by their different mobilities upon native gel electrophoresis. The apoenzyme exhibited markedly reduced enzyme activity which could only be restored by Fe^{2+} and by no other metal. The role of the strictly conserved His-Glu residues was evaluated by site-directed mutagenesis. Substitution of any single amino acid in the four His-Glu motifs with alanine abolished the enzyme activity. With respect to human DOHH, iron chelators will be the lead structures for the development of specific inhibitors.

It has been shown in in vitro assays of human vascular endothelial cells (HUVEC) that small compounds structurally related to dihydropyrimidines, which target the active metalloenzyme, inhibit DOHH in a concentration-dependent manner (Clement et al. 2003). Their efficacy varies widely in the following order: ciclopirox → deferoxamine → 2,2′-dipyridyl → deferiprone → mimosine (IC_{50} 5–200 μM).

Inhibitor studies of purified DOHH from *Plasmodium* have just begun. In the search for novel antiparasitic DOHH inhibitors, compounds related structurally to dihydropyrimidines like the plant amino acid mimosine (Andrus et al. 1998) and the antifungal drug ciclopiroxolamine (Saeftel et al. 2006), have been investigated in *P. falciparum* in vitro cultures. However, when these inhibitors were administered in vivo in rodent models mimicking cerebral malaria, they were either toxic, in case of mimosine, or they did not cure the animals from cerebral malaria.

Alkyl 4-oxo-piperidine 3 carboxylates, which are structurally related to dihydropyrimidines, were found to be the most potent, putative DOHH inhibitors in vitro

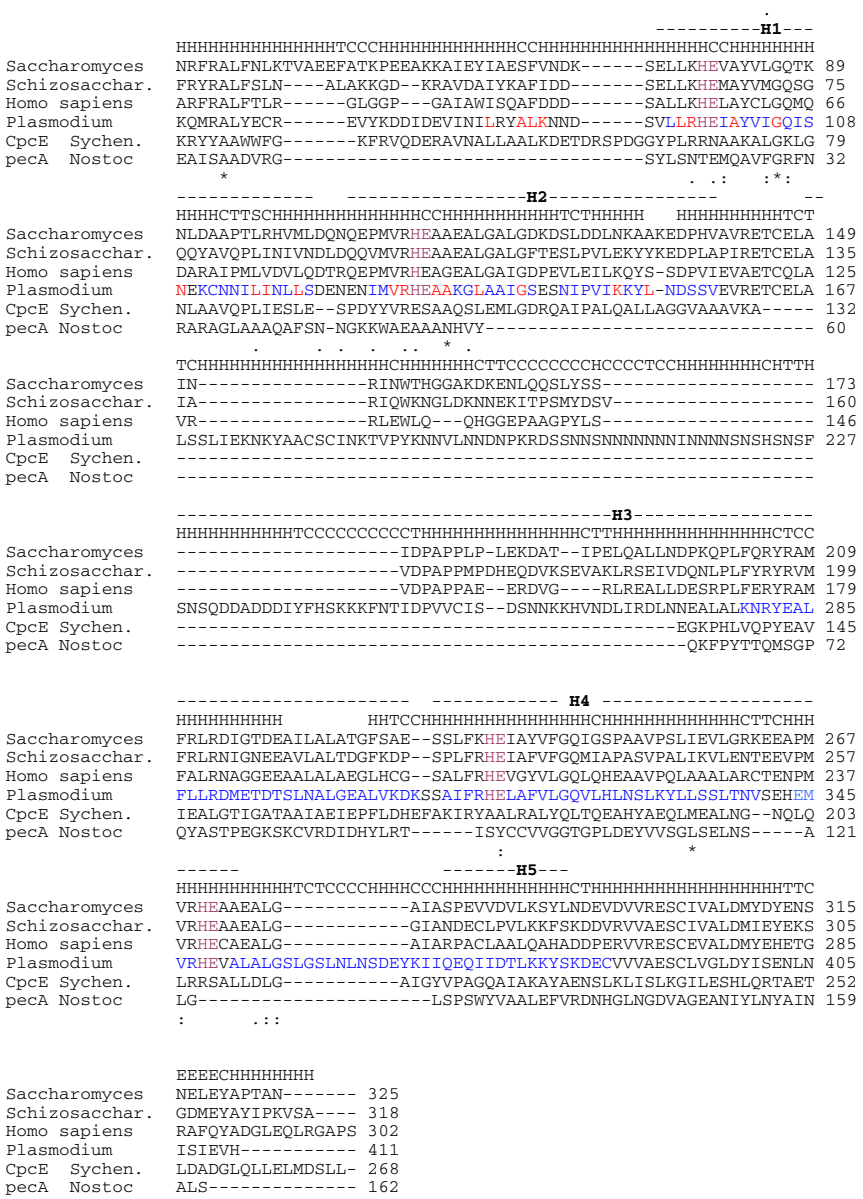

Fig. 2 Multiple amino acid alignment of DOHH proteins from four different eukaryotes (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Homo sapiens* and *Plasmodium falciparum* strain NF54), the homolog of a biliprotein lyase (CpcE) from *Synechococcus elongatus*, and an α-subunit of a cyanobacterial biliprotein (PecA from *Nostoc sp.* PCC7120). The five individual EZ-HEAT repeat domains from *P. falciparum* are numbered and shown above the alignment. Amino acids with *blue capital letters* show various degrees of homology to EZ-HEAT repeats present in proteins involved in energy metabolism and conversion. The most significant amino acid identity to HEAT-repeats in CpcE from *Synechococcus elongates* is found in EZ-HEAT-repeat domains 1 and 2 (amino acid positions 76–179 and 47–184). The identical amino acids are marked in *red color*. Histidine-glutamate motifs are highlighted in *purple*. The secondary structure prediction above the alignment presents *H* for the α-helix, *E* for an extended structure, *T* for a *β*-turn and *C* for the rest and was obtained by the JPred version 3.0 and SCRATCH programmes (Cuff et al. 1998). Gaps (-) were introduced to obtain maximum alignment. *Asterisks* label amino acid identities, colons (:) and dots (.) label amino acid similarities

(Saeftel et al. 2006), since the catalytic activity of the parasitic enzyme seems also to be dependent on Fe^{2+}.

However, alkyl 4-oxo-piperidine carboxylates showed no inhibitory effect on the purified DOHH protein (Kaiser, unpublished results), suggesting that their in vitro activity in *P. falciparum* was based on complexing metal ions from other enzymes rather than complexing the Fe^{2+} of DOHH from the parasite.

In contrast, one compound, which can be considered as an oxime ether of the piperidine aldehyde, is not able to form a complex with a metal ion (Goebel et al. 2008) but shows a low IC_{50} value in vitro and protects mice against cerebral malaria. This suggests that other features of parasitic DOHH might be of importance for the interaction with the drug. However, the drug failed to inhibit purified plasmodial DOHH.

Silencing or deletion of the *dohh* gene is a helpful tool in target evaluation and in elucidation of the biological function of DOHH protein

First evidence about the function of DOHH came from the fission yeast *Schizosaccharomyces pombe* (Weir and Yaffe 2004). A mutation in the *dohh* gene caused defects in mitochondrial morphology, distribution and displayed synthetic defects in growth. In summary, the data demonstrated that in the fission yeast, DOHH is required for the alignment of mitochondria along microtubules.

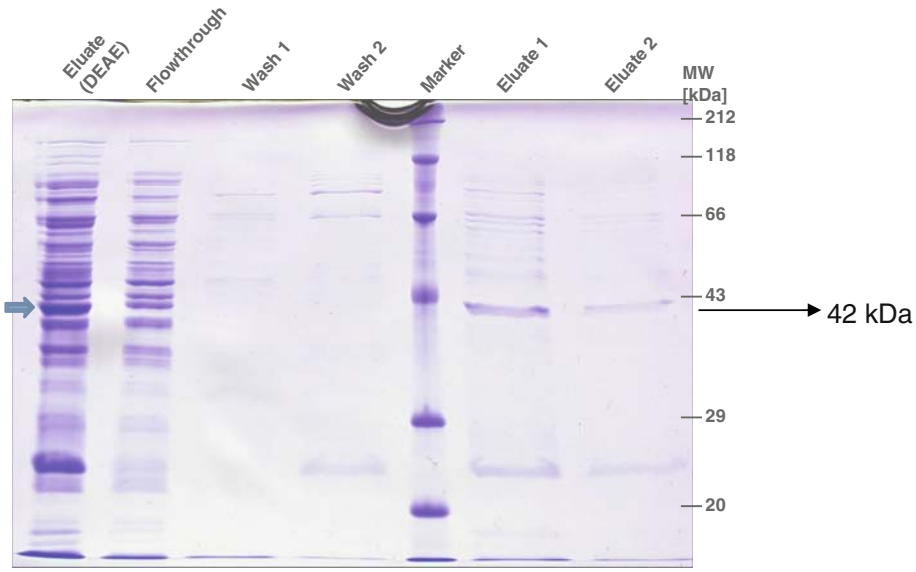

Fig. 3 Native SDS–PAGE of the combined purification of plasmodial DOHH by anion exchange chromatography with a HiTrap DEAE FF column and a nickel-chelate chromatography column. The partially purified eluate which was obtained after anion exchange chromatography was purified on a nickel chelate affinity chromatography column. Elution was performed with 500 mM imidazole in NaH_2PO_4 buffer containing 300 mM NaCl. The high molecular weight bands in the eluate fraction presumably derive from the non-iron containing apoenzyme

In contrast, a homozygous, diploid *dohh* knock-out strain of the budding yeast *Saccharomyces cerevisiae* showed nearly normal growth in comparison to the wild type strain in rich and minimal medium. Since the *dhs* gene is an essential gene and the first step of hypusine modification is absolutely required for cell viability in *Saccharomyces cerevisiae*, the normal growth was unexpected. These data suggested that the deoxyhypusinated eIF5A intermediate could fulfill the function of the hypusinated eIF5A in yeast.

In higher eukaryotes like *Drosophila melanogaster*, the homologous *dohh* gene and its target eIF5A are required for cell growth and the regulation of autophagy (Patel et al. 2009). A mutated deoxyhypusine hydroxylase gene *nero* in *Drosophila* affected cell and organ size. However, *nero* is not required for cell viability.

With respect to *nero* function in relation to *eIF5A*, loss of *eIF5A* causes phenotypes highly similar to *nero* but more severe than *nero*. The Nero protein regulates eIF5A activity. Consistent with this notion, eIF5A is upregulated in *nero* mutants. Inhibition of Nero or eIF5A by RNAi causes a similar impairment in translation elongation. These data support a function of Nero in eIF5A mediated translational control.

The Nero protein is highly conserved in *Drosophila* (Patel et al. 2009). Nero shares 59% amino acid identity with its human ortholog. The four histidine-glutamic acid metal-binding motifs present in human DOHH are fully conserved in *Drosophila*. Interestingly, overexpression of human DOHH carrying mutations in these metal-ion binding motifs failed to complement nero mutations, arguing that these residues are also important for the Nero function in *Drosophila*.

A robust, non-radioactive assay for DOHH activity is necessary for High-Throughput-Screening in the future

To determine DOHH activity, eIF5A, DHS and DOHH have to be purified. The human and bovine proteins have been expressed as GST tagged fusions (glutathione-*S*-transferase) and purified by glutathione affinity chromatography (Park et al. 2006; Huang et al. 2007). Alternatively, the genes of the hypusine pathway from *Plasmodium* were expressed as histidine tagged proteins, using nickel-chelate affinity chromatography as the preferred method of purification (Kaiser et al. 2003, 2007). Although *E. coli* cells lack enzymes of the hypusine pathway, some host proteins which show approximately the same molecular weight, interfere with the purification. In the case of the plasmodial DOHH protein, a weak anionic exchanger column (HiTrap DEAE FF) was combined with nickel-chelate-affinity chromatography (Fig. 3) to solve the problem. However, in native SDS–PAGE electrophoresis high molecular weight bands reappeared, probably due to the presence of the non-Fe^{2+}-containing apoenzyme which is part of human DOHH (Kim et al. 2006).

Instead of the commercial radioactive DOHH activity assay (Kang et al. 1995), two alternative robust assays have been developed.

Recombinant DOHH and non-radioactively modified eIF5A (Dhp) derived from *Plasmodium* were incubated and the modified precursor protein was analyzed by peptide hydrolysis and subsequent GC/MS analysis. Hypusine was found in the assay with purified DOHH enzyme, together with small amounts of deoxyhypusine (Frommholz et al. 2009). We identified the molecular ion $[M]^{+\bullet}$ at 377 (mass/charge ratio) for hypusine and, in contrast to deoxyhypusine,

a molecular fragment of [M–OCH_3] with $[M-31]^+$. A quantification of hypusine by GC/MS was not possible. An alternative, selective and sensitive reversed-phase HPLC method is the derivatization by ortho-phthaldialdehyde (OPA) (Beninati et al. 1990), which can be used for quantification. Hypusine and the amino acids obtained in the peptide hydrolysate are derivatized with OPA to fluorescent isoindol-derivatives. This method could be used for HTS in the future.

Perspectives

Deoxyhypusine hydroxylase is the key enzyme in the biosynthesis of hypusine-containing eukaryotic translation initiation factor 5A (eIF5A), which plays an essential role in the regulation of cell proliferation. DOHH from *Plasmodium* has very peculiar features in comparison to its human ortholog i.e. the presence of a N-terminal signal peptide and HEAT-like repeats which differ in number and amino acid identity from its human counterpart. These characteristics might pave the way for the discovery of selective inhibitors in antimalarial therapy.

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