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Plasmodium dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy

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Abstract

Malaria remains a globally prevalent infectious disease that leads to significant morbidity and mortality. While there are a number of drugs approved for its treatment, drug resistance has compromised most of them, making the development of new drugs for the treatment and prevention of malaria essential. The completion of the *Plasmodium falciparum* genome and a growing understanding of parasite biology are fueling the search for novel drug targets. Despite this, few targets have been chemically validated *in vivo*. The pyrimidine biosynthetic pathway illustrates one of the best examples of successful identification of anti-malarial drug targets. This review focuses on recent studies to exploit the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway of *P. falciparum*, dihydroorotate dehydrogenase (*Pf*DHODH), as a new target for drug discovery. Several chemical scaffolds have been identified by high throughput screening as potent inhibitors of *Pf*DHODH and these show strong selectivity for the malarial enzyme over that from the human host. Potent activity against parasites in whole cell models with good correlation between activity on the enzyme and the parasite have also been observed for a number of the identified series. Lead optimization of a triazolopyrimidine-based series has identified an analog with prolonged plasma exposure, that is orally bioavailable, and which shows good efficacy against the *in vivo* mouse model of the disease. These data provide strong evidence that *Pf*DHODH is a validated target for the identification of new antimalarial chemotherapy. The challenge remains to identify compounds with the necessary combination of potency and metabolic stability to allow identification of a clinical candidate.

Keywords

Malaria; Plasmodium; pyrimidine biosynthesis; dihydroorotate dehydrogenase; drug discovery

Malaria the disease, and the current state of chemotherapy

Malaria is a global disease caused by protozoan Apicomplexan parasites of the *Plasmodium* species and is transmitted by the *Anopheles* mosquito [1,2]. It is endemic in 90 countries, putting over 2 billion people at risk and leading to the deaths of 1–2 million people annually. Pregnant woman and children are the most susceptible to severe disease. Until the early 1950s malaria was endemic in parts of the US and Europe, but it was eliminated through extensive control programs. It is now found primarily in tropical and subtropical countries with the African population most at risk. A number of anti-malarial agents are in clinical use, however the development of resistance to both chloroquine and sulfadoxine-pyrimethamine led to the

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rapid increase in the number of fatalities due to malaria [1,3,4]. Drug resistance has been reported to almost every known anti-malarial agent, underscoring the ease by which parasite populations can adapt and survive. Field isolates from drug-resistant regions of the world appear to acquire resistance to new agents faster than parasites isolated before resistance had developed [5]. The introduction of Artemisinin-based combination therapies (ACT) in conjunction with vector control measures has led to real progress in reducing the burden of the disease [6,7]. Recent reports of Artemisinin resistance in western Cambodia raise the worrying possibility that this class of drugs may also fall to resistance [8].

The path forward to new drug discovery

A significant effort is underway to identify new anti-malarial agents. The discovery effort is being fostered by partnerships formed between nonprofit organizations such as Medicines for Malaria Venture (http://www.mmv.org/), and academic and industrial collaborators, as well as by public organizations such as the US National Institutes of Health [3,9,10]. A number of additional ACTs are in late stage clinical trials, and several novel agents including synthetic trioxalanes (Rbx11160 and OZ439), pyridones developed by GlaxoSmithKline (GSK 932121), and MK4815 licensed to MMV by Merck, are under clinical investigation. The search for new molecular targets is being aided by the completion of the genome sequence for *P. falciparum* [11]. However despite extensive efforts to identify new essential and druggable targets, few have been chemically validated. The identification of mitochondrial electron transport chain cytochrome bc_1 complex as the target for atovoquone represents that most recent discovery of a new target that is validated with clinically proven inhibitors [12–15].

Many of the clinically relevant anti-malarial agents that have a known mechanism of action directly or indirectly affect pyrimidine metabolism. Drugs targeting dihydrofolate reductase (DHFR) or dihydropteroate synthase (e.g. pyrimethamine, cycloguanil, sulfonamides and sulfones) disrupt folate metabolism, which is essential for the formation of thymidine [16]. Atovoquone directly targets the electron transport bc_1 complex in the mitochondria, however this too causes toxicity through disrupting pyrimidine metabolism. This link was first suggested by the observation that Atovoquone treatment causes a reduction in cellular UTP and CTP levels [17,18]. A more recent study showed directly that the activity of the bc_1 complex is essential for providing oxidized ubiqinone to DHODH for the formation of pyrimidines [19, 20]. Inhibitors of thymidylate synthase are also potent anti-malarials, though none have yet reached the clinic [21–24]. These studies suggest that the pyrimidine biosynthetic pathway may be a rich source for the discovery of new anti-malarial agents. This review focuses on efforts to exploit the fourth enzyme in the *de novo* pyrimidine pathway, DHODH for discovery of new chemical species targeting this enzyme for the treatment of malaria.

De novo pyrimidine biosynthesis is essential in malaria

Pyrimidines are essential metabolites that are precursors for DNA and RNA biosynthesis [16]. Cells acquire pyrimidines either through *de novo* synthesis starting from ammonia (derived from L-glu), bicarbonate, and L-asp, or by salvaging preformed pyrimidine bases (uracil, cytosine and thymine) or nucleosides (uridine, thymidine and cytidine). *Plasmodium* species are unusual in that they lack pyrimidine salvage enzymes and the *de novo* pathway provides the only source of pyrimidines for cell growth. In contrast, human cells are able to utilize both pathways. Six enzymes in *Plasmodium* species are required to synthesize UMP, which is then used to generate UTP, CTP, dTMP, and the subsequent additional metabolites of these nucleotides that are required by the cell. These include bifunctional glutamine amidotransferase/ carbamoylphosphate synthetase (GAT/CPS), aspartate carbamoyltransferase (ACT), dihydroorotase (DHOtase), DHODH, orotate phosphoribosyltransferase (OPRT) and orotidine 5’-monophosphate decarboxylase (OMPDC)

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(Figure 1). The activities of the enzymes have been detected in parasite extracts [25,26] and the genes for each have been identified in the parasite genome [11]. *Pf*DHODH has been purified and characterized from the parasite mitochondria [18,27,28], and the gene sequence [29] reported prior to the completion of the malaria genome. The organization of the *Plasmodium* enzymes differs from their mammalian counterparts. In mammals the first four activities (GAT/CPS/ACT/DHOtase) are fused into a single polypeptide, as are the final two (OPRT/OMPDC)[30]. In contrast, for the Plasmodium enzymes, only the first two activities are present in a fused protein (GAT/CPS).

Plasmodium is the only genus amongst the pathogenic apicoplasts to rely solely on *de novo* pyrimidine biosynthesis. *Toxoplasma gondii* has both the *de novo* and salvage pathways, while *Cryptosporidium* utilizes only pyrimidine salvage pathways. The absence of pyrimidine salvage pathways in *Plasmodium* was first biochemically demonstrated by analysis of the incorporation of radiolabeled precursors into nucleic acids [31] and by enzymatic assay [26], and has now been confirmed by the completion of the genome sequence [11]. Orotic acid is the only preformed pyrimidine that can be incorporated by the parasite, and consistent with this observation, 5-fluoroorotate is a potent anti-malarial agent while 5-fluorouracil is not [22,23]. In the absence of salvage enzymes for the incorporation of preformed bases and nucleosides all of the *de novo* biosynthetic enzymes are presumably essential to *Plasmodium* species. However, in order to successfully target an enzyme for a drug discovery program, more than essentiality is required. It is also necessary that the protein target binds small drug-like molecules with high affinity (i.e. the target is “druggable) [32,33], that inhibitors of the target cause cell growth inhibition, and furthermore that selectivity can be achieved between the host and parasite. As summarized below, *Plasmodium* DHODH meets these criteria, providing a strong case for the current focus on this target for drug discovery efforts.

DHODH is a “druggable” target

Human mitochondrial-type DHODH is a clinically validated drug target based on the successful development and approval of leflunomide for the treatment of rheumatoid arthritis [34–36]. Leflunomide (**1**) is an isoxazole-based prodrug, which after administration is rapidly converted to the active metabolite A77 1726 (**2**) (Figure 2). A77 1726 is a potent inhibitor of mammalian DHODH [37–39] and the immunosuppressive activity of Leflunomide is mediated by pyrimidine depletion [40]. Resting lymphocytes can survive on pyrimidine salvage while activated lymphocytes require *de novo* pyrimidine biosynthesis to support their enhanced growth rate, providing a level of selective toxicity towards the target cell population. Other inhibitors of human DHODH with immunosuppressive activity have also been reported, including additional analogs of A77 1726 [41], redoxal [42], S-2678[43] and notably the cinchoninic acid derivative brequinar (**3**) [44–48]. Brequinar was evaluated in clinical trials as a potential anti-cancer agent, however it was never approved for clinical use.

Evolutionary history of DHODH

DHODH belongs to a large super family in the β/α–barrel structural class [30,49]. In different species DHODH localizes to either the cytoplasm (Class 1) or to the inner mitochondrial membrane (or plasma membrane for bacteria) with the catalytic domain oriented towards the inner membrane space (Class 2). The Class 2 enzymes are bound to the membrane through a short membrane spanning sequence and they have been shown to display detergent-dependent kinetics [50]. Most eukaryotes and gram-negative bacteria encode Class 2 enzymes, however the two classes are distributed unevenly across the evolutionary tree, and no simple generalizations can be made. Both human and Plasmodium contain Class 2 mitochondrial enzymes, thus providing the first indication that malarial DHODH was a “druggable” target.

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In contrast, the kinetoplastids [51] and some yeast have Class 1 fumartate-dependent enzymes, for which there is no supporting druggability data.

DHODH catalyzes the flavin mononucleotide (FMN)-dependent oxidation of dihydroorotate to produce orotic acid. Two separate half reactions are required to complete the catalytic cycle: 1) oxidation of dihydroorotate driven by the reduction of FMN and, 2) reoxidation of $FMNH_2$ to regenerate the active enzyme. The reoxidation of FMN is accomplished by one of several potential cofactors depending on the species-specific cellular localization of the enzyme. The Class 1 cytoplasmic enzymes use fumarate or NAD^+ and the mitochondrial enzymes require oxidized ubiquinone (CoQ), thus coupling pyrimidine biosynthesis to the respiratory chain [20,52,53]. The Class 1 enzymes are further classified into Family 1A (fumarate-dependent) and Family 1B (NAD-dependent). For the Family 1A enzymes, fumarate is believed to share the same binding-site as orotate (Type 1A)[54,55]. In contrast the Family 1B enzymes are composed of proteins from two gene products. Electrons are transferred from FMN bound in the DHODH-homologous subunit to an [1Fe-2S] iron-sulfur cluster followed by FAD and NAD, all three of which are bound on a second subunit with similarity to ferredoxin reductase [56,57]. In the Class 2 enzymes CoQ is thought to bind on the opposite face of FMN from orotate. This hypothesis is based on the available structural information, and the observation of ping-pong reaction kinetics [49,58–61]. The kinetic and structural characterization of the family 2 enzymes has been performed with the truncated soluble domain lacking the mitochondrial membrane anchor and the sequence N-terminal to this region. The recombinant protein for the Plasmodium and human enzymes has produced in *E. coli* (e.g. *Pf*DHODH [62]).

Structural analysis of DHODH

Clardy solved the X-ray structures of human DHODH bound to A77 1726 and brequinar providing the first insight into the nature of the inhibitor binding-site in the Class 2 enzymes [49]. Subsequently a number of additional X-ray structures of the human enzyme bound to various inhibitors have been reported (e.g. [63–65]), and the X-ray structure of *Pf*DHODH has been solved bound to both A77 1726 [59] and to novel malarial inhibitors from the triazolopyrimidine series [66], the latter of which will be discussed in detail below. The central structural element of DHODH from all class types is the core β/α-barrel domain. This domain houses the binding site for the FMN cofactor, which is bound near strand β13 at the top of helix α11 (Figure 3). Orotate forms a stacking interaction with FMN on one side, while the opposite side of the orotate binding-site is formed by β11 and surface loops containing Ser-395 and Thr-459. In addition the class 2 enzymes have a largely hydrophobic N-terminal helical domain (α1 and α2) that presumably sits adjacent to the membrane. The inhibitor binding-site, as illustrated for A77 1726, is formed between the N-terminal helices and the β/α-barrel domain, making interactions with helix α3, α11 and strand β5.

Species selective inhibition of DHODH is feasible

Inhibitors of DHODH were recognized early on to show species selective inhibition between even closely related species. Knecht and Loffler found strong species differences in IC_{50} values for both A77 1726 and brequinar between rat and human DHODH [67], and inhibitors of human DHODH were shown to be poor inhibitors of *E. coli* DHODH [68]. Capitalizing on these observations, Copeland used HTS approaches to identify two series of DHODH inhibitors that were selective for the bacterial enzymes: the thiadiazolidinediones, which inhibit *E. coli* DHODH [69], and pyrazoles that have selective activity on the enzyme from *Helicobacter pylori* [70,71]. Comparison of the X-ray structures of the unliganded *E. coli* Class 2 DHODH [61] with the human enzyme bound to A77 1726 or brequinar [49] shows that the amino acid residues in the inhibitor binding-site are not conserved between the two enzymes, explaining

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the species-selective binding of these inhibitors. Our group showed that species-selective inhibitor binding extended to the *P. falciparum* enzyme. Potent inhibitors of human DHODH were found to be poor inhibitors of *Pf*DHODH [62]. The subsequent X-ray structure of *Pf*DHODH by Clardy's group showed that A77 1726 is bound in the same binding pocket in *Pf*DHODH and the human enzyme, but the amino acid residues that contact the ligand are not conserved between the species [59] (Figures 3 and 4).

Genetic evidence that *Pf*DHODH is an essential enzyme

The differences in localization and cofactor specificity between DHODH from divergent species were recently exploited by the Vaidya group to provide important insight into the tight link between the mitochondrial electron transport chain and pyrimidine biosynthesis in the malaria parasite [19,20]. A transgenic *P. falciparum* cell line was constructed that expressed yeast fumarate-dependent DHODH in the parasite cytoplasm, thus bypassing the need for the mitochondrial enzyme (Figure 1). This cell line was resistant to atovoquone, suggesting that at least for *in vitro* growth, the main function of the bc_1 complex is to provide oxidized CoQ for the generation of orotic acid by DHODH. These studies provided an elegant genetic confirmation that DHODH is an essential enzyme in *P. falciparum*.

Summary of the case for PfDHODH as a target for drug discovery efforts. P. faliciparum

DHODH catalyzes an essential reaction and no functional bypass exists in blood stage parasites to overcome the loss of this activity. Based on homology with the human enzyme, malaria DHODH is a "druggable" target, and the lack of conservation in the inhibitor binding-site across species suggests that the identification of species-selective inhibitors was feasible. Furthermore while the lack of pyrimidine salvage pathways in *Plasmodium* makes it vulnerable to inhibition of this pathway, the presence of salvage pathways in human cells affords additional selectivity. In order to exploit *Pf*DHODH for the development of new anti-malarials, high-throughput screening (HTS), medicinal chemistry on human selective DHODH inhibitors, and structure-based methods have been used to identify species selective inhibitors of *Pf*DHODH. The most successful of these approaches has been HTS.

Novel and selective inhibitors of P. falciparum DHODH discovered by HTS

Our group published the first reported HTS against *P. falciparum* DHODH [72]. An end point dye-based assay was adapted to 384 well plate format and was used to screen a 220,000 compound library for inhibitors of *Pf*DHODH. This assay couples the reoxidation of CoQ with the reduction of dichloroindophenol (DCIP), which can be monitored at 600 nm. The library was screened using a single concentration point of 3 μM and 1249 hits (defined by >60% inhibition) were identified, representing a hit rate of 0.6%. A preliminary dose response against *Pf*DHODH was determined and the initial hits were counter screened against human DHODH at the same concentrations. The identified hits were almost uniformly selective for the *Plasmodium* enzyme. We choose compounds with an IC_{50}<0.6 μM for further analysis and our published hits are described below.

Phenylbenzamides, ureas and naphthamides

The most common chemical classes identified by this screen were phenylbenzamides **4**, ureas **5** and naphthamides **6** [72] (Figure 5 and Table 1). These compounds inhibit *Pf*DHODH with an IC_{50} between 0.02 – 0.8 μM and show selectivity versus human DHODH ranging from 70–12,500-fold. The most potent compounds were also the most selective (e.g. compound **4**). Despite the potent enzyme activity, these inhibitors have no anti-malarial activity and we chose not to advance the compounds in these series.

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Triazolopyrimidine series

Our screen also identified a potent and selective triazolopyrimidine-based compound DSM1 (**7**), which has similarly potent activity against *P. falciparum* 3D7 in whole cell assays while displaying stringent species selectivity (IC_{50} *Pf*DHODH= 0.047 μM; IC_{50} *h*DHODH > 200 μM; EC_{50} *Pf* 3D7 cells = 0.079 μM [73]) (Table 1 and Figure 5). This compound series was chosen for progression and it has formed the basis of an ongoing lead optimization program by our group. A simple and inexpensive 3-step synthetic route was developed that allowed us to synthesize a number of analogs of DSM1 from commercially available amines to replace the naphthyl portion of the molecule [66,73,74] (Table 2). These studies showed that 1) the unsubstituted bridging nitrogen N1 was essential for activity, 2) introduction of heteratoms into the naphthyl ring reduced potency, 3) the naphthyl could be replaced by larger aromatic and hydrophobic ring systems including anthracene and substituted phenyl rings, 4) phenyl rings at the naphthyl position required substitution at the para position (CF_3 >Br>Cl>Me>F), with ortho substitution leading to complete loss of activity, and 5) replacement of C6 with ethyl or CF_3 reduced potency, as did addition of a Me to C2. A good correlation was observed between enzyme activity and whole cell potency throughout the series providing strong evidence that DHODH is the cellular target of these inhibitors.

The X-ray structures of *Pf*DHODH bound to three inhibitors from the triazolopyrimdine-based series DSM1(**7**), DSM2 (**13**) and DSM74 (**22**) were solved by our group, providing the first structures of *Pf*DHODH bound to a parasite selective, potent inhibitor (Figure 3)[66]. The triazolopyrimidine series inhibitor binding-site is composed of two sites: 1) a completely hydrophobic site, which binds the naphthyl, anthracenyl or phenyl-trifluoromethyl, and 2) the triazolopyrimidine pocket that contains the only residues capable of H-bond interactions. Both sites provide a significant amount of binding energy to the inhibitor series; π-stacking interactions are observed between the naphthyl and two Phe residues (227 and 188), and H-bonds between H185 and R265 and the triazolopyrimidine ring are present in all three structures. The triazolopyrimidine functionality overlaps the binding site of the β-hydroxy enamide portion of A77 1726 observed in the previously reported *Pf*DHODH structure [59]. In contrast, the naphthyl binding-site accesses a previously unidentified pocket created by a significant rotation of Phe188 that moves this residue into a position that overlaps the phenyl portion of A77 1726. These data provide a structural explanation as to how *Pf*DHODH is able to bind multiple chemical classes with high affinity.

The structural basis for the species selective binding of the triazolopyrimidines towards *Pf*DHODH was also evident from these structures (Figures 3 and 4). Some of the key amino acid differences that lead to species selectivity include, the replacement of *h*Ala-59 and *h*Pro-364 with *Pf*Phe-188 and *Pf*Met-536, which closes off the hydrophobic pocket that binds the biphenyl of brequinar in human DHODH, and the substitution of *h*Thr-63 and *h*Met-111 with *Pf*Gly-192 and *Pf*Leu-240, which creates the naphthyl pocket in *Pf*DHODH.

Most importantly, the X-ray structures of the triazolopyrimdine series provided us with significant insight into the emerging structure-activity relationships (SAR), and with a blueprint for further modification of this chemical series. Comparison of the ligand-bound DHODH structures to the small molecule crystal structures provided insight into the importance of the bridging nitrogen (N1). The small molecule structures showed that the intrinsic electronic configuration of the triazolopyrimidine ring favors charge delocalization from the bridging nitrogen (N1) onto the pyridine nitrogen (N5). Intrinsic dipoles in the ring promote formation of energetically favorable H-bond interactions with the protein between His185 and N1, and between Arg265 and N5. Triazolopyrimidine analogs with S or O replacing N1 lack this intrinsic dipole and are inactive as inhibitors, while mutation of His185 or Arg265 to Ala also reduced inhibitor binding, supporting the importance of these interactions. Heteroatom substitution into the naphthyl ring reduced activity, consistent with the completely

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hydrophobic nature of the naphthyl binding-pocket on the enzyme. Ortho-substituted phenyl rings are inactive and our analysis of the X-ray structure shows that the contacts made between the protein and the ortho positions on the aromatic ring are too close to allow substitution at this position. Finally a narrow channel leading from C5 on the inhibitor towards the FMN cofactor was observed, suggesting that this pocket might be exploited for future optimization of the lead series.

Substituted pyrrole and thiofuran analogs

A second high throughput screen to identify *Pf*DHODH inhibitors was reported by Clardy and Wirth at Harvard using a small molecule library of 208,000 compounds from Genzyme [75]. This screen was also run using the DCIP assay with a fixed inhibitor concentration of 10 μM. The hit rate in this screen was 0.3% and 55 compounds were identified with an IC_{50} below 1 μM on the enzyme. Five scaffolds inhibited *P. falciparum* 3D7 growth in whole cell assays at submicromolar concentrations (Figure 5 and Table 1). All 5 inhibitors were selectively active against the parasite enzyme over the human enzyme. The most potent compound **8** has an IC_{50} = 40 nM on *Pf*DHODH, and they generated a small number of analogs from this lead to establish preliminary SAR (Table 3). While none of the analogs showed greater potency than the original hit, the activity versus the enzyme and the parasite showed similar trends supporting *Pf*DHODH as the cellular target. The cellular target was definitively established by characterizing the DHODH inhibitors against transgenic parasites harboring the cytoplasmic yeast DHODH, as established by the Vaidya Lab [19]. Parasites transfected with yeast DHODH showed significant resistance to the *Pf*DHODH inhibitors, establishing the mechanism of action.

Medicinal chemistry to evolve human DHODH inhibitors towards PfDHODH selectivity

Structure-based methods and the synthesis of brequinar and A77 1726 analogs have also been used as strategies to identify inhibitors of *Pf*DHODH by a group at the University of Leeds. The group initially explored a series of brequinar derivatives. Brequinar is an exceptionally poor inhibitor of *Pf*DHODH (IC_{50} > 500 μM), and the best analog identified in the study only had mid micromolar range activity on *Pf*DHODH (IC_{50} = 40 μM), and furthermore showed no selectivity versus the human enzyme [76]. Their attempts to generate potent and selective *Pf*DHODH inhibitors from the A77 1726 scaffold were also unsuccessful. They synthesized a series of 26 analogs using the *Pf*DHODH A77 1726 co-crystal structure to aid in analog design. The best inhibitor had micromolar activity against *Pf*DHODH (IC_{50} = 4 μM), and these compounds showed in almost all cases far better activity against human DHODH [63]. This group also used the molecular design program SPROUT with the *Pf*DHODH structure bound to A77 1726 to identify potential inhibitory scaffolds of *Pf*DHODH [77]. Based on this analysis a series of 6 amide derivatives of anthranilic acid were prepared, with none showing better than mid micromolar activity against *Pf*DHODH. Finally this group utilized a previously described tetracyclic amine that had been shown to have immunosuppressive activity similar to brequinar and A77 1726 as a starting point to generate analogs with potential activity against *Pf*DHODH [78]. Tricyclic amine derivatives of this compound were shown to have submicromolar activity on *Pf*DHODH (IC_{50} = 0.2 – 0.4 μM) and these inhibitors do show selectivity for the P. *falciparum* enzyme (200 –1200-fold). The most potent of these **12** (Figure 5 and Table 1) has micromolar activity against *P. falciparum* 3D7 (EC_{50} = 2 μM).

The mechanism of *Pf*DHODH inhibition

The location of the CoQ binding site on DHODH is presently a point of speculation. Competitive inhibition kinetics for some human DHODH inhibitors, including brequinar [79], coupled with the structural data showing that the binding-site of A77 1726 and brequinar is adjacent to FMN, led to the suggestion that the CoQ binding-site overlaps the inhibitor

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binding-site [49]. However, there is no crystallographic data to demonstrate where CoQ binds. Insight into the relationship between the inhibitor binding-site and the CoQ binding-site was obtained by steady-state and pre-steady state kinetic analysis of the *Pf*DHODH inhibitor binding-site mutant enzymes [80]. Our studies suggested that CoQ and inhibitors (e.g. A77 1726, DSM1, **4**) do not share an overlapping binding site. A series of mutant enzymes replacing residues in the inhibitor binding-pocket with Ala were evaluated, including mutants of H185, R265, F188 and F227. These mutations decreased inhibitor affinity, consistent with their contribution to the energetics of inhibitor binding. In contrast, steady-state analysis showed that the K_m^{app} for CoQ was not significantly affected by these mutations. Secondly, the pre-steady-state kinetic analysis showed that the mutations reduced the rate of the CoQ-dependent flavin oxidation step but they did not significantly alter the K_d^{ox} for CoQ (the dissociation binding constant for CoQ in the oxidation step of the reaction). The rate of the DHO-dependent reductive half-reaction was also unchanged. DSM1 and **4** also blocked the CoQ-dependent oxidative half-reaction but not the DHO-dependent step. Thus the mutations affect inhibitor binding, and both the inhibitors and the mutations affect the electron transfer between CoQ and FMN, but mutation of this binding site was not coupled to changes in the apparent CoQ affinity. This discrepancy suggests that CoQ and the inhibitors do not bind the same site. Based on these data we proposed two models that explain the inhibition kinetics: 1) the inhibitors block electron transfer between flavin and CoQ or, 2) they act by stabilizing a conformation that excludes CoQ binding. X-ray structure analysis of DHODH bound to CoQ would provide substantial clarification of the mechanism of DHODH inhibition by all inhibitors that bind this site. Clardy and Wirth evaluated this same set of mutant enzymes and found that these mutations also reduced the binding affinity of the inhibitors identified in their screen [75]. This result establishes that all classes of inhibitors identified to date by HTS against *Pf*DHODH share an overlapping binding site.

Identification of PfDHODH inhibitors with in vivo anti-malarial activity

The identification of potent and species selective *Pf*DHODH inhibitors that are able to inhibit parasite growth in whole cell assays has provided several validated hits that can form the basis of lead optimization programs with the goal of identifying a clinical candidate. Of the identified series, our group has progressed the triazolopyrimidines to the early lead stage by the application of an iterative lead optimization strategy [74]. The design of new analogs with better *in vivo* properties is being informed by *in vitro* metabolism and pharmacokinetic analysis in mice, in addition to the evaluation of inhibitor potency.

The original HTS hit DSM1, while showing potent activity against *P. falciparum* in whole cell assays, has no activity against *P. berghei* in the standard mouse model. Reasonable plasma levels were achieved after a single oral dose, however we found that multiple doses led to decreased plasma levels, suggesting that DSM1 maybe a metabolic inducer. This result explained the lack of *in vivo* activity. Additionally potency differences between *P. falciparum* and *P. berghei* DHODH contributed to the lack of efficacy for some inhibitors in the series. A series of substituted phenyl replacements for the naphthyl moiety were synthesized and analyzed in human liver microsomes to evaluate metabolic stability (Table 2). From these studies we identified DSM74 (**22**)(Table 2 and Figure 6). This compound shows prolonged plasma exposure after multiple oral doses, and it significantly suppressed parasetimia in a 4-day mouse efficacy test using the *P. berghei* model (95% and 71% suppression on day five for b.i.d. and q.d. dosing respectively). DSM74 was well tolerated and no signs of overt toxicity were observed. These data provide the first proof of concept that *Pf*DHODH inhibitors can function *in vivo* to affect parasetimia, and they provide strong validation of *Pf*DHODH as a target for new-antimalarial drug discovery.

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Progress targeting other enzymes in the de novo pyrimidine biosynthetic pathway for drug discovery

In addition to efforts to target *Pf*DHODH some in roads have also been made towards characterizing other enzymes in the pathway for their potential to lead to new chemotherapy. Carbonic anhydrase, which is needed to generate HCO_3^- for the first step in pyrimidine biosynthesis has been expressed and purified as the recombinant enzyme. Benzenesulfonamide derivatives with low micromolar activity on the enzyme (K_i = 0.18 μM) and some activity on *P. falciparum* in whole cell assays (EC_{50} = 1 μM) and against *P. berghei in vivo* have been reported [81]. Dihydroorotase, the third enzyme in the de novo pathway, has been expressed as the recombinant protein in *E. coli*, and characterization of the enzyme shows it is a monofunctional protein differing from the mammalian enzyme, which is part of a multisubunit complex [82]. The most studied enzyme after *Pf*DHODH is orotidine 5'phosphate decarboxylase (OMPDC), which catalyzes the last step in the pathway forming UMP. Both the structure and mechanism of OMPDC have been the subject of intense research because of the difficult chemistry and the high catalytic proficiency of the enzyme [83]. The Plasmodium enzyme has been shown to form a complex with orotate phosphoribosyltransferase, the penultimate step in UMP biosynthesis [84]. Enzymatic properties of both enzymes are affected by formation of the heterotetramer. Several X-ray structures of *P. falciparum* ODCase have been reported, including the unliganded enzyme [85] and the enzyme bound to several mononucleotide derivatives that are covalent inhibitors of the enzyme [86]. For orotate phosphoribosyltransferase, kinetic isotope effects and computational methods have been used to predict the transition state structures for both the Plasmodium and human enzyme [87]. This is a powerful method that can provide important insight into the design of tight binding inhibitors.

Conclusion

Pyrimidine biosynthesis is a highly privileged pathway for drug discovery against *Plasmodium* parasites based on the high proportion of known anti-malarials targeting this pathway. The reliance of the parasite on *de novo* pyrimidine biosynthesis makes it highly vulnerable to inhibitors targeting the pathway. DHODH catalyzes the fourth step in *de novo* pyrimidine biosynthesis and both genetic and chemical studies have provided conclusive evidence that it is an essential enzyme and a druggable target in malaria. HTS methodology has proved a successful strategy to identify potent and highly species selective inhibitors of *Pf*DHODH. X-ray structures of *Pf*DHODH bound to inhibitors have provided insight into the structural basis for species selectivity and are a valuable asset for continued efforts to optimize the identified leads. The triazolopyrimidine series has been progressed from the hit stage into early lead optimization through an iterative lead optimization strategy that allowed us to identify analogs with good metabolic stability, and which have demonstrated *in vivo* efficacy. We conclude that *Pf*DHODH is a very promising target for the identification of novel anti-malarial agents, and if the current discovery efforts are successful work on this target will allow chemophores that are entirely different from current drugs to enter the clinics. The tools are now in place, and lead optimization efforts are underway to progress the identified series, several of which are within the MMV portfolio (www.mmv.org). It now remains for compounds with the necessary combination of potency and metabolic stability to be identified so that at least one series can be progressed to a clinical candidate. Final validation of the target awaits the discovery of a clinically useful drug.

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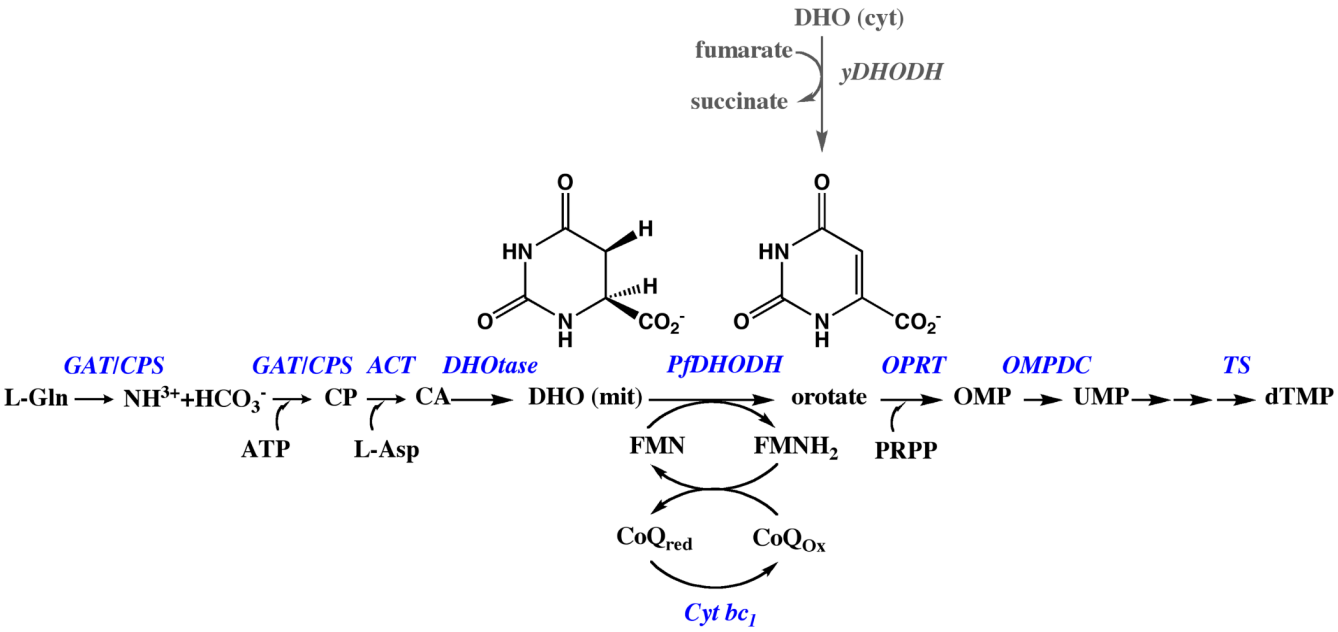

Fig. 1.

De novo pyrimidine biosynthetic pathway in Plasmodium. The pathway involves six enzymes: a bifunctional glutamine amidotransferase (GAT) and carbamoyl-phosphate synthetase (CPS), aspartate carbamoyltransferase (ACT), dihydroorotase (DHOtase), dihydroorotate dehydrogenase (DHODH), orotate phosphoribosyltransferase (OPRT) and orotidine-5'-monophosphate decarboxylase (OMPDC). L-Gln, L-glutamine; CP, carbamoyl phosphate; Asp, L-aspartic acid; CA, carbamoyl aspartate; DHO, dihydroorotate; OA, orotic acid; OMP, orotidylate; UMP, uridylate. Enzyme names are displayed in blue. The rescue pathway used to bypass mitochondrial *Pf*DHODH in transgenic parasites harboring yeast DHODH is displayed in grey.

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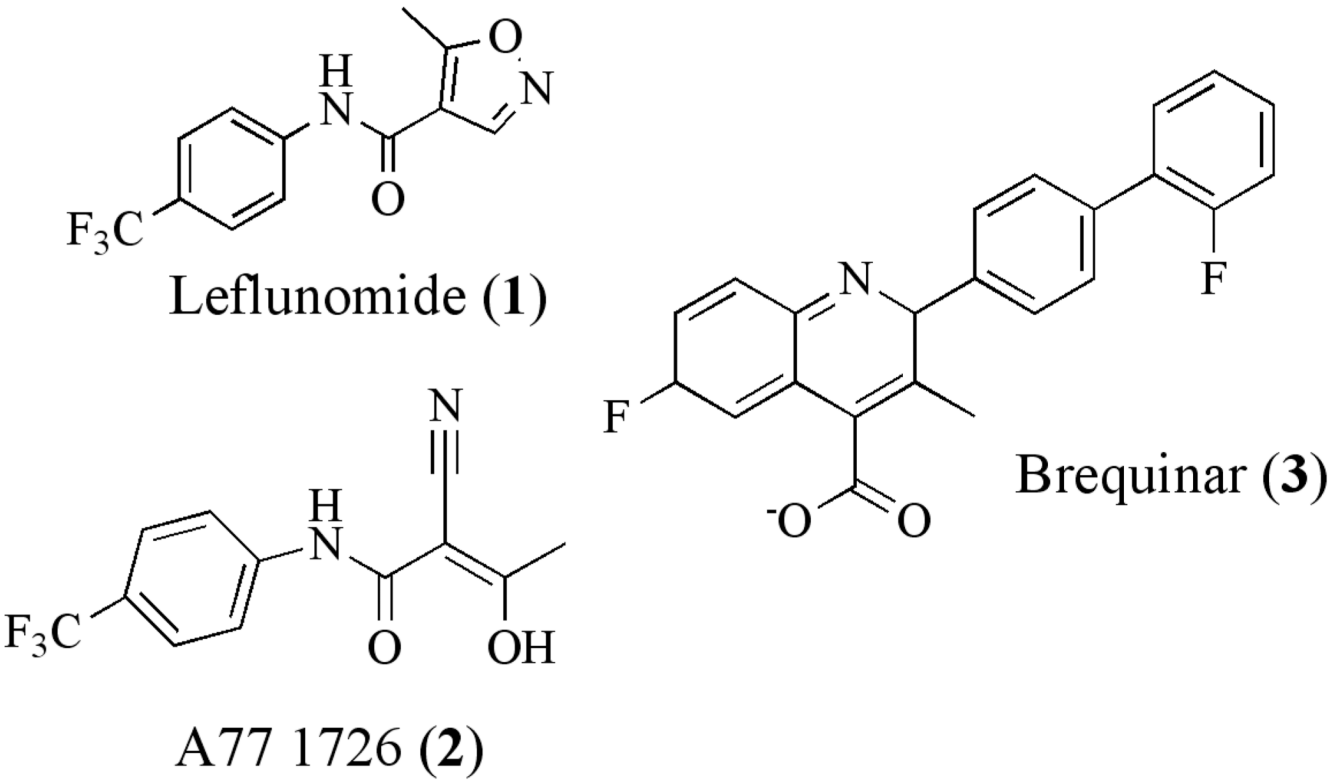

Fig. 2.
Inhibitors of human DHODH

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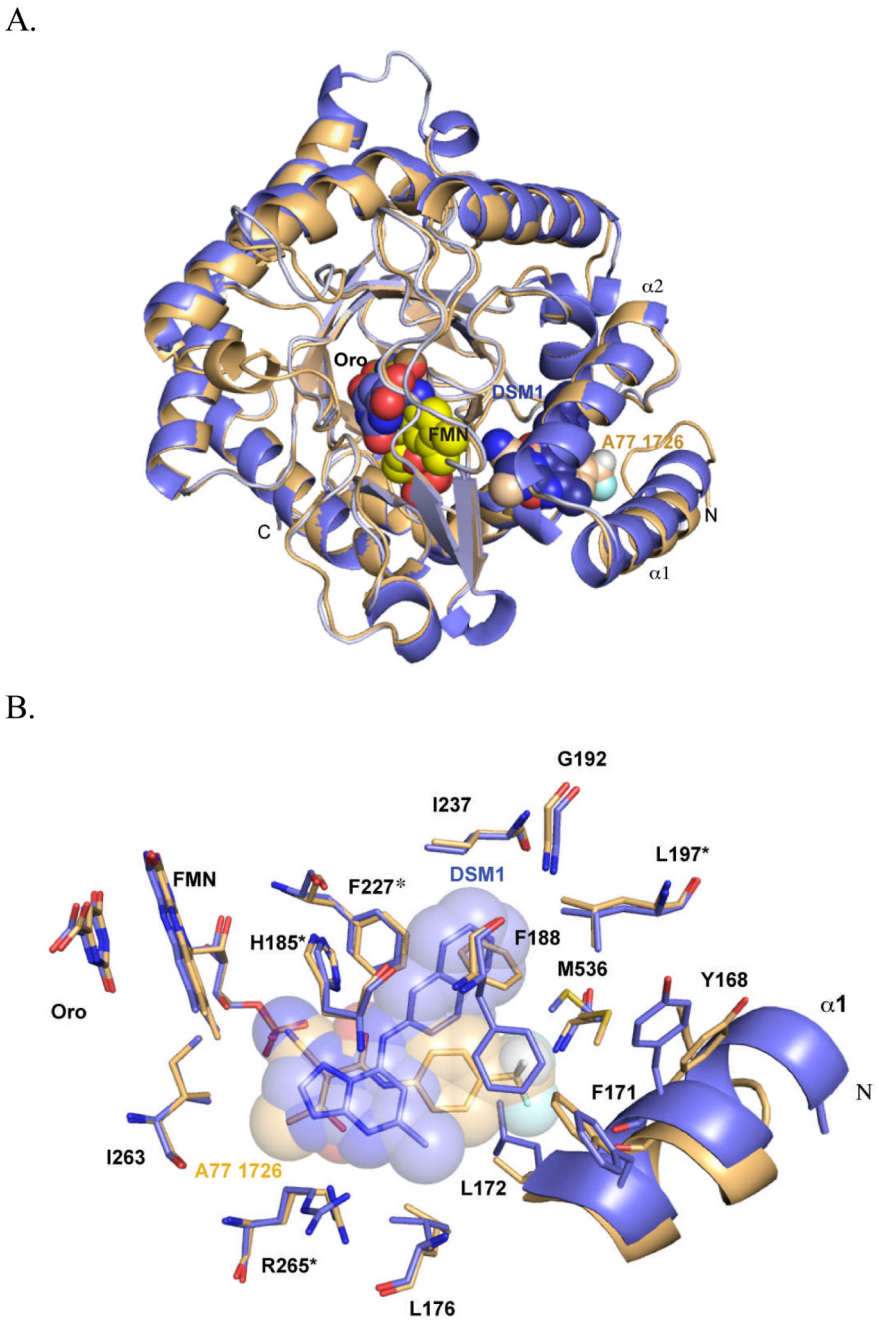

Fig. 3.

X-ray structure of *Pf*DHODH. A. Ribbon diagram of an alignment of the structures bound to A77 1726 (tan; pdb 1TV5) and DSM1 (purple; pdb 3I65). B. A77 1726 and DSM1 inhibitor binding sites. Residues marked with * are conserved in human DHODH, while the remainder of the shown residues are variable between *Plasmodium* and human DHODH. Structures were displayed in PyMol (Delano Scientific)

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```
                 160       170       180       190       200       210
Pfalciparum  ESYNPEFFLYDIFLKFCLKYIDGEICHDLFLLLGKYNILPYDTSNDSIYACTNIKHLDFI 218
Pberghei     ESYNPEFFMYDVFLDFCLNYVDSEVCHDLFLLLGKYGLLPYDTSNDSVYATSDIKNLNFI 185
Human        MATGDERFYAEHLMPTLQGLLDPESAHRLAVRFTSLGLLPRARFQDSDMLEVRVLGHKFR 89

                 220       230       240       250       260       270
Pfalciparum  NPFGVAAGFDKNGVCIDSILKLGFSFIEIGTITPRGQTGNAKPRIFRDVESRSIINSCGF 278
Pberghei     NPFGVAAGFDKNGICIDSILKLGFSFIEIGTITPKPQKGNNKPRIFRDVENKSIINACGF 245
Human        NPVGIAAGFDKHGEAVDGLYKMGFGFVEIGSVTPKPQEGNPRPRVFRLPEDQAVINRYGF 149

                 280       290       300       310       320       330
Pfalciparum  NNMGCDKVTENLILFRKRQEEDKLLSKHIVGVSIGKNKDTVNIVDDLKYCINKIGRYADY 338
Pberghei     NNIGCDKVTENLINFRKKQEEDKLLSKHIVGVSIGKNKHTENIVDDLKYSIYKIARYADY 305
Human        NSHGLS-VVEHRLRARQQKQAKLTEDGLPLGVNLGKNKTSVDAAEDYAEGVRVLGPLADY 208

                 340       350       360       370       380       390
Pfalciparum  IAINVSSPNTPGLRDNQEAGKLKNIILSVKEEIDNLEKNNIMNDESTYNEDNKIVEKKNN 398
Pberghei     IAINVSSPNTPGLRDNQESNKLKNIILFVKQEINKIEQIGHNGET--------------- 350
Human        LVVNVSSPNTAGLRSLQGKAELRRLLTKVLQERDGLRR--------------------- 246

                 400       410       420       430       440       450
Pfalciparum  FNKNNSHMMKDAKDNFLWFNTTKKKPLVFVKLAPDLNQEQKKEIADVLLETNIDGMIISN 458
Pberghei     ----------------FWMNTIKKKPLVFVKLAPDLENSEKKKIAQVLLDTGIDGMIISN 394
Human        ----------------------VHRPAVLVKIAPDLTSQDKEDIASVVKELGIDGLIVTN 284

                 460         470       480       490       500       510
Pfalciparum  TTTQIND--IKSFENKKGGVSGAKLKDISTKFICEMYNYTNKQIPIIASGGIFSGLDALE 516
Pberghei     TTINKMD--IKSFEDKKGGVSGKKLKDLSTNLISDMYIYTNKQIPIIASGGILTGADALE 452
Human        TTVSRPAGLQGALRSETGGLSGKPLRDLSTQTIREMYALTQGRVPIIGVGGVSSGQDALE 344

                   520       530       540       550       560
Pfalciparum  KIEAGASVCQLYSCLVFNGMKSAVQIKRELNHLLYQRGYYNLKEAIGRKHSKS-----   569
Pberghei     KIEAGASVCQLYSCLVFNGVKSAIQIKREFNNALYQKGYYNLREAIGKKHSNA-----   505
Human        KIRAGASLVQLYTALTFWGPPVVGKVKRELEALLKEQGFGGVTDAIGADHRR------   396
```

Fig. 4.

Amino acid sequence alignment of *Pf*DHODH, *Pb*DHODH and human DHODH. Secondary structure elements are indicated by color outline; blue, α-helix and green, β-sheet. Residues within 4Å of DSM1 in the inhibitor binding-site are highlighted by bold lettering.

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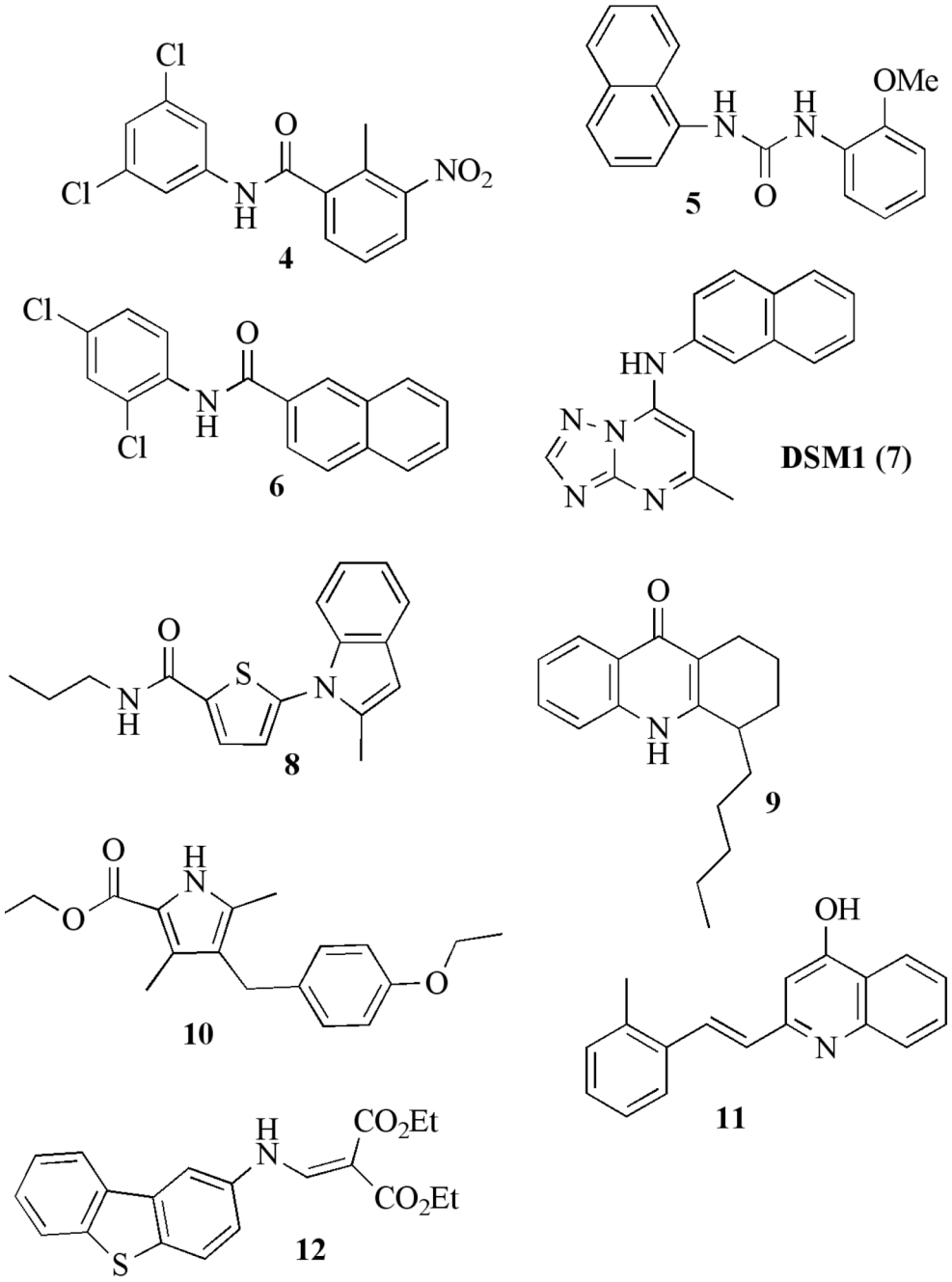

Fig. 5.
Species selective inhibitor chemophores of *P. falciparum* DHODH with submicromolar activity.

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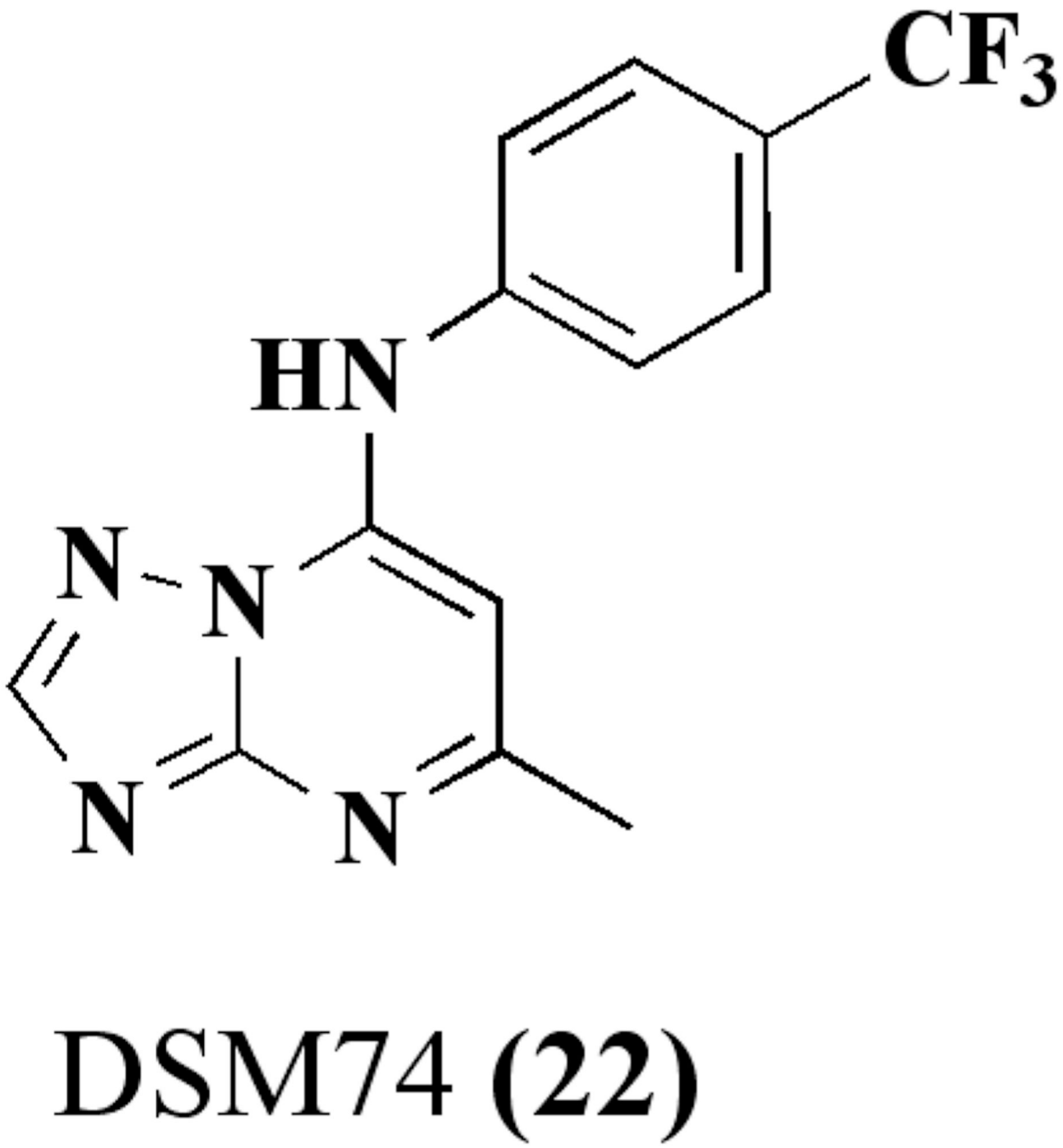

Fig. 6.
P. falciparum DHODH inhibitors with demonstrated *in vivo* activity.

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Table 1

Representative early hits derived from HTS and medicinal chemistry programs

compound	*Pf*DHODH (IC_{50}) μM	Human DHODH (IC_{50}) μM	Fold selectivity
4	0.016	200	12,500
5	0.23	110	500
6	0.05	>50	>1000
7	0.047	>200	>4000
8	0.042	>30	>700
9	0.34	>30	>90
10	0.083	>30	>360
11	0.93	>30	>30
12	0.16	30	>190

Compounds 4–11 were assayed at the following conditions: CoQ_D = 20 μM;DHO = 200 μM, DCIP = 100–120 μM, [DHODH] = 10 nM;**4–6**[72], **7** [73], 8–11 [75];compound **12**, conditions were instead: CoQ_D = 100 μM;DHO = 200 μM, DCIP = 60 μM, [DHODH] = 5 nM [78].

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Table 2

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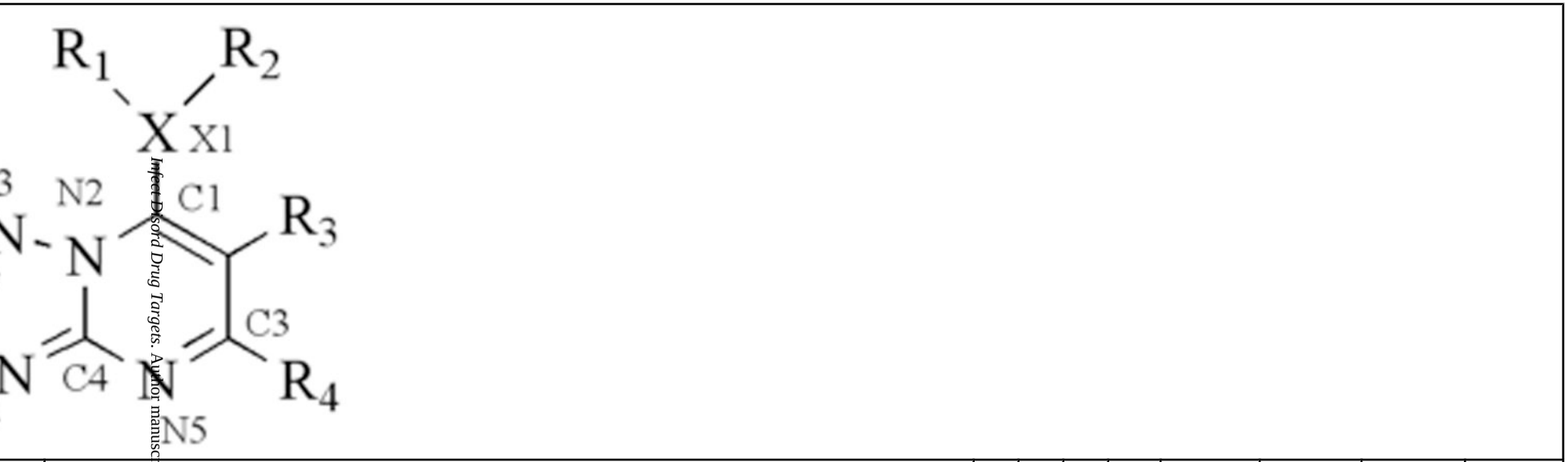
resentative triazolopyrimidine-based derivatives against DHODH and *P. falciparum*



R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	0.047	0.23	0.079	0.84

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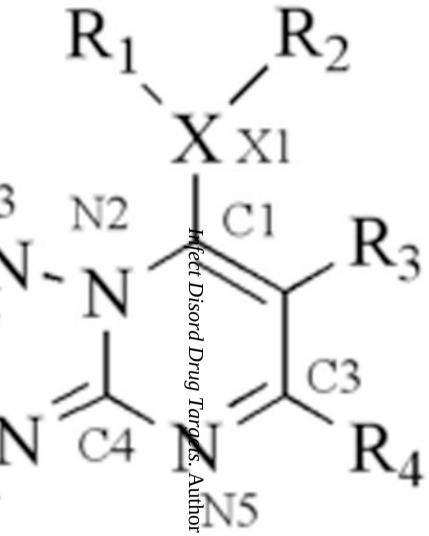
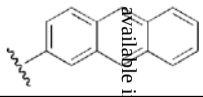


R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	0.056	3.7	0.19	0.7
	H	H	Me	N	>100	>10	>100	n.d.

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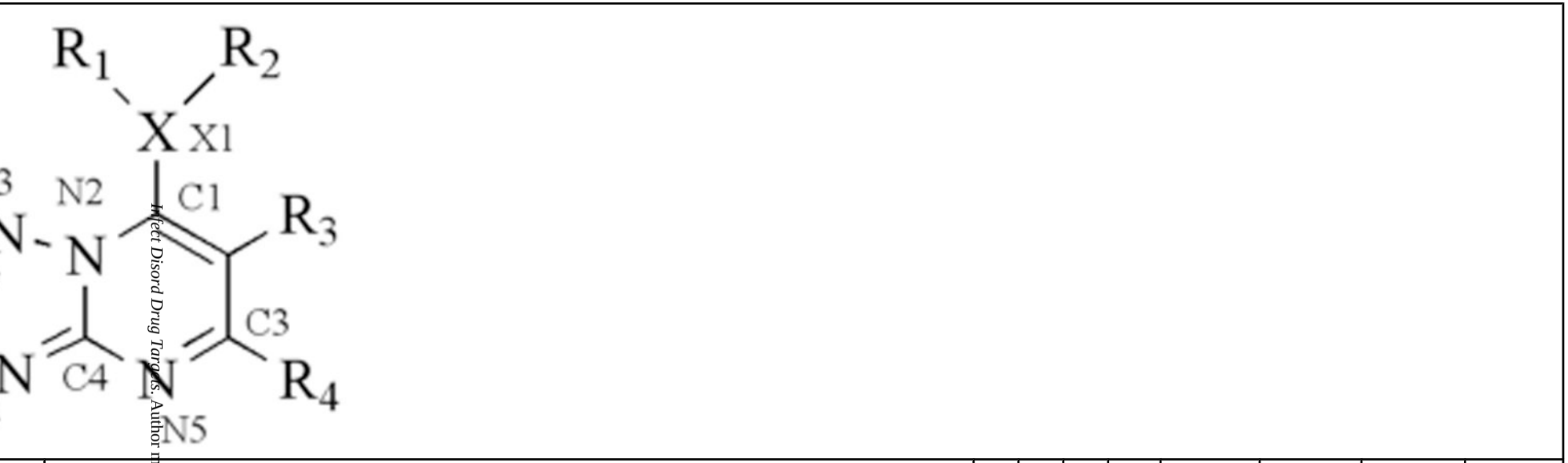

	R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
SM16		H	H	Me	S/ O	>100	n.d.	>100	n.d.

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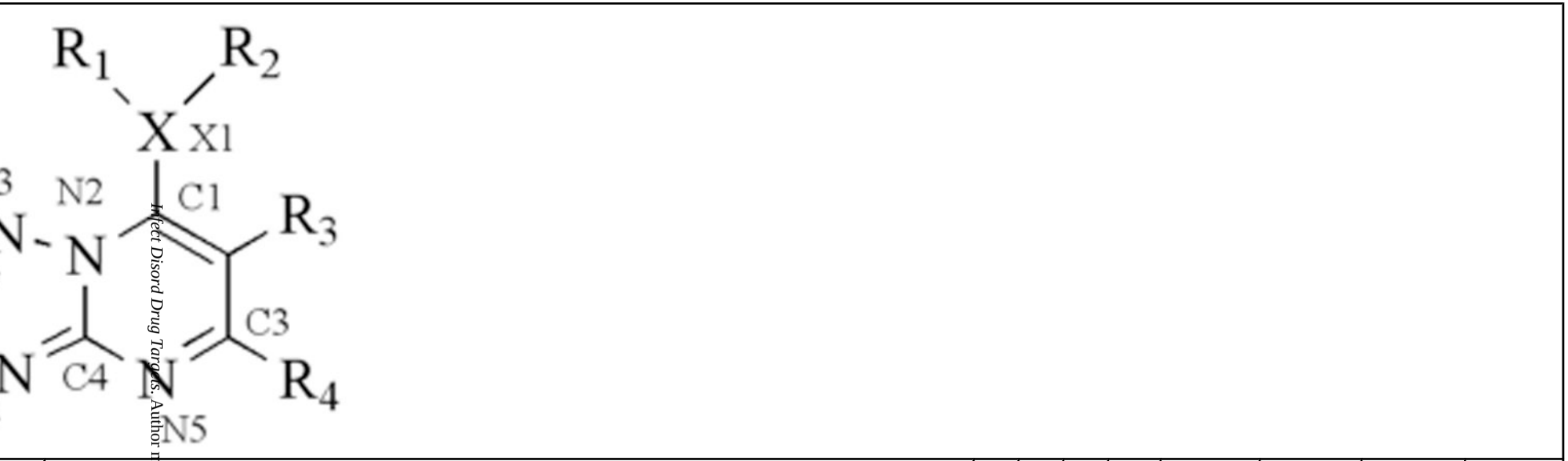
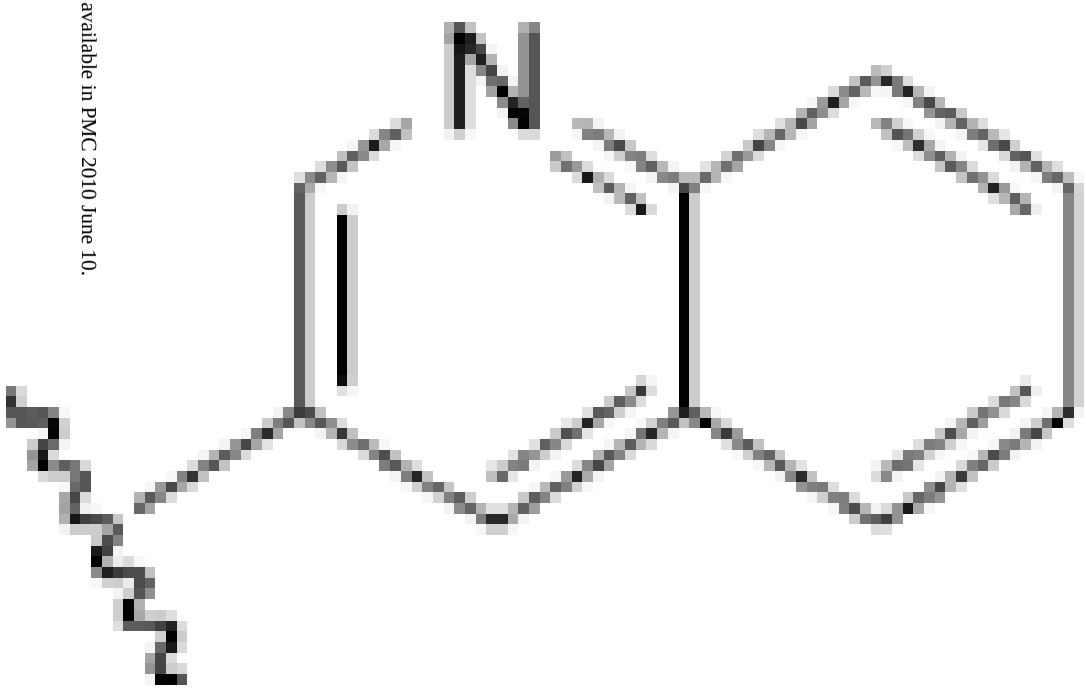


R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	1.7	n.d.	1.6	n.d.

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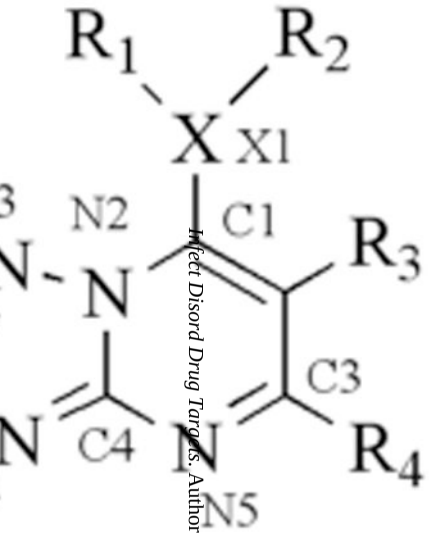
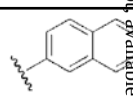
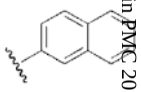


R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	Me	Me	N	0.16	n.d.	0.55	n.d.
	Me	H	Me	N	3.0	n.d.	16	n.d.

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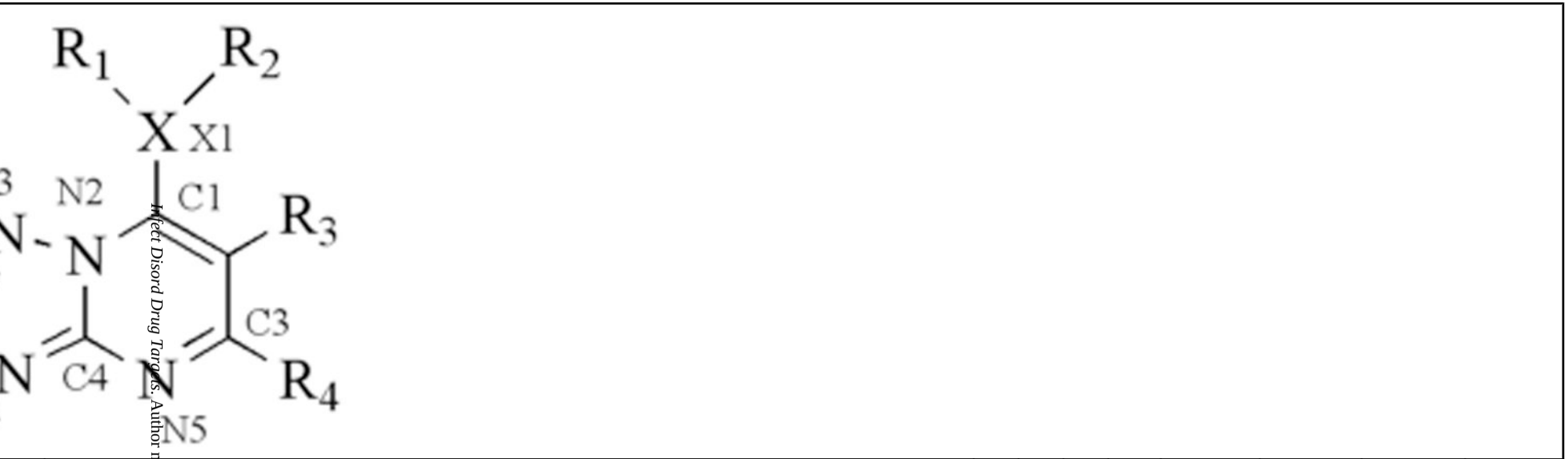

R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Et	N	0.19	n.d.	0.31	n.d.

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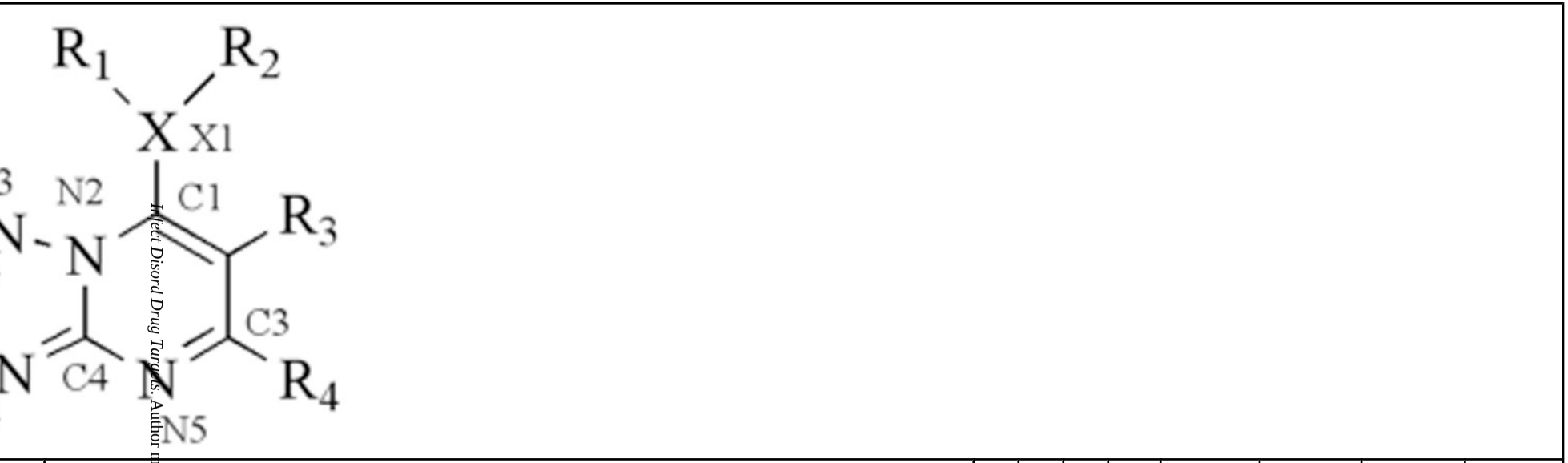
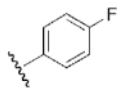
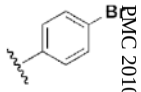
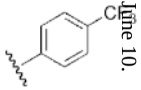
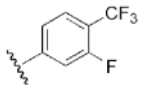
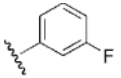


R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	19	>100	50	n.d.
	H	H	Me	N	0.78	1.3	4.2	n.d.
	H	H	Me	N	0.28	0.38	0.34	0.29
	H	H	Me	N	0.077	0.26	1.3	0.21
	H	H	Me	N	9.2	>100	34	0.5

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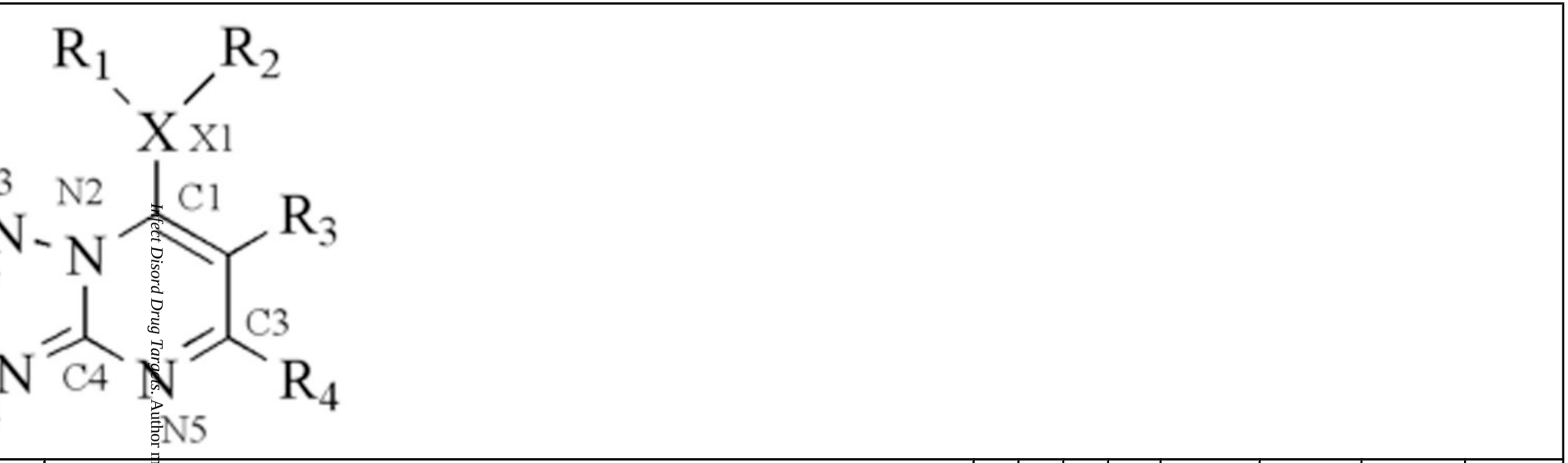
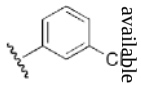


R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	1.4	31	8.5	0.68

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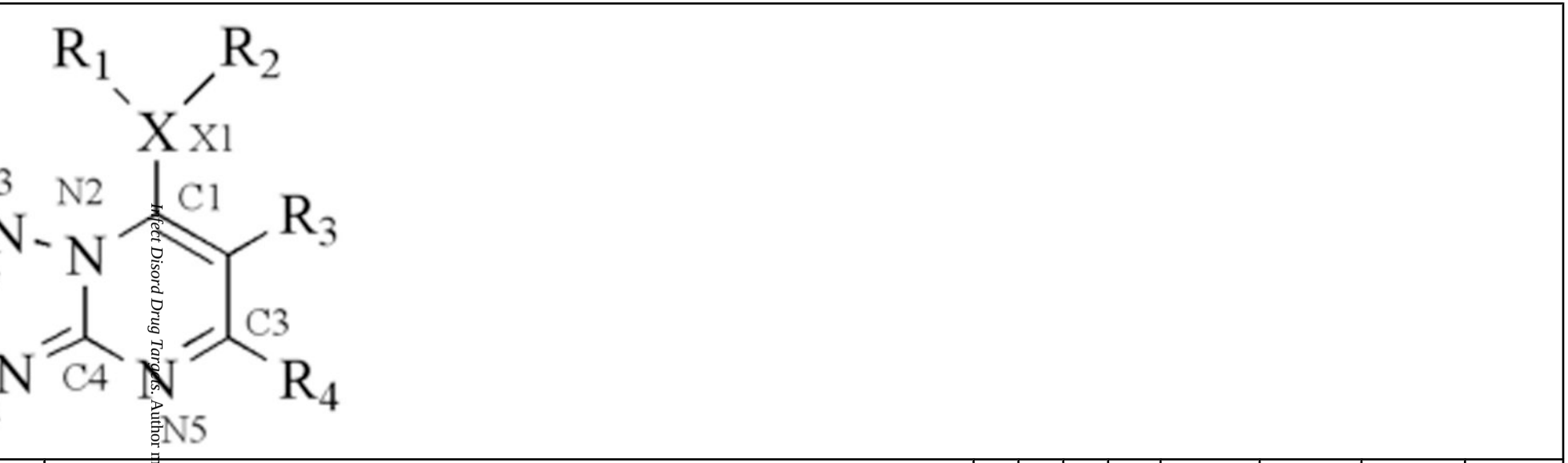

R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
	H	H	Me	N	>100	>100	>50	n.d.

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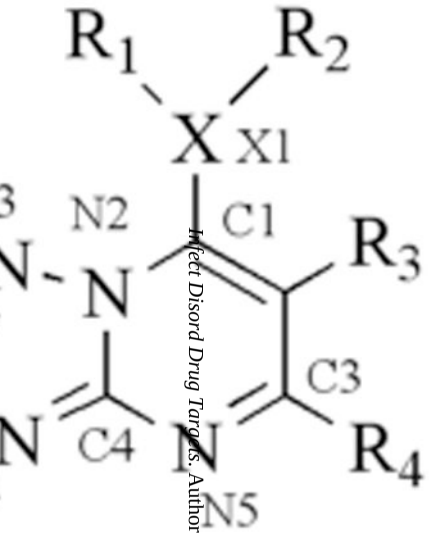

	R1	R2	R3	R4	X	IC_{50}(μM) *Pf*DHODH	IC_{50}(μM) *Pb*DHODH	EC_{50}(μM) *Pf* 3D7 cells	Pred E_h human microsome
		H	H	Me	N	>100	>100	>25	n.d.

$_{50}$ versus human DHODH >100 μM for all compounds. Data taken from [66,73,74]

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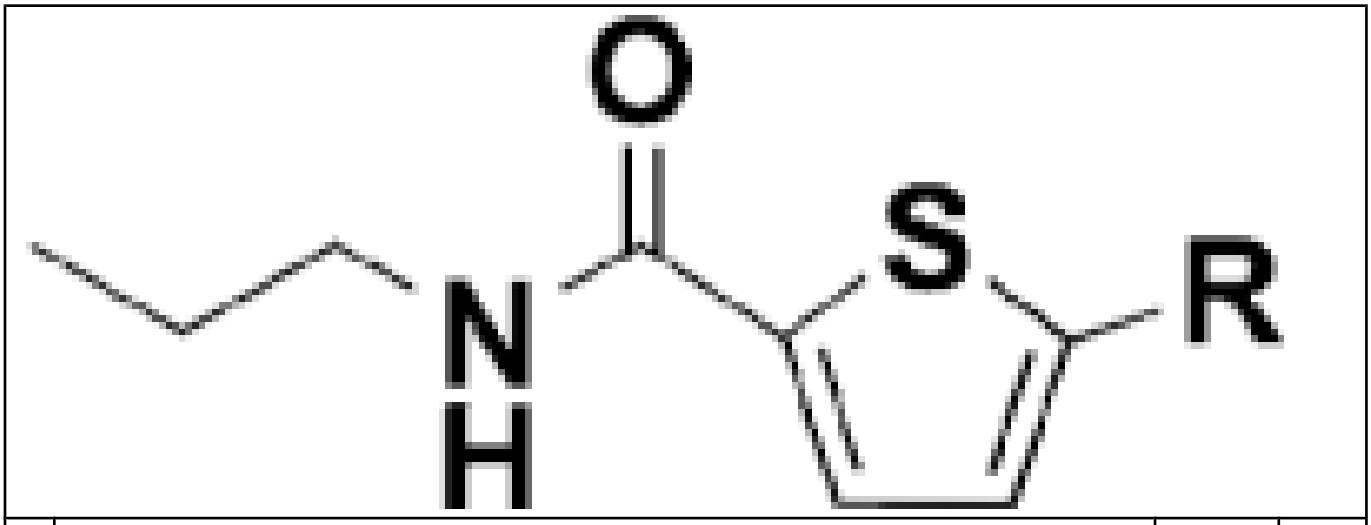
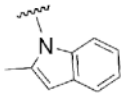
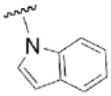
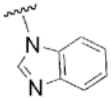
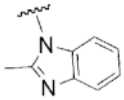
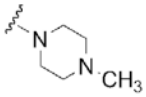
Table 3

Structure activity of 2,5 substituted thiofurans.



	Structures	*Pf*DHODH IC_{50} (μM)	*Pf* 3D7 cells EC_{50} (μM)
8		0.042	0.49
28		0.46	0.71
29		0.7	0.47
30		0.17	0.63
31		>30	>10
32		>30	>10

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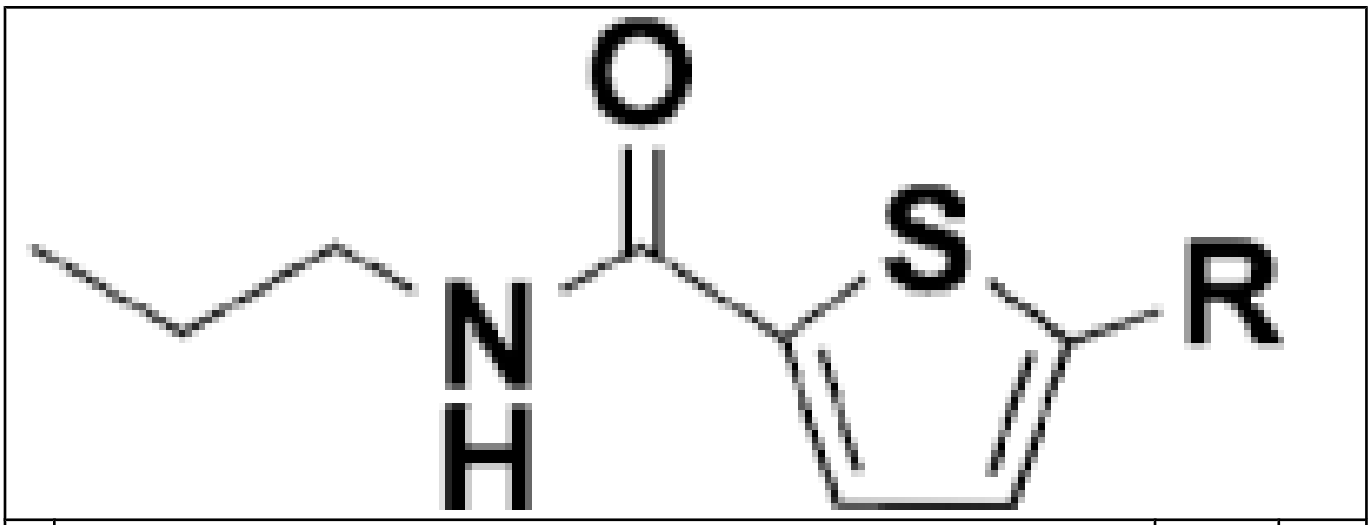
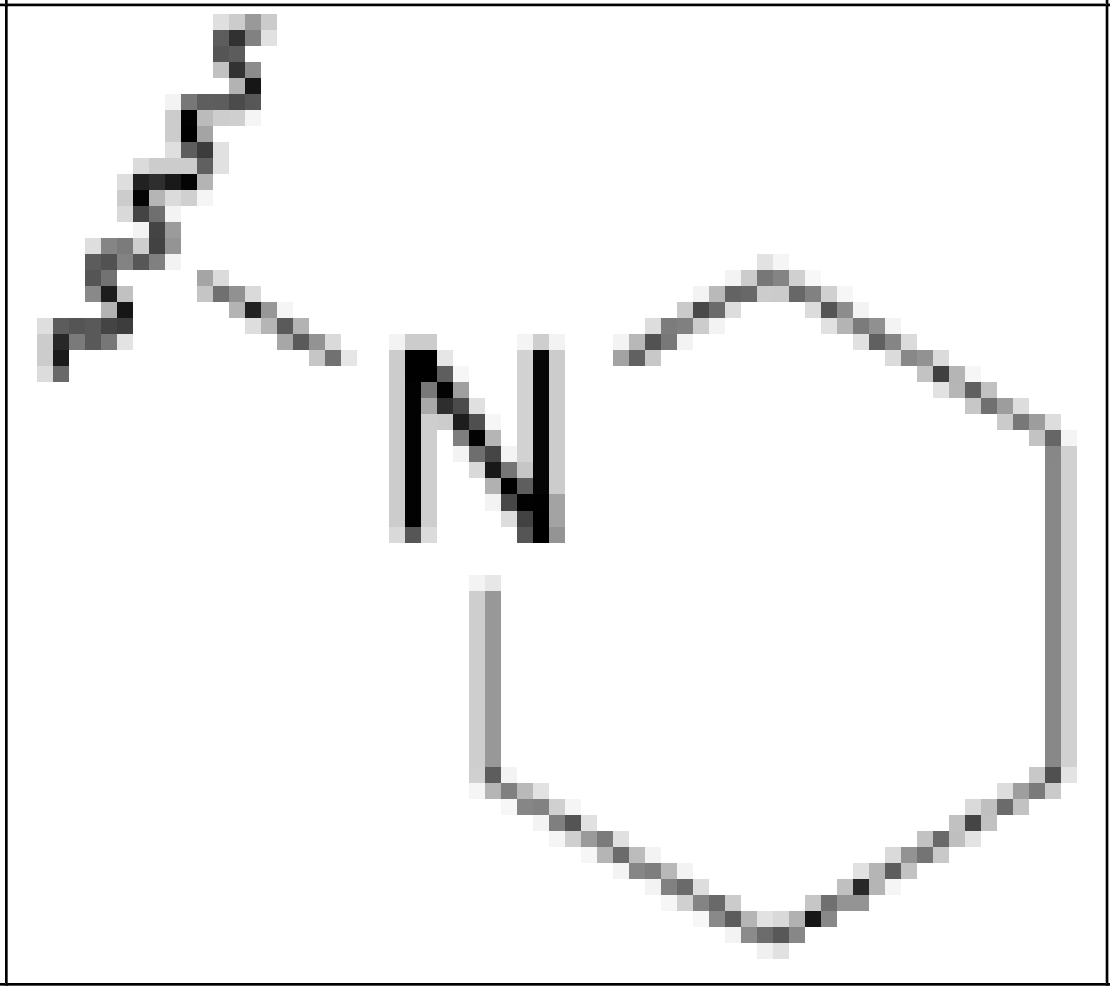


	Structures	*Pf*DHODH IC_{50} (μM)	*Pf* 3D7 cells EC_{50} (μM)
33		>30	>10

Data taken from [75]. Assay conditions were described in Table 1

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