**Targeting the Mitochondrial Electron Transport Chain of *Plasmodium falciparum*: New Strategies towards the Development of Improved Antimalarials for the Elimination Era**

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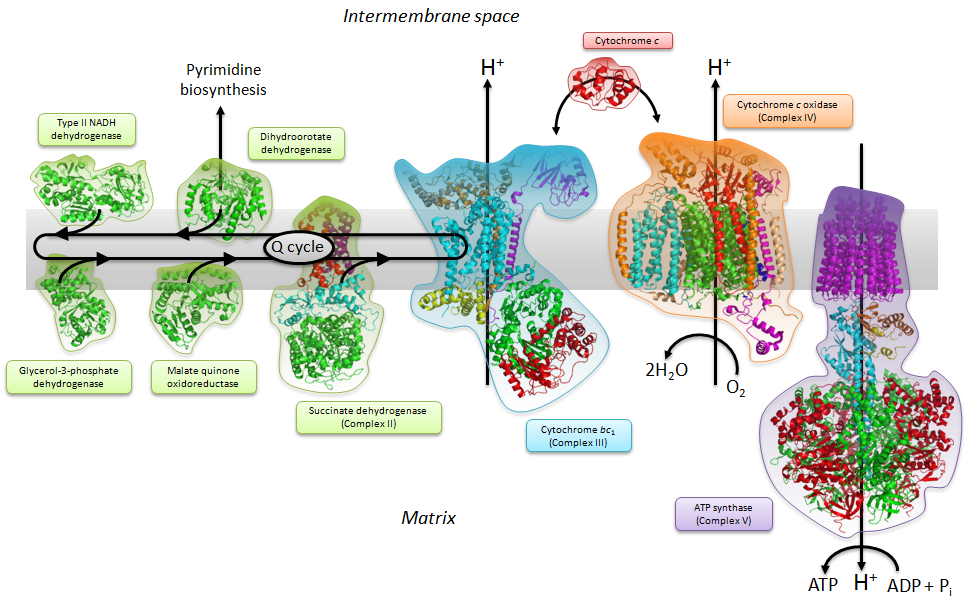
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***Abstract***

Despite intense efforts, there has not been a truly new antimalarial, possessing a novel mechanism of action, registered for over 10 years. Through a novel mode of action, it is hoped that the global challenge of multi-drug resistant parasites can be overcome, whilst developing drugs that possess prophylaxis and/or transmission-blocking properties, towards an elimination agenda. Many target-based and whole-cell screening drug development programmes have been undertaken recently and here we present an overview of specific projects focused on targeting the parasite’s mitochondrial electron transport chain (ETC). Medicinal chemistry activity has largely focused on inhibitors of the parasite cytochrome *bc*1 complex (Complex III) including acridinediones, pyridones and quinolone aryl esters, as well as inhibitors of dihydroorotate dehydrogenase (DHODH) that includes triazolopyrimidines and benzimidazoles. Common barriers to progress and opportunities for novel chemistry and potential additional ETC targets are discussed in the context of the target candidate profiles (TCP) for uncomplicated malaria.

**Introduction to the *Plasmodium* mitochondrial electron transport chain (ETC)**

*Plasmodium*, the causative agent of malaria can be considered the most important parasitic disease in man, with greater than 200 million cases every year and nearly 1 million deaths reported in 2011 [[1](#_ENREF_1)]. It has been acknowledged that the mitochondrion of Plasmodiaplays a critical and essential role in the parasite’s life cycle. Significantly, there are several molecular and functional differences between the parasite’s mitochondrion and the mitochondrion of human cells, and for this reason the organelle is a target for medicinal chemistry projects wishing to exploit these differences towards selective toxicity[[2-4](#_ENREF_2)].



**Figure 1:** The *Plasmodium* mitochondrial electron transport chain (ETC).

The electron transport chain (ETC) of intraerythrocytic malaria parasites is believed to contain five dehydrogenases, namely NADH:ubiquinone oxidoreductase (*Pf*NDH2), succinate:ubiquinone oxidoreductase (complex II or SDH), glycerol-3-phosphate dehydrogenase (G3PDH), the malate quinone oxidoreductase (MQO) and dihydroorotate dehydrogenase (DHODH). Although the functional contribution of these dehydrogenases to the ETC is still not at present completely understood, *Pf*NDH2 and MQO are not found in human mitochondria and DHODH displays distinct molecular differences compared to its human homolog (described below). The dehydrogenase activity serves, at least in part, to provide electrons to the downstream complexes, namely ubiquinol:cytochrome *c* oxidoreductase (complex III or cytochrome *bc*1) and cytochrome *c* oxidase (complex IV) with ubiquinone (Coenzyme Q) and cytochrome *c* functioning as electron carriers between the complexes. [[3](#_ENREF_3)] The ATP synthase (complex V) is not reported to generate ATP (unlike its mammalian counterpart), but is nevertheless proposed as an essential component, possibly acting as a proton leak for the ETC (Fig. 1)[[5-7](#_ENREF_5)].

***Plasmodium falciparum bc1 complex***

Targeting the ETC of the human malaria parasite has already been shown to be a successful chemotherapeutic strategy. *P. falciparum* mitochondria use a different homolog of ubiquinone (CoQ8) than their mammalian host [[8](#_ENREF_8),[9](#_ENREF_9)], and several antimalarial drugs show specificity for parasite CoQ, including the hydroxynaphthoquinones [[10-12](#_ENREF_10)]. Atovaquone, an inhibitor of complex III (or *bc*1 complex), is currently the only drug targeting *bc*1 in clinical use [[13](#_ENREF_13),[14](#_ENREF_14)] (Figure 3). The catalytic core of the *bc*1 complex is composed of three subunits; cytochrome *b* (43 kDa), cytochrome *c*1 (27 kDa) and the Reiske iron-sulfur protein ([2Fe2S]ISP, 21 kDa), with these three subunits participating directly in electron transfer pathway (Figure 2a). The function of the remaining subunits is not fully understood but they are likely to contribute to complex stability and the assembly process. The *bc1* complex contains two distinct quinone-binding sites, the quinol oxidation site Qo and the quinone binding site Qi. These binding sites are located at opposite sides of the membrane and are linked by a transmembrane electron-transfer pathway (Figure 2). The overall structure of *bc1* is highly conserved between species; however unusual structural features have been observed in the *P. falciparum* Q0 site, such as a four residue deletion in the cd2 helix, which may help drive drug selectivity [[5](#_ENREF_5)]. Quinol antagonists such as the natural antibiotics antimycin (Q1) and stigmatellin (Qo) can potently inhibit the *bc1* complex, abolishing ΔP of the enzyme and collapsing the mitochondrial membrane potential, however they are highly toxic and so unsuitable for therapeutic use. Studies have shown that **1** (atovaquone) is a competitive inhibitor of the Q0 binding site [[15](#_ENREF_15),[16](#_ENREF_16)]. Currently *bc1* remains an underexploited drug target and there is an opportunity for second generation inhibitors with improved pharmaceutical properties, lower cost of goods and activity against atovaquone resistant strains of malaria [12]. The development of atovaquone and other drug discovery programmes that target the *Plasmodium* *bc*1 complex are discussed in detail in **Section 1**.

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**Figure 2: Figure 2 a)** Ribbon model (grey) of the homodimeric structure of the yeast cytochrome *bc*1 complex (PDB code 3CX5). Cytochrome *b*, cytochrome *c*1 and the Rieske protein from one monomeric unit are represented in green, cyan and orange respectively. Hemes of cytochrome *b* and cytochrome *c*1 are shown in red wireframe, with the iron (pink) and sulfur (yellow) atoms of the Rieske [2Fe2S] cluster represented in spacefill. The position of the inner mitochondrial membrane lipid bilayer is approximated as a cartoon, with 'p' and 'n' referring to the positive and negative sides with respect to proton translocation. **b)** The structure and Q-cycle mechanism of the catalytic core of the *bc*1 complex. Cytochrome *b*, cytochrome *c*1 and the Rieske protein are represented in green, cyan and orange ribbons respectively. Haems *b*l and *b*h of cytochrome *b* and *c*1 of cytochrome *c*1 are shown in red wireframe. The [2Fe2S] cluster of the Rieske protein is represented in yellow/pink spacefill. Electron transfers to and from ubiquinol (QH2) and ubiquinone (Q) are represented by yellow arrows. Proton movements are indicated by white arrows.

***PfNDH2***

The role of PfNDH2 within the mETC is not fully understood and is still the focus of some debate. *Pf*NDH2 is unlikely to be involved with proton pumping, due to the absence of any transmembrane domains; however it is thought that its activity may indirectly contribute to the formation of an electrochemical transmembrane potential [[17](#_ENREF_17),[18](#_ENREF_18)]. There is however conflicting data with regards to the essentiality of this enzyme during the intraerythrocytic stage of the parasite. Chemical validation of the target includes the use of selective inhibitors such as **2** (CK-2-68) (Figure 3) that were shown to exhibit nanomolar activity against *Pf*NDH2 (but not *bc*1 or DHODH) with corresponding *P. falciparum* kill[[19](#_ENREF_19)]*,* however a genetic knock-out of ndh in rodent malaria (*P. berghei*), was shown to be viable[[20](#_ENREF_20)]. A drug discovery project targeting *Pf*NDH2 at the late lead preclinical phase of the MMV (Medicines for Malaria Venture, [www.mmv.org](http://www.mmv.org)) discovery portfolio is described in **Section 2.**

***PfDHODH***

Within the ETC *PfDHODH* is believed to be involved in pyrimidine biosynthesis, the process of which is essential for parasite survival. It is widely accepted that unlike mammalian cells, the blood stage malaria parasite relies mainly on glycolytic energy metabolism and so depends on the *de novo* pyrimidine biosynthesis which is essential for the formation of DNA and RNA, glycoproteins and phospholipids [6]. The low enzyme activities mentioned previously are consistent with the largely glycolytic carbon and energy metabolism of the blood stage parasite. Genetic sequencing data has revealed that the genes encoding enzymes from the pyrimidine biosynthetic pathway have been conserved, whilst those responsible for salvaging pyrimidines have not [[21](#_ENREF_21)]. This indicates that malaria parasites rely on *de novo* pyrimidine biosynthesis in order to meet its metabolic requirements and cannot salvage preformed pyrimidine bases or nucleosides as with mammalian cells [[2](#_ENREF_2),[3](#_ENREF_3)]. Additional oxidoreductases such as dihydroorotate dehydrogenase (*Pf*DHODH) are present which play an important role in this pyrimidine biosynthesis, catalysing the oxidation of dihydroorotate to orotate. The resulting pair of electrons formed in this oxidation reaction is then fed into the ETC, through flavin mononucleotide co-factor to ubiquinone, which itself is generated at the cytochrome *bc1*complex, thus forming the link between metabolism and the electron transport chain [[22](#_ENREF_22),[23](#_ENREF_23)]. In general, the ETC inhibitors tend to resemble ubiquinol/ubiquinone whereas *Pf*DHODH inhibitors show more diverse scaffolding [[2](#_ENREF_2)], the most effective being compound **3** (5-fluoroorotate) and its derivatives [[24](#_ENREF_24)] (Figure 3). 5-Fluoroorotate given in combination with atovaquone has proved to be more efficient than either compound alone, improving potency and decreasing drug resistance frequency [[25](#_ENREF_25)]. Drug development programmes involving inhibition of *Pf*DHODH are described in **Section 3**.

***Other Components***

Other ETC components have known inhibitors but are relatively underexplored with regard to drug discovery programmes. SDH has shown sensitivity to a number of inhibitors, such as 5-substituted 2,3-dimethoxy-6-phytyl-1,4-benzoquinone derivatives, **4** (plumbagin) and **5** (licochalcone A) [[26](#_ENREF_26)] (Figure 3). ATP synthase is another possible drug development target. It has previously been shown that **6** (Almitrine), originally developed as a respiratory stimulant has activity against *Plasmodium* ATP synthase and whole cell *P.falciparum*. [[27](#_ENREF_27)]Targeting ATP synthase within the ETC of tuberculosis has recently proven to be a successful strategy in the development of the FDA approved drug **7** (bedaquiline, TMC207) for the treatment of multi-drug resistant tuberculosis (Figure 3) providing hope that this may prove to be a valid drug development target for malaria in the future .[[28](#_ENREF_28)]



**Figure 3:** Structure and mode of action of selected ETC inhibitors.

A potent ETC inhibitor with the correct pharmacokinetic profile may provide a drug which meets the current target candidate profile (TCP) for the treatment of uncomplicated malaria (Table 1). Currently there are three projects based around inhibition of ETC components in the MMV pre-clinical pipeline. The ETC inhibitors within the MMV pipeline aren’t without their challenges. Quinolones have inherently poor solubility which may lead to issues with bioavailability and food effects. Safety problems have been encountered with previous ETC inhibitors (pyridones) that have reached advanced phases of the drug development pipeline. It is however hoped that lessons learnt from this will lead to earlier identification of issues, should they be present. The strengths of ETC inhibitors however lie in their clinical potential for use in the treatment and prophylaxis of malaria and in their potential to block transmission.

**Table 1:** Target Candidate Profile for a Single Exposure Radical Cure for the Treatment of Acute Uncomplicated Malaria.

|  |  |  |
| --- | --- | --- |
| ***Parameter*** | ***Minimum*** | ***Ideal*** |
| Clinical anti-relapse activity | > Chloroquine | > 14 days of primaquine |
| Transmission blocking | No enhancement of infectivity | Yes |
| Bioavailability/food effect | >30% / tolerable | >80%/no significant effect |
| Dosing regimen | Oral, once a day for 3 days | Once |
| Safety | No significant SAEs | No SAEs or AEs |
| Hemolysis in G6PD – deficient patients | Dose identified – change at day 7 of <2.6 g/l | Therapeutic dose – change at day 7 of <2.6 g/l |
| Pregnancy | Not contraindicated in 2nd and 3rd trimester | Not contraindicated |
| Formulations | Co-formulated tablet – adults  Dispersible tablets - pediatrics | Co-formulated tablet – adults  Dispersible tablets - pediatrics |
| Cost of treatment course | ≤ $5.00 – adults  ≤ $1.25 – infants (under 2). | ≤ $1.00 – adults  ≤ $0.25 – infants (under 2). |
| Shelf life | ≥ 2 yr | 5 yr |

***1. Inhibition of Plasmodium falciparum bc1***

The Plasmodium falciparum *bc*1 complex is currently the only component of the electron transport chain with a clinically used antimalarial drug associated with it. These investigations have resulted in the clinical development and use of **1** (atovaquone) to treat malaria. The rapid emergence of resistance to atovaquone has however resulted in it being used as a combination therapy with proguanil (Malarone TM). The cost of this combination therapy has proven to be prohibitive in its widespread use in resource poor, disease endemic areas. Its primary use is as a prophylactic antimalarial for people travelling to these endemic areas.

More cost effective inhibitors that overcome the resistance issue are highly sought after. The development of *Plasmodium* cytochrome *bc*1 inhibitors was reviewed by Barton *et al* in 2010 and so here we shall concentrate on what has emerged to be the more pertinent aspects of that review and advances since 2010.[[29](#_ENREF_29)]

* 1. ***Atovaquone and other hydroxynapthquinones***

Over half a century of research by many groups and extensive investigations into the antiparasitic properties of numerous structurally related compounds resulted in atovaquone becoming the first clinically used *Plasmodium* cytochrome *bc*1 inhibitor.[[30-35](#_ENREF_30)] Atovaquone’s development as an antimalarial drug began due to a sudden shortage of quinine following the outbreak of World War 2. [[36](#_ENREF_36)] In an attempt to find an alternative, thousands of structurally diverse compounds were interrogated, a large number of which were hydroxynaphthoquinone-like structures. Some of which had modest antimalarial activity in ducks infected with *Plasmodium lophurae*. A robust lead optimisation programme was thus embarked upon which generated over 300 quinones, with some demonstrating improved activity in the duck assay when compared to quinine. Upon administration to malaria patients it was however discovered that due to poor absorption and rapid metabolism these compounds had no antimalarial activity.[[37](#_ENREF_37),[38](#_ENREF_38)] All attempts to resolve these issues to produce an orally active analogue were unsuccessful both at the time and when the issues were re-addressed in the 1960s.[[39](#_ENREF_39)] The 1960’s investigation did however lead to the development of intravenously administered **8** (lapinone), which when given by this route had activity against *Plasmodium vivax* (Figure 5A).[[40](#_ENREF_40)]

In the 1980s a group at the Wellcome Research Laboratories reinvestigated the use of quinones as antimalarial agents. The development of test systems using the human parasite *P.falciparum in vitro* and *in vivo* in *Aotus* monkeys lead to the generation of more meaningful biological data sets. This study was designed to develop a quinone with good metabolic stability in humans in addition to good antimalarial activity against *P. falciparum*. In order to achieve this numerous 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone analogues (for example, **9** and **10**) were synthesised with substitution on the metabolically labile 4’ position of the cyclohexyl ring.[[41](#_ENREF_41),[42](#_ENREF_42)] A large number of these quinones had a potency of ~1 nM against *P. falciparum in vitro* but the majority of these compounds were still metabolically unstable. Only one analogue from the series, **1** (atovaquone) was found to be inert to human liver microsomes (Figure 4A).[[43](#_ENREF_43)] [101]

**1** (Atovaquone) has a mode of action involving competitive inhibition of the Qo sight of the *bc1* complex.[[15](#_ENREF_15)] A fixed dose combination of **1** (atovaquone) with proguanil (MalaroneTM) is currently used clinically. A mis-sense point mutation at position 268 in cytochrome *b*, exchanging tyrosine for serine (Y268S) or, less frequently, asparagine (Y268N) has been linked with MalaroneTM drug failure.[[44-48](#_ENREF_44)] Position 268 in cytochrome *b* is highly conserved across all phyla and is located within the “ef” helix component of the Qo site which is putatively involved in ubiquinol binding. Growth IC50 curves of this atovaquone-resistant phenotype are up to 1000-fold greater than susceptible strains. However, a ~40 % reduction in the Vmax of the *bc*1 complex is also noted indicating a substantial fitness loss within the parasite.[[16](#_ENREF_16)]

Whilst there is strong literature precedence stating that atovaquone administration as a monotherapy leads to the rapid emergence of *de novo* resistance, the underlying cause for this observation has not yet been identified.[[49](#_ENREF_49),[50](#_ENREF_50)] There are several plausible explanations for this which include pharmacokinetic considerations which relate to the physicochemical properties of atovaquone and considerations relating to the molecular target such as the effect of an increased mutation rate of mitochondrially-encoded genes such as cytochrome *b* compared to nuclear encoded genes.[[51](#_ENREF_51)] In addition to this, there has been a report that generation of an *in vitro* atovaquone-resistant parasite line possessing wild-type cyt *b* has been possible in a laboratory setting.[[52](#_ENREF_52)] Work is still ongoing to fully elucidate the mechanism underlying the atovaquone-resistant phenotype in this strain.

In an attempt to overcome the poor bioavailability associated with atovaquone El Hage *et al* designed compounds where the 3-hydroxyl functional group of atovaquone was replaced by more lipophilic ester and ether groups.[[53](#_ENREF_53)] These attempts were however largely unsuccessful giving compounds with similar properties to atovaquone.

Hughes *et al* were however more successful with their development of a potent hydroxynaphthoquinone inhibitor **12** (NQ3) from **11** (S-10576).[[54](#_ENREF_54)] **11** (S-10576) was a highly selective against *Plasmodium bc*1 but was inactive in humans due to rapid metabolic degradation. Here, their strategy of incorporating trifluoromethyl groups into branched and straight chain alkyl groups on the quinoid carbon-carbon double bond in combination with a methyl group on the aromatic ring produced compounds that overcame the metabolic instability (Figure 4B).

***1.2. Pyridones***

Pyridones have been known to possess antimalarial activity since the 1960’s when **13** (clopidol)was shown to have activity against chloroquine resistant strains of *P.falciparum*. [102] **13** (Clopidol) also maintains activity against atovaquone-resistant strains suggesting that pyridone derivatives may bind at a different site to that of atovaquone in the Qo pocket of the *bc1*complex.

In 2006 GlaxoSmithKline (GSK) reported the pre-clinical evaluation of a new class of antimalarial 4(1H) pyridines targeting the *bc1*complex.[[55](#_ENREF_55)] Research initiated in the former Wellcome laboratories and continued by GSK resulted in the development of a series of disubstituted derivatives of clopidol as described by Yeates *et al* (Figure 4C).[[56](#_ENREF_56)] Incorporation of an atovaquone-like bicyclic side chain into the pyridone template gave the initially promising candidate **14** (GW844520). However its development was terminated by the MMV due to unexpected cardiotoxicity.[[57](#_ENREF_57)] Further development of the template gave **15** (GSK932121) which was found to be highly potent both in vitro and against multidrug resistant strains of *P.falciparum* in a novel murine *P.falciparum* model.[[58](#_ENREF_58),[59](#_ENREF_59)] **15** (GSK932121) entered Phase I clinical trials in 2008 however the trial was again suspended due to the simultaneous discovery of toxicity issues in rats dosed with the phosphate ester prodrug of **15** (GSK932121). The toxicity was attributed to higher systemic exposure of the parent drug which was later confirmed when similar toxicity was observed in rats dosed with the parent drug by the intraperitoneal route. It is known that pyridone analogues can demonstrate significant differences in ADME properties. This in conjunction with their potent activity *in vitro* and *in vivo* against both resistant strains and liver stages may possibly warrant further investigation of the series.

* 1. ***Acridinediones and acridones***

Acridinediones are known to be potent antimalarials and their mode of action has been proven to be heme-binding and prevention of crystallisation to hemozoin. Mode of action investigations of the two hydroacridinediones **17** (floxacrine) and **16** (WR249685) showed that **16** (WR249685) had higher *in vitro* antimalarial activity than **17** (floxacrine) (IC50 = 15 nM vs. IC50 = 140 nM for **17** (floxacrine)) yet **17** (floxacrine) bound to heme 20 times better.[[60](#_ENREF_60)] Subsequently **16** (WR249685 was found to inhibit the Plasmodium *bc*1 complex (IC50 = 3 nM) with far superior selectivity than other *bc*1 inhibitors (therapeutic index TI ≥ 4600, compared to **1** (atovaquone) TI = 24 and pyridone **14** (GW844520) TI = 5)(Figure 4D).

Acridones, initially isolated as intermediates in the synthesis of a series of heme-complexing compounds have also been found to possess a similar dual mechanism of action. Riscoe *et al* have synthesised a number of highly potent acridone ether derivatives (compounds **18** and **19**) to gain a better understanding of the antimalarial SAR.[[61](#_ENREF_61)] The high potency demonstrated by these compounds is a reflection of the dual binding ability of these compounds. These molecules have the ability to bind to *Plasmodium bc*1 as well as disrupting hemozoin formation. **20** (T3.5) (Figure 4D) is a more recent example of this approach. **20** (T3.5) incorporates a heme targeting scaffold and a chemosensitisation functionality into one molecule.[[62](#_ENREF_62)]

* 1. ***Quinolones***

There has been a large amount activity in the development of quinolones to target the *plasmodium bc*1 complex in recent years. In 2008 quinolone inhibitors were shown to bind to the Qo site of the cytochrome *bc*1 complex. Several alkyl and alkoxy 4(1H)-quinolones were synthesised in an effort to ascertain the SAR around these compounds that would provide a potent and selective inhibitor. [[63](#_ENREF_63)] More recently development of these compounds to contain diarylethers has led to the discovery of **23** (ELQ-300). **23** (ELQ-300) is a selective potent inhibitor of the *Plasmodium bc*1 complex that has been selected as a pre-clinical candidate by the MMV. The biological activity of the backup compound within this series **24** (P4Q-391) has also been fully evaluated for its biological activity.[[64](#_ENREF_64)]

**23** (ELQ-300) was developed from **21** (Endochin). **21** (Endochin) was first described by Salzer and co-workers more than 70 years ago as a potent antimalarial in avian models of the disease but this activity did not translate to activity in humans due to metabolic instability.[[65](#_ENREF_65)] **21** (Endochin) contains a long alkyl chain at the three position on the quinolone core which is responsible for its metabolic instability, replacement of this alkyl chain with the side chain from the previously described pyridone **14** (GW844520) gave **22** (ELQ-271) which demonstrated improved metabolic stability. Further structure-activity manipulations lead to **23** (ELQ-300) which demonstrated a greatly improved selectivity ratio for *Plasmodium bc*1 over human *bc*1 (Table 2). **23** (ELQ-300) was also shown to have no effect on intracellular ATP levels in two different mammalian cell lines whereas **22** (ELQ-271) caused a concentration dependent decline in ATP levels. **24** (P4Q-391) containing a fluorine in the diaryl ether side chain was selected as the backup compound for the series as it also demonstrated promising potency and target selectivity (Figure 4E).

**Table 2:** Selectivity indexes of selected diaryl quinolones in comparison to atovaquone.[[64](#_ENREF_64)]

|  |  |  |  |
| --- | --- | --- | --- |
| Compound | IC50 (nM) | | Selectivity Index |
| Human Cytochrome *bc*1 | *P.falciparum* cytochrome *bc*1 | Human/  *P.falciparum* |
| **22** (ELQ-271) | 1750 | ND | ND |
| **23** (ELQ-300) | >10000 | 0.56 | >18000 |
| **24** (P4Q-391) | >10000 | 1.0 | >10000 |
| **1** (atovaquone) | 460 | 2.0 | 230 |

Following full biological evaluation **23** (ELQ-300) was selected as the pre-clinical candidate as it had superior antiplasmodial activity *in vitro* and *in vivo* against blood and liver stages of malarial parasites as well as improved selectivity. This class of compounds does however have its limitations. Aqueous solubility is poor and subsequently this has an effect on the pharmacokinetics of the drug. As the dose in mice and rats is increased, the bioavailability decreases in line with solubility limited absorption. This has implications for *in vivo* toxicity testing as it may not be possible to establish the maximum tolerated dose if not enough drug can be taken on board and therefore the therapeutic index cannot be determined. Formulation approaches are currently in progress to address this.

Other groups have also investigated quinolones as *P.falciparum bc*1 inhibitors. Da Cruz *et al* recently reported the findings of a drug screen of 1037 existing drugs on *Plasmodium* liver stages.[[66](#_ENREF_66)] The most potent inhibitor of the liver stages (IC50 = 2.6 nM) was found to be **25** (decoquinate) both *in vitro* and *in vivo* (Figure 4F). Further investigation into its mode of action revealed it to specifically and selectively inhibit the parasites mitochondrial *bc*1 complex.

Cowley *et al* have investigated quinolone esters and through extensive probing of the SAR have proven that targeting the *P.falciparum bc*1 complex can lead to highly potent antimalarial compounds with the lead compound **26** (RCQ) having an IC50 of 0.46 nM (Figure 5F).[[67](#_ENREF_67)] Docking studies *in silico* at the yeast Qo site demonstrated a key role for residues His182 and Glu272 in the recognition of these highly potent inhibitors.



**Figure 4:** Structures and SAR of current *P.falciparum* *bc*1 inhibitors.

**2. Inhibition of *Plasmodium falciparum* Type II NADH:Quinone Oxidoreductase (*Pf*NDH2)**

*Pf*NDH2 has only one known inhibitor, **27** (hydroxy-2-dodecyl-4-(1H)-quinolone (HDQ)) and work on this target has only recently been undertaken by Biagini *et al*.[[68](#_ENREF_68)] A high-throughput screen (HTS) against *Pf*NDH2 was set up using recombinant *Pf*NDH2 expressed in a heterologous expression system in *Escherichia coli* NADH dehydrogenase knockout strain ANN0222 (*nuoB:: nptI-sacRB, ndh::tet*), eliminating background NADH:quinol oxidoreductase activity. An assay protocol suitable for HTS was optimised and validated for screening with the *Z*’ ranging from 0.66 to 0.9 and a signal/background >10.[[69](#_ENREF_69)] **27** (HDQ) in combination with a range of chemoinformatics methods was used to rationally select 17000 compounds for HTS. Identification of several distinct chemotypes was possible from the screen. Brief investigations into these chemotypes lead to the selection of the quinolone core as the key target for SAR development and rapid selection of **2** (CK-2-68) as a lead for further development.[[70](#_ENREF_70)]

Compounds with a mono aryl group at the 2- position (compound **28**) were the primary focus of the SAR investigations; however it was soon clear that activity below 500 nM against the 3D7 strain of *P.falciparum* could not be achieved. Improvements in both antimalarial and *Pf*NDH2 activity were seen as the SAR moved towards replacement of the more metabolically vulnerable HDQ-side chain with the closer HDQ analogues incorporating a longer biaryl/phenoxy biaryl side chain. Additional structural alterations including the introduction of a methyl substituent at the 3-position allowed the generation of over 60 compounds as exemplified by **2** (CK-2-68)with activity of 31 nM against 3D7 and 16 nM against *Pf*NDH2 (Figure 5A). Preliminary animal studies involving **2** (CK-2-68) indicated that a reduction in ClogP and enhancement in aqueous solubility was required in order to administer the drug in a suitable vehicle for oral dosing without the need for a pro-drug approach. Heterocycle incorporation into the quinolone side chain gave a series of compounds containing a pyridine group within the side chain. The pyridine group reduces CLogP, improves aqueous solubility and makes salt formation possible. Other strategies investigated included the incorporation of protonatable groups within the side chain, the use of polar heterocycles in the side chain, the placement of a polar group centrally in the side chain with a lipophilic group at the terminal end and extending the terminal end of the side chain using polar heterocycles .[103]

These structural modifications led to analogues **30** (SL-2-64) and **31** (SL-2-25).[[19](#_ENREF_19)] **31** (SL-2-25) has IC50s in the nanomolar range versus both the enzyme and whole cell *P. falciparum* (IC50 =15 nM *Pf*NDH2; IC50 = 54 nM (3D7 strain of *P.falciparum* ) with notable oral activity of ED50/ED90 of 1.87/4.72 mg/kg versus *Plasmodium berghei* (NS Strain) in a murine model of malaria when formulated as a phosphate salt. **30** (SL-2-64) the 7-fluoro analogue, also has antimalarial activity against the 3D7 strain of *P.falciparum* (IC50 = 75 nM) and (*Pf*NDH2 IC50 =4.2 nM) with an ED50/ED90 of 2.6/6.5 mg/kg (Figure 5A). [[19](#_ENREF_19)]

Whilst the initial drug discovery efforts were focused on optimisation of activity versus *Pf*NDH2, it was revealed, during hit to lead development, that optimised structures with single digit nanomolar activity versus the primary target were also active at the parasite *bc*1 complex. This dual inhibitory effect is also seen with the starting point for this program, **27** (HDQ) suggesting that the quinolone pharmacophore is a privileged scaffold for inhibition of both drug targets. Such multi-target drugs are increasingly seen as having therapeutic benefit over drugs acting exclusively at one site.

Manipulation of the quinolone core to impart some selectivity is possible (Figure 5B). A direct comparison of 3-aryl [104] and 2-aryl quinolones can be made from the compounds depicted in Figure 5B. This clearly depicts a loss of *Pf*NDH2 activity when moving from 2-aryl to 3-aryl examples with **32** (CK-2-67) having 16 nM *Pf*NDH2 activity with this dropping to 492 nM for 3-aryl quinolone **33** (WDH-1U-4). All analogues depicted in Figure 5B demonstrate good levels of antiparasitic activity. The potent oral activity of 2-pyridyl quinolones underlines the potential of this template for further lead optimization studies, the lead compound from this series is currently in the MMV discovery pipeline.



**Figure 5:** Rational design of 2-diheteroaryl quinolones to target *Pf*NDH2.

**3. Inhibition of *Plasmodium falciparum* dihydroorotate dehydrogenase (DHODH)**

Pyrimidines are essential metabolites, required for DNA and RNA biosynthesis and the biosynthesis of phospholipids and glycoproteins. Unlike mammalian cells, the malaria parasite cannot salvage preformed pyrimidines and they need to be acquired through *de novo* biosynthetic pathways.

The fourth step of pyrimidine biosynthesis is catalysed by DHODH, a flavin mononucleotide-dependent enzyme. Inhibitors of human DHODH have proven efficacy for the treatment of rheumatoid arthritis, with an approved compound **34** (Leflunomide) (Arava TM, active metabolite **35** (A77 1726)) on the market, **36** (Brequinar) is an antitumor and immune suppressive agent thus demonstrating that DHODH is a ’’druggable target’’(Figure 6). The inhibitor binding pocket has extensive variation in amino acid sequence between the human and malarial enzymes, providing the structural basis for the identification of species-specific inhibitors.[[23](#_ENREF_23)]



**Figure 6**: Human DHODH inhibitors

Although potent Human DHODH inhibitors, **34** (Leflunomide) and **36** (Brequinar) demonstrated poor inhibition against *Pf*DHODH, the skeletons provided a starting point for the drug development of novel *Pf*DHODH inhibitors. However, early quinolone-based inhibitors designed around the brequinar skeleton lacked any inhibitory activity against *Pf*DHODH and it was evident that a significantly different template would be required for species selective inhibition. The isolation of X-ray crystal structure of *Pf*DHODH enzyme co-crystallised with **35** (A77-1726) has allowed for more rational structure based molecular design of *Pf*DHODH inhibitors.[[71](#_ENREF_71)] Recently Vyas *et al* have reviewed DHODH inhibitors, as a result this article looks briefly at the most pertinent aspects of that review and focuses in more depth on *Pf*DHODH inhibitors developed since 2011.[[72](#_ENREF_72)]

**3.1 Triazolopyrimidine based DHODH inhibitors**

In an attempt to discover novel scaffolds, an HTS program was initiated by Philips M.A. *et al* with the aim of screening a diverse library of 220,000 drug-like molecules. [[73](#_ENREF_73)] Several classes of *Pf*DHODH inhibitors were identified including a series of halogenated benzamide/naphthamides and urea based compounds containing naphthyl or quinolinyl substituents. In total 13 candidates displayed <100nM potency against the enzyme and showed at least 100 fold selectivity, however most had weak antimalarial activity in the whole cell assay.

From this initial screen, a triazolopyrimidine-based compound **37** was discovered as a *Pf*DHODH inhibitor with low nanomolar activity in the whole cell based assay. Compound **37** was identified as the most promising lead candidate (IC50 *Pf*DHODH = 47nM) and it is >5000 fold selective when compared to human enzyme. [[74](#_ENREF_74)] A series of triazolopyrimidine analogues of **37** were synthesised and tested against the enzyme and the parasite in whole cell assay (Table 3). These compounds showed a wide range of IC50 values against *Pf*DHODH (0.05 to >200nM) and showed strong correlation with potency against the parasite in the whole cell assay. Preliminary SAR shows i) R and R1 alkyl substituents can be modified with a modest decrease in activity, ii) substitutions at R2 results in loss of potency, iii) introduction of heteroatoms on or in the naphthyl ring reduced activity iv) naphthalene ring attached at 1-position reduced activity v) replacement of naphthalene with a smaller phenyl group significantly reduced activity but the larger anthracene group was tolerated. [[74](#_ENREF_74)]

**Table 3**: Structure and activity of the Triazolopyrimidne based series against *Pf*DHODH and *P.falciparum* whole cell assay.[[74](#_ENREF_74)]



To provide an insight into the structural basis for potent *Pf* specific inhibitor **37**, the first X-ray crystal structure of *Pf*DHODH bound to triazolopyrimidine inhibitors was reported.[[75](#_ENREF_75)] The conformational flexibility of triazolopyrimidines resulted in an unexpected binding mode identifying a new hydrophobic pocket on the enzyme. The malarial enzyme has the flexibility to form two different inhibitor binding pockets (*Pf*-naphthyl pocket and the *Pf*-A77 phenyl pocket). This flexibility may explain why the enzyme can accommodate a number of diverse chemical scaffolds.

Compound **37** was found to have poor *in vivo* activity in the mice model (*P. berghei*) explained by a combination of poor plasma exposure and reduced potency against *Pb*DHODH. [[76](#_ENREF_76)] The lead compound **37** was optimised by including various phenyl substituted groups in place of the naphthyl. Forty new compounds were synthesised and it was found that a *p*-trifluoromethyl phenyl group was optimal. This optimisation increased the metabolic stability of the compound and suppressed the growth of *P.berghei* in mice after oral administration.

The X-ray structure of **37** and **46** bound to *Pf*DHODH demonstrated that the binding pocket for the aryl amine is completely hydrophobic and unable to form any H-bonding interactions and also shows that the pocket between aryl amine and the aromatic ring binding site is narrow, consistent with the observation that ortho substituents on the phenyl ring are not tolerated. Further efforts in optimising the aromatic functionality to improve potency and *in vivo* properties led to two new triazolopyrimidnes with substituted phenyl moieties (compounds **47** and **48**), showing good plasma exposure and better efficacy in the *P.berghei* mouse model than compound **46** from the series (Figure 8).[[77](#_ENREF_77)] The substitution of 3,5-difluoro-4-CF3 and 4-SF5 for 4-CF3 resulted in 2-3 fold increased activity against *Pf*DHODH possibly due to increase in hydrophobicity.

Although these metabolically stable triazolopyrimidines are able to suppress parasites in a mouse model of infection, these compounds lacked the potency required of a clinical development candidate. The enzyme bound structure of **37** and **46** showed that the triazolopyrimidines filled most of the available binding pocket but a channel between the C2 position of the triazolopyrimidine ring was available that could potentially accommodate additional functionality. The further medicinal chemistry advancement included incorporation of alky, haloalkyl, ether and amine groups at C2 position. This modification yielded several potent compounds with better pharmacokinetic profile and *in vivo* efficacy in mouse models than the previous compounds. Incorporation of CF2CH3 at the C2 position significantly improved the potency, plasma exposure profile, efficacy and maintained excellent selectivity. The compound **49** was identified as a preclinical candidate and is currently in the MMV pre-clinical pipeline (Figure 7).[[78](#_ENREF_78)]

More recently, as part of a backup program around this scaffold, the authors have explored heteroatom rearrangement of the triazolopyrimidine ring and replacement of -NH linker with an amide linker. Replacement of pyridine nitrogen N5 with carbon and –NH with –NHCO reduced potency, whereas, substitution of the triazolopyrimidine ring with imidazolopyrimidine (imidazo[1,2-a]pyrimidine ) was well tolerated and gave 4-fold more potent compound **51**.[[66](#_ENREF_66)]



**Figure 7:** Development of triazolopyrimidine based DHODH inhibitors

**3.2 Benzimidazolyl thiophene-2-carboxamides**

Booker M.L. *et al* have identified benzimidazolothiophene-2-carboxamide **52** as novel DHODH inhibitor from a HTS screening of 208,000 diverse compounds. [[79](#_ENREF_79)] The overall structure of *Pf*DHODH complexed with **52** is similar to the previously reported structures of *Pf*DHODH bound to the triazolopyrimidine inhibitors (*e.g.* **37** (DSM1)) and **35** (A77 1726).Further optimisation of the hits obtained from the HTS screen by incorporation of electron withdrawing groups on the phenyl ring identified compounds with double digit nanomolar potency against DHODH from *P.falciparum*, *P.berghei* and *P.vivax* whilst lacking activity against the human enzyme. Structural modifications around the secondary amide confirmed the cyclopropyl group to be optimal as it occupies a defined hydrophobic pocket and further optimisation on the substitution at 4-position of benzimidazole ring identified compounds **53**-**55** with improved potency (Figure 9A). These compounds had a profound inhibitory effect on growth of the 3D7 strain of P.falciparum. [[80](#_ENREF_80)] Compound **53** proved *in vivo* efficacy both in *P.berghei* and *P.falciparum* animal models was possible. Although potent, this compound produced significant inhibition of *CYP2D6* as well as the cardiac hERG channel, rendering it somewhat less desirable as a development candidate. Later analogues, **54** and **55**, were selected on the basis of equivalent activity, and more favourable cytochrome P450 and hERG inhibition properties when compared with **53**.

On the basis of the efficacy observed in the three mouse models of malaria, acceptable safety pharmacology risk assessment and safety toxicology profile in rodents, lack of potential drug- drug interactions, acceptable ADME/pharmacokinetic profile, and projected human dose, compound **55** was identified as a potential drug development candidate.[[81](#_ENREF_81)]

**3.3 S-Benzyltriazolopyrimidines**

Fishwick C.W.G and Johnson A.P *et al* have used triazolopyrimidine as input for the ROCS (rapid overlay of chemical structure) application to identify novel chemical scaffolds that may be potential DHODH inhibitors. A ROCS screening was performed on the Maybridge chemical screening library of 30,000 compounds. From the top-ranked 500 compounds, 10 compounds were selected and tested for activity against *Pf*DHODH and *h*DHODH, resulting in compound **56** as the most active with an IC50 of 1uM against *Pf*DHODH (Figure 9B). [[82](#_ENREF_82)]

**3.4 Biaryl Carboxamides**

A detailed comparison of x-ray crystal structures of *Pf*DHODH and *h*DHODH bound to **35** (A77-1726) revealed subtle differences, particularly in the dimensions and topography of the hydrophobic ubiquinone channels. In particular, the channel within the human enzyme is flattened by the protrusion of a methyl group from A59 in the region occupied by the aromatic ring of the bound inhibitor. Therefore, the shape of this cavity appears to require inhibitors of this type to be planar, whereas the same region within the plasmodium derived enzyme is much less congested so can accommodate somewhat cylindrical shaped inhibitors. Fishwick C.W.G and Johnson A.P *et al* applied *de novo* molecular design program SPROUT to design novel and selective inhibitors of *Pf*DHODH and identified 20 different small molecule templates. The most attractive of these, for which predicted binding affinities were in the micromolar range were simple amides of anthranilic acid, if they adopt a non-planar arrangement (conformation **b**) between the amide unit , attached aryl groups and the carboxylic acid moiety. In contrast, planar versions (conformation **a**) of this type of inhibitor are predicted to interact best with the human enzyme (Figure 8). A small number of amides were synthesised and tested against *Pf*DHODH and *h*DHODH. *N*-methyl compounds **57** and **59** exhibit a higher affinity for plasmodium enzyme and adopt the conformation **b** than those found for the *N*-unsubstituted derivatives (compounds **58** and **60**) which adopt the conformation **a** (Figure 9C). Although these compounds showed only modest affinity for the *Pf*DHODH enzyme they offer a good starting point for further optimisation, and this approach could be used in conjunction with HTS screening. [[83](#_ENREF_83)]



**a**

**b**

Intermolecular

H-bonding

‘Twisting’ – may maximize interaction within hydrophobic cavity of *Pf*DHOD

**Figure 8:** Conformations of –NH and –NMe biaryl carboxamides

**3.5 Brequinar analogues**

Boa *et al* synthesised a series of 2-phenylquinoline-4-carboxylic acid derivatives related to brequinar and evaluated them as inhibitors of *Pf*DHODH and antimalarial agents. **36** (Brequinar) itself binds to a channel in human DHODH but doesn’t inhibit *Pf*DHODH. Selected compounds **61-63** were evaluated for inhibition of *Pf*DHODH and they were found to be more active than **36** (brequinar) (Figure 9D). These new compounds selectively inhibit *Pf*DHODH over hDHODH. [[84](#_ENREF_84)] This study demonstrates that parasite-specific inhibitors can be identified and should serve as template for the design of more potent, selective inhibitors in the future. The positive selectivity results for **63** infers that these derivatives may bind in a totally different orientation to that of **36** (brequinar) or that they occupy a different site.

**3.6 *N*-Substituted salicylamides**

Fritzson *et al* have identified *N*-substituted amides of salicylic acid with moderate inhibitory activity against the *Pf*DHODH enzyme. The SAR of this class of compound was analysed and identified showing that the biphenyl ethyl substituted amide (compound  **65**) increased the enzyme inhibition 2-fold when compared to the phenyl ethyl amide (compound **64**). Further optimisation by substitution of salicylic phenyl ring with 5’-chloro group improved the enzyme binding affinity and maintained good selectivity for *Pf*DHODH inhibition (compound **66**) (Figure 9E).[[85](#_ENREF_85)]

**3.7 Trifluoromethyl Phenyl Butenamide derivatives (Leflunomide analogues)**

The authors, Fishwick C.W.G and Johnson A.P. *et al* have applied a software package, SPROUT-LeadOpt for structure-based drug discovery and lead optimisation to improve the binding of **35**, the active metabolite of Leflunomideby utilising the hydrogen bond potential of the ‛head-group’ and modifying the ‛tail’ to fill the hydrophobic pocket of the binding pocket in the *P.falciparum* and *human* DHODH. The methyl group was found to be optimal on the head group and for the tail group replacing the CF3 group with a phenyl ring improved the binding affinity which was further increased by introducing the electron withdrawing groups like Cl and CF3 on the biphenyl moiety. All these inhibitors had an enhanced levels of inhibition for both *Pf*DHODH and *h*DHODH enzymes compared to that of **35** (A77-1726). The majority of them were selective for *h*DHODH which may indicate that variations in the hydrophobic tail can at best give modest selectivity for *Pf*DHODH *vs* *h*DHODH (compound **68**)(Figure 9F). [[86](#_ENREF_86)]

**Figure 9:** Structures and SAR of current *Pf*DHODHinhibitors.

***Conclusion & future perspective***

The mitochondrial electron transport chain of *P.falciparum* is a validated drug development target as proven by the development of the *Plasmodium bc*1 inhibitor atovaquone and its current clinical use. More recent key developments within this area include the selection of ELQ-300, a *Plasmodium falciparum* *bc*1 inhibitor for preclinical development, the pre-clinical drug development of triazolopyrimidine based DHODH inhibitors and the publication of the first *Pf*NDH2 quinolone inhibitors. All three of the aforementioned programmes are currently within the MMV’s development pipeline.

The ETC is an attractive target owing to the multi-stage activity of its inhibitors, however barriers to development of novel inhibitors includes poor solubility/exposure, the likelihood of resistance development and safety (cardiotoxicity). The lesser investigated elements of the ETC such as SDH and ATP synthase may also provide alternative drug development pathways.

***Defined Key Terms***

**Reiske iron-sulfur protein** – components of cytochrome *bc*1 complexes and cytochrome b6f complexes which were first discovered and isolated by John S. Rieske and co-workers in 1964.[[87](#_ENREF_87)] It is a unique [2Fe-2S] cluster in that one of the two Fe atoms is coordinated by two histidine residues rather than two cysteine residues. They have since been found in plants, animals, and bacteria with widely ranging electron reduction potentials from -150 to +400 mV.

**Proton pumping –** a proton pump uses energy to transport protons from the matrix of the mitochondrion to the inner and outer mitochondrial membranes. It is an active pump that allows the formation of a [concentration gradient](http://en.wikipedia.org/wiki/Gradient) along the inner mitochondrial membrane, because there are more protons outside the matrix than inside. The proton pump does not create energy, but forms a gradient that stores energy for later use.

**Krebs Cycle –** is a series of chemical reactions used by all aerobic organisms to generate energy through the oxidization of acetate derived from carbohydrates, fats and proteins into carbon dioxide. In addition, the cycle provides precursors including certain amino acids as well as the reducing agent NADH that is used in several biochemical reactions.

***De novo* biosynthesis –** refers to the synthesis of complex molecules from simple molecules such as sugars or amino acids, as opposed to their being recycled after partial degradation.

**ADME** - is an acronym in pharmacokinetics and pharmacology for **a**bsorption, **d**istribution, **m**etabolism, and **e**xcretion, and describes the disposition of a pharmaceutical compound within an organism.

***Executive Summary***

**Introduction to the *Plasmodium* Mitochondrial Electron Transport Chain**

* The electron transport chain (ETC) of intraerythrocytic malaria parasites is believed to contain five dehydrogenases, namely NADH:ubiquinone oxidoreductase (PfNDH2), succinate:ubiquinone oxidoreductase (complex II or SDH), glycerol-3-phosphate dehydrogenase (G3PDH), the malate quinone oxidoreductase (MQO) and dihydroorotate dehydrogenase (DHODH).
* The dehydrogenase activity serves, at least in part, to provide electrons to the downstream complexes, namely ubiquinol:cytochrome c oxidoreductase (complex III or cytochrome *bc*1 and cytochrome *c* oxidase (complex IV) with ubiquinone (Coenzyme Q) and cytochrome c functioning as electron carriers between the complexes.
* The ATP synthase (complex V) is not reported to generate ATP (unlike its mammalian counterpart), but is nevertheless proposed as an essential component, possibly acting as a proton leak for the ETC.
* The review concentrates on the inhibition of *Pf*NDH2, cytochrome *bc*1 and *Pf*DHODH.

**Inhibition of *Plasmodium falciparum bc*1**

* Atovaquone is the only drug in clinical use that targets the electron transport chain. Its mode of action is known to be inhibition of the *bc*1 complex.
* New, low cost inhibitors are required that can overcome atovaquone resistance.
* Drug development programmes based around hydroxynapthoquinones, pyridones, acridinediones, acridones and most recently quinolones (ELQ300) are discussed.

**Inhibition of *Plamodium falciparum* Type II NADH: Quinone Oxidoreductase (*Pf*NDH2)**

* Inhibition of *Pf*NDH2 is relatively under explored with quinolones bring the main area of drug development.
* Inhibitors of *Pf*NDH2 have been shown to have a dual mechanism of action which may provide an advantage in the fight against resistance.

**Inhibition of *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH)**

* Triazolopyrimidine based DHODH inhibitors have been identified as pre-clinical drug development candidates.
* Other classes of inhibitors under development include benzimidazoyl thiophene-2-carboxamides, *S*-benzyltriazolopyrimidines, biaryl carboxamides, brequinar analogues, *N*-substituted salicylamides and leflunomide analogues.

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