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New Opportunities in the Structure-based Design of Anti-Protozoan Agents

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Abstract: Since the recent renaissance of phenotypic screening in the field of protozoan drug discovery, is there still an opportunity for the structure-based design of new anti-protozoan agents? Target-based approaches should be used in parallel to phenotypic screening to strengthen the pipeline of anti-protozoan agents. We give an overview of the protozoan drug discovery landscape highlighting four protein targets of interest: cytochrome bc_1, dihydroorotate dehydrogenase, dihydrofolate reductase and calcium-dependent protein kinase 1. We discuss recent structure-based design efforts to inhibit these targets, reviewing their crystal structures and their ability to accommodate potent and selective compounds. Finally, we discuss future opportunities to apply structure-based methods to promising molecular targets within protozoan parasites discovered using chemical genomics.

Keywords: Apicomplexan drug discovery, CDPK1, Cytochrome bc_1, DHFR, DHODH, Structure-based design.

Apicomplexan parasites and the challenges of protozoan drug discovery

The phylum Apicomplexa is composed of a highly diverse group of obligate intracellular parasites which are responsible for a number of human infectious diseases including babesiosis (Babesia), malaria (Plasmodium), cryptosporidiosis (Cryptosporidium parvum), Cyclosporiasis (Cyclospora cayetanensis), Isosporiasis (Isospora belli) and toxoplasmosis (Toxoplasma gondii) [1, 2]. These parasites actively invade and replicate within host cells, modifying their host cells to prevent immune clearance and increase survival and dissemination [3]. Such diseases effect >500 million people worldwide each year, mainly in the developing world, resulting in 438,000 deaths for malaria alone in 2015 [4].

One of the challenges in targeting apicomplexan parasites is that they have multiple life-stages of infection within their human host [5]. This has implications when selecting molecular targets as not all validated targets will remain essential across all relevant life stages since metabolic states and cell machinery can differ [6]. The majority of drug discovery programmes targeting protozoa, a broad definition of human parasites of Apicomplexa, has centred on treatments for malaria. Together with toxoplasmosis and cryptosporidiosis, which share some antimalarial drug targets, these parasitic diseases affect human health to a serious degree and will therefore be the focus of this review.

Malaria is caused by infection with one of the species in the genus Plasmodium that are transmitted to humans via a bite from the female Anopheles mosquito [7]. Of the six parasite species that infect humans [5], Plasmodium

falciparum (Pf, 60-75% of the global malaria burden) and Plasmodium vivax (Pv, 25-40% of global burden) are responsible for the majority of malaria cases. The symptoms associated with malaria such as fever, joint pain, vomiting, anaemia, and chills can be attributed to the blood stages of the replication cycle of the parasite. The majority of deaths are due to cytoadherance of infected erythrocytes to the inside of capillaries causing a blockage of blood flow, which is particularly life-threatening if cerebral [8].

Toxoplasma gondii is a readily transmitted, promiscuous parasite that is estimated to affect up to a third of the world's population [9]. Although infections in humans are usually asymptomatic and self-limiting within immuno-competent individuals, they are potentially fatal in immuno-compromised patients (AIDS, cancer, organ transplant) [10]. Toxoplasmic encephalitis (TE) is a leading cause of death amongst AIDS patients when HAART is not obtained or affordable [11]. Transmission to humans is usually via an environmental route (e.g. ingesting contaminated meat/water), but transplacental transmission can occur in pregnant women leading to congenital infections with serious consequences for the unborn baby: miscarriage/still-birth and chronic degradative ocular lesions. Again, the existence of multiple life stages of the T. gondii parasite within the human host presents a challenge in treating toxoplasmosis [6]. Although the tachyzoite stage is responsible for the acute symptoms, it is the bradyzoite stage, responsible for forming highly resilient cysts in the brain and central nervous system (CNS), which poses the greatest challenge regarding treatment, as without elimination of these cysts recrudescence of the disease is possible. The chronic nature of the infection can cause serious complications when combined with the long term nature of many immuno-compromising conditions, as many patients may develop intolerance to treatments. Current treatments of the acute infection are largely based upon antimalarial agents including combinations of pyrimethamine & sulfadiazine, spiramycin and atovaquone (Table 1) which target the tachyzoite stage of the infection [12]. There are currently no effective treatments to eliminate bradyzoites.

Another opportunistic apicomplexan is Cryptosporidium which can be life-threatening in an immuno-compromised individuals and young children; it is responsible for approximately 15-20% of cases of diarrhoea in African children [13]. Unlike malaria, this parasite does not require another organism to act as an infection vector, with transmission largely a result of environmental contamination, particularly contaminated water. Unlike Plasmodium and Toxoplasma, the basic biology of Cryptosporidium is poorly understood and drug screening is difficult [14]. Clinical treatment for cryptosporidiosis is limited to one FDA-approved drug, nitazoxanide (Table 1), which is not effective in immuno-compromised patients [2].

Drug	Target	Efficacy [5, 15]	Combination Partner
Malaria		EC_{50} (strain)	
Artemisinin Artemether Artesunate	Unknown, several proposed mechanisms of action including interference with parasite heme-detoxification pathways and inhibition of PfATP6.	15 nM (Dd2) 3.1 nM (K1)	
Lumefantrine	Unknown	0.45 nM (W2)	Artemether
Sulfadoxine	Dihydropteroate synthetase (DHPS)	30 nM (3D7)	Artesunate or pyrimethamine
Pyrimethamine	Dihydrofolate reductase (DHFR)	23 nM (3D7)	Artesunate and sulfadoxine
Quinine[#] Amodiaquine* Mefloquine* Pyronaridine* Chloroquine* Piperaquine*	Not fully elucidated; believed to be related to hemoglobin degradation.	10.8 nM (W2) 4.4 nM (W2) 7 nM (W2) 9.5 (W2) 19 nM (3D7) 12.5 nM (W2)	[#]Dihydroartemisinin or *artesunate
Proguanil	Disrupts the inner membrane potential of the mitochondria.	7400 nM	Atovaquone
Atovaquone	Cytochrome bc_1 complex (III)	0.53 nM (W2)	Proguanil
Toxoplasmosis			
Pyrimethamine	See Malaria	0.8 μM (RH)	Sulfadiazine or clindamycin [16]
Atovaquone	See Malaria		
Spiramycin	Ribosome	23 μM (RH)	
Cryptosporidiosis			
Nitazoxanide [17]	Interference with the pyruvate:ferredoxin oxidoreductase (PFOR)	3.9 μM [18]	

Table 1. Frontline treatments for infections by the apicomplexans Plasmodium, Toxoplasma and Cryptosporidium.

Many of the first-line treatments for these apicomplexan parasites (Table 1) suffer from major drawbacks including the spread of resistance to anti-malarial drugs, poor tolerance of drugs to treat toxoplasmosis and a lack of effective medicines for cryptosporidiosis. Currently available drugs are unable to treat the dormant form of these parasitic diseases (e.g. P. vivax hypnozoites/T. gondii bradyzoites). To address this therapeutic need and to keep pace with the threat of drug resistance, it is essential that protozoan drug discovery programmes develop new medicines with either novel scaffolds and/or modes of action.

A lack of drug targets and the renaissance of phenotypic screening

There are few clinically validated targets for known anti-protozoan agents [19, 20]. Despite many efforts during the genomic era in target-based high-throughput screening for the discovery of novel anti-infectives, most newly-identified targets proved unsuccessful as they were not essential for the survival of the parasite [21]. Consequently,

much of the recent drug discovery progress for the treatment of protozoan infections has focussed on phenotypic screening [22, 23]. Pharmaceutical companies such as GlaxoSmithKline (GSK) and Novartis, together with St Jude Children's Research Hospital, have released phenotypic screening data against the blood stage of Plasmodium in the hope of seeding antimalarial drug discovery [24]. Over six million compounds have been tested to date, 25,000 of which had IC_{50} values <1 µM against Plasmodium [25]. The pinnacle achieved by this research is the creation of the 'Malaria Box' and the 'Pathogens Box' from Medicines for Malaria Venture (MMV). Each is a collection of 400 drug-like compounds with either potent antimalarial activity, selected from the 25,000 antimalarial hits [26], or anti-protozoan activity, selected as interesting leads against a variety of pathogens by a panel of experts. These 'boxes' are freely available upon request and have been screened in vitro against many molecular targets by the worldwide scientific community in order to establish the mode of action (MoA) of these phenotypic hits. These datasets also seed drug discovery against other protozoan parasites, T. gondii and Entamoeba histoltica [27], Cryptosporidium [28], Trypanosomatids [29] as well as chemogenomic efforts to identify targets through machine learning techniques [19].

A recent publication [30] relating to the theme of this article is the identification of MMV008138 from the Malaria Box which targets isoprenoid biosynthesis via the non-mevalonate (MEP) pathway. This biosynthetic pathway is inhibited by the antibiotic fosmidomycin (**1**, Fig. 1), a potent antimalarial, targeting the second enzyme 1-deoxy-d-xyluose-5-phosphate reductoisomerase (IspC) in this pathway [31]. Whilst the production of isoprenoid precursors has previously been identified as the only essential function of the apicoplast organelle in P. falciparum blood-stage parasites [32], MMV008138 (**2**, Fig. 1) is the first inhibitor of P. falciparum 4-diphosphocytidyl-2C-methyl-d-erythritol cytidylyltransferase (PfIspD), the third enzyme in the pathway, to be identified [33]. Unfortunately, no crystal structure of PfIspD has yet been solved, although structures do exist for Mycobacterium tuberculosis and Escherichia coli IspD [34]. IspD has been less extensively studied than IspC, the target of fosmidomycin, but is known to possess a polar active site, common amongst MEP enzymes [34]. An interesting feature of IspD from the eukaryote Arabidopsis thaliana is the identification of an allosteric pocket adjacent to the active site which binds 7-hydroxytriazolopyrimidine compounds [35] (**3**, Fig. 1) highlighting the possibility that MMV008138 acts on PfIspD through a similar MoA.

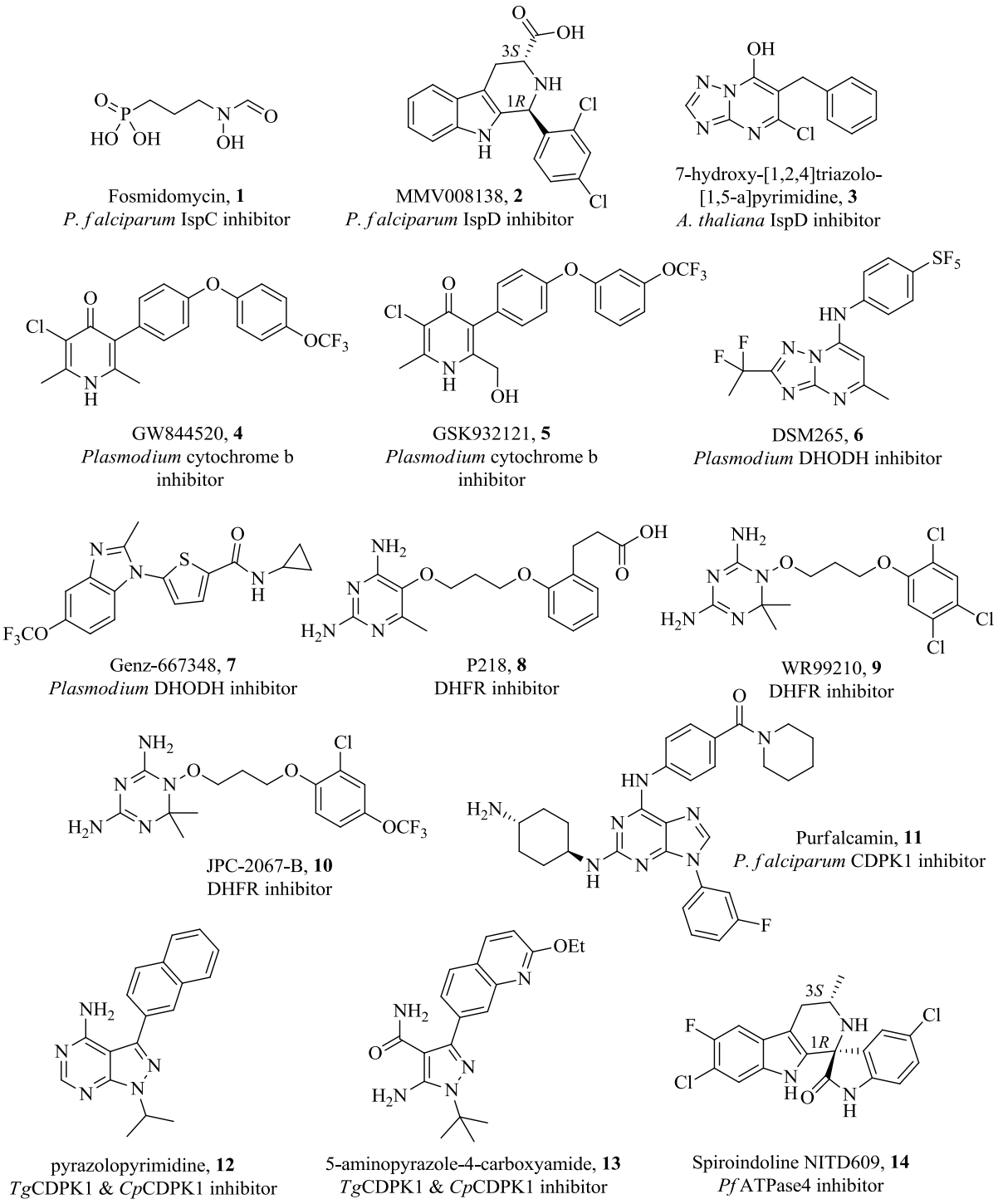

Fig. (1). Structures of inhibitors mentioned in this review.

Opportunities for structure-based design in the discovery of antiprotozoal agents

Structure-based techniques use the structural knowledge of a protein target to drive drug discovery and development by optimising both enzyme and parasite inhibition in parallel, whilst controlling selectivity if a human orthologue is present. The advantage here is that a design hypothesis can be defined from the outset and a smaller number of compounds need only be synthesised. This type of target-based approach is most successful

when paired with molecular targets that have been validated, usually via phenotypic screening. Target-based approaches can help develop drugs that attack other stages of a parasite's life cycle [25]. This is important in the context of treating Plasmodium and Toxoplasma since there is a need for new medicines to block reactivation by dormant parasites in the liver (P. vivax hypnozoites) or central nervous system and brain (T. gondii bradyzoites).

Herein, we describe four targets of interest to the structure-based community within parasite drug discovery: cytochrome bc_1, dihydroorotate dehydrogenase, calcium-dependent protein kinase 1 and dihydrofolate reductase. These represent current targets to be pursued in the development of next generation of anti-protozoan drugs. A key feature of these molecular targets is that they are not specific to one type of apicomplexan parasite.

Cytochrome bc_1 (complex III)

Cytochrome bc_1 is the third complex in the mitochondrial electron transport chain (mtETC) which has been the focus of intense drug discovery research over the last 10 years [36]. The mtETC in Plasmodium is responsible for (i) maintaining an electro-potential across the inner membrane necessary for transport and (ii) regeneration of ubiquinone to support pyrimidine biosynthesis [37]. Both are essential functions which, when inhibited, result in parasite death. Cytochrome bc_1 (complex III) carries out energy transducing electron transfer through the reduction and oxidation of ubiquinone and ubiquinol respectively. The redox recycling of ubiquinone (CoQ) is called the Q-cycle which is catalysed by the two active sites present in the cytochrome b subunit, known as the Q_o (oxidation) and Q_i (reduction) sites respectively [38]. Both substrate binding sites are located within the hydrophobic transmembrane portion of the complex, on opposite sides of the cytochrome b subunit. Therefore, short proton transfer pathways are required for proton uptake and release during ubiquinone/ubiquinol redox-coupled reactions.

The antimalarial drug atovaquone inhibits cytochrome bc_1 at the Q_o site, a therapeutic target amongst apicomplexan parasites. It is used as part of the combination therapy Malarone with proguanil (Table 1) for the prophylactic treatment of malaria. Atovaquone is also an effective inhibitor of T. gondii in a murine model [39] and for the treatment of babesiosis [40]. The central ring system is a 2-hydroxyquinone which is an analogue of the substrate ubiquinone. However, mutations at the Q_o site which cause drug resistance are seen with a high frequency [41].

In 2015 Capper et al. [42] solved two bovine cytochrome bc_1 crystal structures (PDB ID 4D6T and 4D6U) bound to GSK's clopidol derivatives GW844520 and GSK932121 [43] (**4** and **5**, Fig. 1), determining that these

compounds bind at the Q_i (reduction) site (Fig. 2A). This discovery confirms that the Q_i site is druggable and facilitates new structure-based design opportunities at this binding site. Many recent compound series based on 4-(1H)-quinolone/4-pyridone scaffolds are also proposed to bind to the Q_i site [44]. This motif appears to be a privileaged scaffold for the inhibition of cytochrome bc_1 in both Plasmodium and Toxoplasma [44-48]. There are a number of advantages to targeting the Qi site: (i) targeting a novel site within a validated target increases confidence that inhibition of this target will have the desired phenotypic response; (ii) there are no existing resistance mechanisms. This has been demonstrated with various putative Q_i inhibitors which show no cross resistance to atovaquone-resistant parasites (Tm90-C2B) [45].

Analysis of the Q_i site within the bovine crystal structures (4D6T and 4D6U) suggests an amphiphilic nature: a deep narrow pocket flanked by residues with the ability to hydrogen bond, next to a hydrophobic pocket adjacent to a solvent-exposed region (Fig. 2). Within the hydrophilic environment, the amino acid residues Asp-228 and His-201 are seen to form hydrogen bonds with GW844520/GSK932121 in the bovine crystal structure. There would also appear to be an opportunity for a hydrogen bonding interaction with the Ser-205 (Fig. 2A).

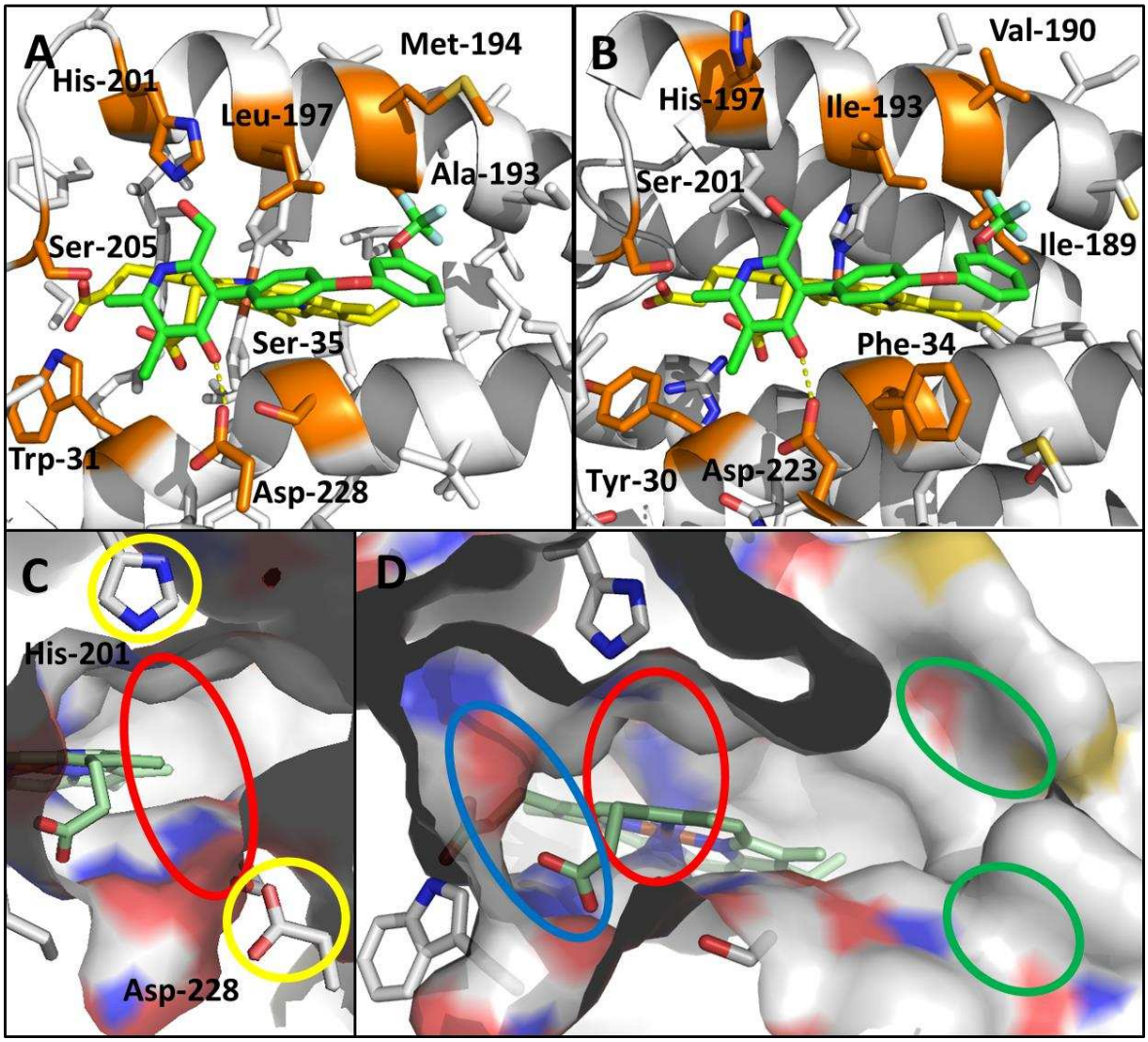

Fig. (2). (A) The crystal structure of GSK932121 (**5**, green) bound within bovine Q_i site with residues in closest proximity (orange). (B) Inhibitor GSK932121 (**5**, green) positioned inside a homology model of the T. gondii Q_i site. (C) 4-(1H)-Pyridone binding pocket (red) with key binding residues, His-201 and Asp-228 (yellow). (D) Q_i site pocket showing areas occupied by larger 4-(1H)-quinolones (blue), adjacent to the binding site of the 4-(1H)-pyridone motif (red), and the two hydrophobic pockets (green).

The overall sequence identity between the bovine cytochrome bc_1 crystal structure and the human orthologue is high (~70%) but a much lower sequence conservation exists between the mammalian and parasite cytochrome structures (~40%). Within the Q_i site sequence identity between mammalian and parasite is comparable to the complex as a whole (~40%) (Fig. 3). Analysis of the 4D6U/4D6T crystal structures revealed that the most significant difference is Ser-35 in bovine (and human), which makes a hydrogen bond with the carbonyl oxygen of the 4-pyridone ring (Fig. 2B). This amino acid residue is Phe-30/34 in Plasmodium/Toxoplasma and may have significant effects on ligand binding conformations. This is supported by work on a similar series of compounds based upon a larger quinolone scaffold. In these compounds, it was observed there was a delicate balance between

compounds inhibiting at the Q_o site and the Q_i site, and that this could be effected by subtle changes in the compounds structures. However, the rationalisation discussed was based on a 'flipped' conformation of the inhibitor within the binding site which was based on a homology model of the Plasmodium cytochrome bc_1 [44]. In this 'flipped' conformation the NH of the quinolone interacted with Asp-223 as opposed to His-197. Obtaining a crystal structure for either Plasmodium or Toxoplasma with a bound inhibitor would determine the precise mode of binding.

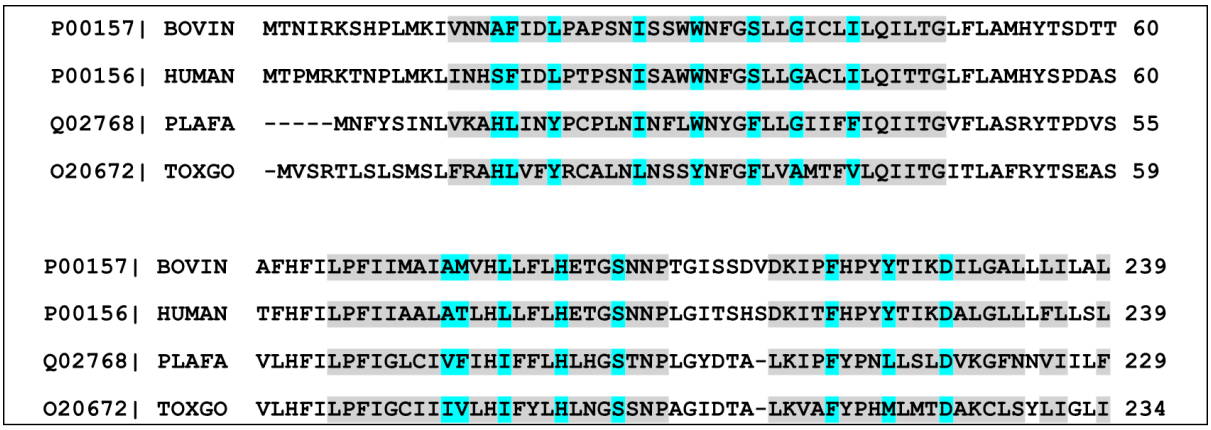

Fig. (3). Sequence alignment of the Q_i site from bovine, human, Plasmodium and Toxoplasma species. Grey residues form the binding site (within 20 Å of a bound inhibitor) with blue residues in closer proximity (within 4 Å).

A promising result of the Q site selectivity of 4-(1H)-quinolone compounds was the discovery that inducing resistance for the Qi specific compounds was challenging [49]. These observations strongly suggest that either a mutation within this site must carry a high fitness cost or that there is limited capacity within this site for viable mutations. Both of these conclusions are promising indicators of a reduced propensity for the development of resistance in the enzyme itself. If this is the case the possibility of a combination of Q_i and Q_o inhibitors may require an infeasible fitness cost for the parasite target to develop resistance [42].

Overall, the discovery of inhibitors selective for this site, the availability of high resolution crystallographic data, an indication of a low propensity to develop resistant mutations and its existence as a validated target for protozoan parasites, makes this a promising new target with a great deal of potential for a structure-based design approach.

DHODH – targeting pyrimidine biosynthesis using structure-based methods

Dihydroorotate dehydrogenase (DHODH) catalyses the fourth and rate-limiting step in the de novo pyrimidine biosynthesis pathway. Pyrimidines are essential metabolites required for DNA and RNA biosynthesis and are acquired through either a salvage or de novo pathway. Whilst higher eukaryotes use both pathways to significant levels, the picture is more complex amongst apicomplexan parasites. Plasmodium only uses the de novo pathway highlighting its essential function [7], whereas Cryptosporidium is deficient in de novo capabilities and relies on the salvage pathway [14]. Toxoplasma utilizes both pathways and inhibition of the de novo pathway leads to a reduction in acute virulence of the T. gondii parasite in mice, highlighting its potential as a point of chemotherapy [50]. To date, due to the essential nature of the de novo pathway, only Plasmodium DHODH has been the focus of significant drug discovery research. This has been facilitated by the availability of crystal structures and the subtle differences between the human and P. falciparum DHODHs which allow the development of selective inhibitors [51, 52]. Inhibition of T. gondii DHODH has been investigated using a cross-section of known DHODH inhibitors, but as yet only modest inhibitors have been identified and with no selectivity for TgDHODH [53]. A crystal structure of TgDHODH would greatly aid its potential as a target for chemotherapy.

Since the first crystal structure of PfDHODH was solved (PDB ID 1TV5) [54], a number of research groups have embarked on the structure-guided design and optimisation of PfDHODH inhibitors [8, 52, 55, 56]. The most advanced programme has been the development of triazolopyrimidines as potent PfDHODH inhibitors (IC_{50} <50 nM) conducted by Phillips at the University of Texas Southwestern, in collaboration with Monash University, GSK Tres Cantos and MMV [57]. This has led to DSM265 (**6**, Fig. 1) which has just completed a Phase II clinical trial, exemplifying the potential of target-based approaches to the field of antimalarial drug discovery. The development of this compound has been well documented elsewhere and readers are directed to the latest publication [58] for more information. Despite the success of DSM265 (**6**), back up PfDHODH inhibitors with novel chemical structures are required to mitigate for the potential shortcomings of DSM265 during clinical trials. A research programme at the University of Leeds is being conducted to address this, using structure-based de novo design tools.

The binding site of PfDHODH inhibitors is dominated by hydrophobic contacts (Fig. 4A). Key hydrogen-bond contacts used by the triazolopyrimidines and other chemical series are the amino acid residues Arg-265 and His-185, highlighted in Figure 4A. Two inhibitor classes have been crystallised with PfDHODH to date: the aforementioned triazolopyrimidines (PDB ID 3SFK) [59] and the thiophene-2-carboxamides (PDB ID 3O8A)

[60], The structures reveal that the inhibitor binding pocket is directly adjacent to the FMN cofactor (shown in red in Fig. 4A) which can be divided into two portions: (i) the space immediately next to the FMN which is generally fully occupied by structurally-diverse PfDHODH inhibitors (i.e. the triazolopyrimidine ring in Fig. 4B, black circle). The hydrogen-bond contacts Arg-265 and His-185 are located in this space, as well as a number of hydrophobic contacts Val-532, Ile-272 and Ile-273 (white residues/surface, Fig. 4); (ii) a hydrophobic pocket either above (blue circle) or adjacent (green circle). The hydrophobic pocket above the triazolopyrimidine ring is occupied by a substituted aniline which makes van der Waal contacts with Leu-240, Leu-531 and Met-536, and an edge-on π-stack interaction with Phe-227 (blue residues/surface, Fig. 4). The pocket highlighted by the green residues/surface contains the benzimidazole group of Genz-667348 (**7**, Fig. 1) [61], which interact with Tyr-168, Leu-172 and Cys-175 via van der Waal contacts. The difference in binding between these two chemical series is governed by the plasticity of the inhibitor binding site and in particular residue Phe-188. The differing position of this residue in the two crystal structures is highlighted in Figure 4A, where its position in 3O8A (green) is located within the hydrophobic pocket which accommodates the aniline ring of the triazolopyrimidine series. Conversely, it is flipped down towards the benzimidazole pocket in 3SFK (blue). This structural knowledge can play a crucial role in designing future inhibitors that have advantages over the current generation of compounds. Finally, given the described plasticity of the inhibitor binding site, new and alternative binding modes could also be discovered through further cycles of high throughput screening (HTS) against PfDHODH and solving the co-crystal structures of hit compounds.

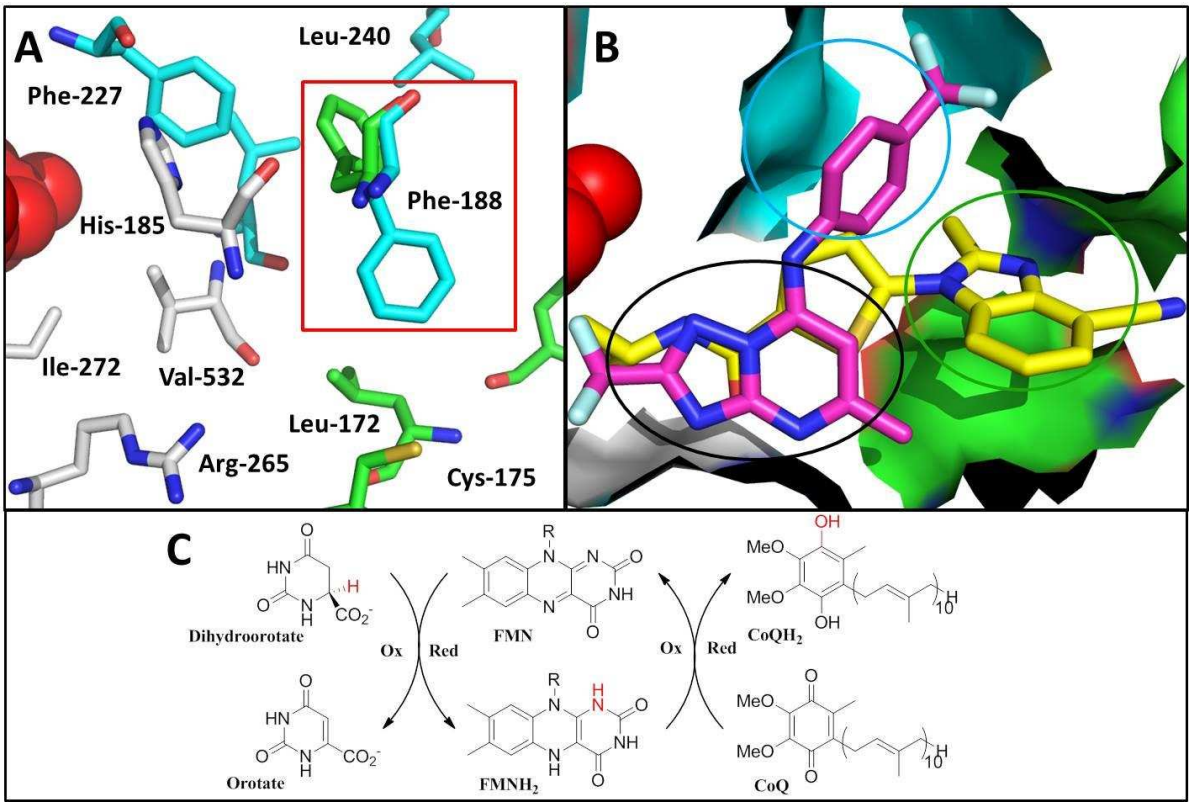

Fig. (4). PfDHODH inhibitor binding site highlighting key features. (A) Residues involved in binding multiple compound series are shown in white, including the hydrogen bond contacts, Arg-265 and His-185. Residues involved in binding the triazolopyrimidine and thiophene-2-carboxamide series are shown in blue and green respectively, FMN cofactor in red. The orientation of the Phe-188 residues from two crystal structures has been highlighted with a red box. (B) Overlay of the two chemical series showing the common binding pocket (black circle) and points of diversion (blue and green circles). The residue colour coding is matched between panels (A) and (B). (C) Redox reactions catalysed by DHODH via a 'ping-pong' mechanism. CoQ is provided by cytochrome bc_1.

DHFR – revival of a resistance-compromised target

Dihydrofolate reductase (DHFR) is a clinically validated antimicrobial and anticancer drug target [41]. DHFR inhibitors such as pyrimethamine (Table 1) have also been the mainstay for treating Plasmodium, Toxoplasma and Eimeria apicomplexan parasite infections. DHFR catalyses the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) via the oxidation of coenzyme NADPH. Tetrahydrofolates are essential building blocks for the de novo synthesis of purines, amino acids and thymidylic acid required for the proliferation of many protozoan parasites [62]. An unusual feature of DHFR in apicomplexans is that it is a bifunctional protein, associated with thymidylate synthetase (TS), whereas these are separate monofunctional enzymes in humans [41]. The dhfr-ts gene which encodes for this bifunctional protein is present in Plasmodium, Toxoplasma and Cryptosporidium [14].

Pyrimethamine-resistance in P. falciparum is widespread and is particularly severe in endemic areas [23]. Clincial isolates of P. falciparum indicate a combination of four point mutations (N51I, C59R, S108N and I164L) which leads to a reduction in the binding affinities of pyrimethamine. The crystal structures of the wild type, double mutant (C59R/S108N) and quadruple mutant (N51I/C59R/S108N/I164L) P. falciparum DHFR enzymes were solved by Yuvaniyama et al. [63] revealing the S108N mutant to make close contacts with the NADPH cofactor and rigid p-chlorophenyl group of pyrimethamine (Fig. 5A). Armed with this structural information Yuthavong et al. [20] developed the preclinical candidate P218 (**8**, Fig. 5B and C) which is highly efficacious, orally available and inhibits both wild-type and clinically relevant mutated forms of PfDHFR (K_i values 11.6 and 7.4 nM respectively). This seminal work sets the precedent for future generations of parasite DHFR inhibitor to combat resistance.

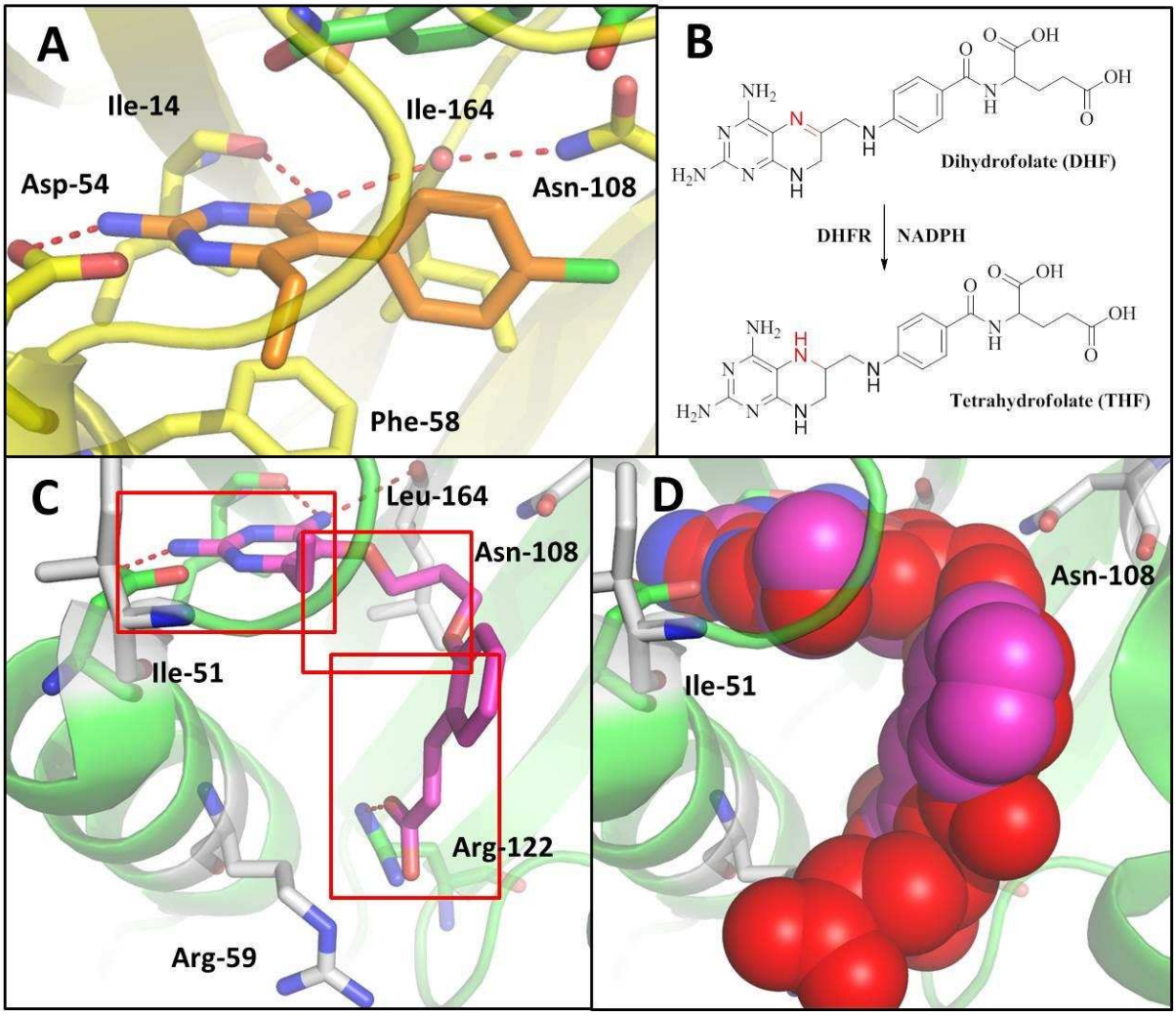

Fig. (5). PfDHFR inhibitor binding of pyrimethamine and P218 (**8**). (A) Binding of pyrimethamine (orange) to double mutant PfDHFR (C59R/S108N) showing hydrogen bond contacts (red) and the close proximity of N108 to NADPH (green) and the p-chlorophenyl ring of pyrimethamine (orange). (B) Reaction catalysed by DHFR. (C) Binding of P218 (**8**, purple) to

quadruple mutant (N51I/C59R/S108N/I164L) showing the three key aspects of next-generation PfDHFR inhibitors: heterocycle, flexible linker and selectivity pocket (red boxes). (D) Overlay of dihydrofolate (red) and P218 (purple) showing how the binding of P218 is within the substrate envelop. The orientations of PfDHFR are consistent throughout.

The DHFR binding site can then be divided into three section: i) the hetercyclic region ii) the flexible linker iii) the selectivity pocket (Fig. 5C). Many diamino-heterocycles can served as good scaffolds for DHFR inhibition (triazines, pyrimidines, pteridines, quinazolines and pyridopyrimidines) by mimicking the pteridine ring of the dihydrofolate substrate. The propyldiether linker present in P218 (**8**) was first seen on WR99210 (**9**, Fig. 1), a triazine-based PfDHFR inhibitor, the development of which was terminated due to low bioavailability and toxicity [64]. This flexible linker is crucial in retaining activity against mutant parasite DHFRs by avoiding a steric clash with S108N. The selectivity pocket contains a conserved Arg-122 (Arg-70 in human DHFR) which binds P218 (**8**) via charge-mediated hydrogen bonds [20]. There are subtle differences in the residues surrounding Arg-122 which allow scaffolds to be fine-tuned for selectivity of PfDHFR over the human orthologue, whilst maintaining this hydrogen bond contact.

Pyrimethamine is a potent inhibitor of T. gondii DHFR (IC_{50} 140 nM) and is used as the frontline treatment for toxoplasmosis. WR99210 (**9**) and related triazine JPC-2067-B (**10**, Fig. 1) are have been investigated as highly effective inhibitiors of TgDHFR (IC_{50} <10 nM) and T. gondii in vitro and in vivo [65, 66]. JPC-2067-B (**10**) is more efficacious than pyrimethamine against T. gondii tachyzoites and is cidal in cell culture. All three T. gondii clonal types and isolates (RH, Me49 and VEG) have identical amino acid sequences for TgDHFRs, highlighting its conserved nature as a promising target for anti-Toxoplasma agents [66].

The key to successfully identifying novel DHFR inhibitors such as P218 (**8**) lies with designing compounds which bind within the substrate envelope (Fig. 5D) [20]. This is broadly defined as the three-dimensional space filled by the dihydrofolate (DHF) substrate. Any mutations of residues proximal to this envelope are likely to arise at a fitness cost since they may effect binding of the substrate [67]. This detailed structural knowledge of PfDHFR can inform the next generation of DHFR inhibitors by providing a rational design principal, with the goal of delaying the on-set of DHFR resistance.

Calcium-dependent protein kinase 1

Calcium-dependent protein kinases (CDPKs) play a crucial role in the activation of the calcium-signalling pathways and the regulation of the life cycle in many apicomplexan parasites, including Plasmodium, Toxoplasma

and Cryptosporidium [68]. Whilst apicomplexans contain a diverse family of CDPKs, several individual protein kinases such as CDPK1 and CDPK4 have important roles [69]. Kato et al. [70] determined that the pfcdpk1 gene was essential for parasite viability and have performed a target-based HTS campaign against P. falciparum CDPK1 to identify 2,6,9-trisubstituted purine-based, ATP competitive inhibitors (**11**, Fig. 1) [23]. CDPK4 has been shown to be essential for the sexual reproduction and mosquito transmission of P. berghei [71]. The T. gondii and Cryptosporidium parvum orthologues of Plasmodium CDPK4 are TgCDPK1 and CpCDPK1 [72]. These are attractive targets in Toxoplasma and Cryptosporidium since there is no direct mammalian orthologue in humans and they contains a small 'gatekeeper' residue which is rare in mammalian kinases[73] and canonical CDPKs (and all protein kinases in T. gondii) [74]. The availability of TgCDPK1 and CpCDPK1 crystal structures facilitates the structure-based design of antiprotozoal agents against these protein targets.

Substituted pyrazolopyrimidines (**12**, Fig. 1), so-called 'bumped kinase inhibitors', have been identified as attractive compounds for further development as antiprotozoal agents since TgCDPK1 is sensitive to inhibition by pyrazolopyrimidine derivatives (IC_{50} <100 nM) but mammalian protein kinases show very low sensitivity [74]. Ojo et al. published the X-ray crystal structures of two pyrazolopyrimidines (**12**) in complex with TgCDPK1 (PDB IDs 3I7C and 3I7B) [75]. The 4-amino group and N-5 of the pyrazolopyrimidine scaffold bind to the hinge-binding region, as a donor and acceptor respectively (Fig. 6A). The Ar and R groups fill the hydrophobic pocket and ribose binding pocket respectively and are key for the selective inhibition of parasite CDPK1s. Between these pockets is the 'gatekeeper' residue Gly-128. This small 'gatekeeper' residue is unique to T. gondii and C. parvum which allows inhibitors to access the Ar hydrophobic pocket located at the rear of the CDPK1 catalytic cleft (Fig. 6A). In most mammalian kinases, access to this hydrophobic pocket is severely restricted due to a larger 'gatekeeper' residue.

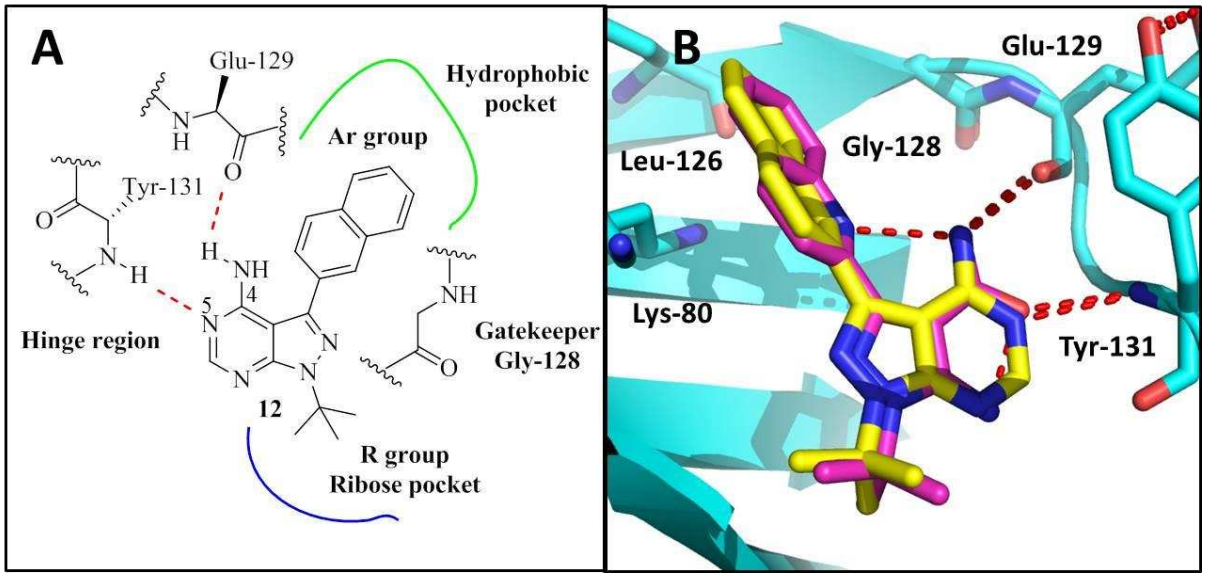

Fig. (6). TgCDPK1 in complex with pyrazolopyrimidine (**12**) and 5-aminopyrazole-4-carboxyamide (**13**). (A) Schematic of pyrazolopyrimidine (**12**) showing key features of binding to TgCDPK1. (B) Overlay of **12** (yellow) and **13** (pink) within the ATP binding site of TgCDPK1 (blue) using PDB IDs 3I7C and 4M84.

Zhang and co-workers exploited this structural knowledge to 'scaffold-hop' from the pyrazolopyrimidine (**12**) to a 5-aminopyrazole-4-carboxyamide (**13**, Fig. 1) scaffold [76]. This compound series was rationally designed to mimic the pyrazolopyrimidines, retaining the relative orientations of the Ar and R groups and maintaining the hydrogen bond contacts (Fig. 6). The 4-carboxamide group replaces the 4-amino/N-5 of the pyrazolopyrimidine scaffold, with a 5-amino group forming an intramolecular hydrogen bond to orientate the 4-carboxamide group into a favourable conformation. This compound series displayed potent inhibition of both TgCDPK1 and CpCDPK1 (IC_{50} <100 nM), with select compounds showing >1000 fold selectivity against the Src kinase family, low toxicity to mammalian cells (EC_{50} >30 μM) and with submicromolar activity against T. gondii. A representative 5-aminopyrazole-4-carboxyamide from the series was crystallised with TgCDPK1 (PDB ID 4M84) to confirm the design rationale [76]. An overlay of the both compound series is shown in Figure 6B. Further work to improve the physiochemical and pharmacokinetic properties of the 5-aminopyrazole-4-carboxyamides has been described [73], and indicates that inhibition of this target holds great promise in chemotherapy strategies for treating Toxoplasma and Cryptosporidium.

Conclusion and future opportunities for target-based approaches

The success of targeting DHFR and DHODH using structure-based design continues to strengthen the antimalarials pipeline, but new targets are also required to help combat resistance. The sequencing of the Plasmodium genome promised new apicomplexan targets, but as yet none have become clinically relevant. Cell-

based screening is one way of finding new molecular targets; [22] the strength of this approach relies on the continual development of forward chemical genomics. As the protein target of a small molecule discovered in a cell-based screen is initially unknown, the elucidation of which protein is inhibited requires further experimentation. Current chemical genomics methods include the pairing of phenotypic screening with in silico compound activity profiling [77] and mode of action studies [78]. In silico profiling uses a guilt-by-association principle to predict which cellular pathways and/or protein targets is being inhibited by a selected compound, by comparing against known actives. Mode of action studies use genetically modified and drug-resistant parasites strains to identify compounds with cross-resistance to known actives/drugs profiles. Further investment by the chemistry and biology community into forward chemical genomics approaches would be hugely beneficial for protozoan drug discovery.

A recent example is the discovery of spiroindolone NITD609 [79] (**14**, Fig. 1) which blocks protein synthesis by inhibiting P-type cation-transporter ATPase4 (PfATP4). Rottmann et al. optimised NITD609 (**14**) from a phenotypic screening hit from a library of natural products into a preclinical candidate in 3 years [22] before the target was identified. PfATP4 is also the target of other chemically distinct series, such as the aminopyrazoles, therefore highlighting its potential as a critical antimalarial drug target [80]. Zhou et al. has explored ATPase4 in T. gondii showing that spiroindolone NITD609 (**14**) is cidal for tachyzoites in vitro (IC_{50} 1 μM), reduces parasite burden in mice by 90% at 100 mg/kg (b.i.d) and that T. gondii likely shares an ATP4 target [81]. While crystal structures of ATP4 are known, the inhibitor binding site(s) are yet to be determined which would facilitate structure-based design.

Structure-based drug design remains an important approach in apicomplexan targeted drug discovery. The targets described above are all amenable to structural methods of inhibitor design. This approach can provide the creation of novel structural inhibitors using bespoke design tools, chemical probes for compound screening and can identify second-generation compounds that can overcome resistance. Continual research into neglected diseases such as apicomplexan infections addresses unmet clinical needs for both the developed and developing world.

Conflict of Interest

The authors declare no conflicts of interest. The authors thank the following for funding: University of Leeds 110 Anniversary Scholarship (JAG), Medical Research Council (MJM).

List of Abbreviations

b.i.d. = bis in die (twice a day)

FDA = Food & Drug Administration

HAART = Highly active antiretroviral therapy

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