Structure-Based Drug Discovery for *Plasmodium falciparum*

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Abstract: X-ray crystallography is a technique which is finding increasing utility in the effort to find new antimalarial drugs. This is in spite of the serious difficulties often encountered in obtaining sufficient quantities of protein to crystallize. This review provides an overview of the *Plasmodium falciparum* proteins which have been crystallized with bound inhibitors and the methodology employed in the heterologous expression of these proteins. Lactate dehydrogenase, plasmepsin II, and triosphosphate isomerase are the most advanced targets of structure-based drug design, but nine other *P. falciparum* proteins have been crystallized with inhibitors as well, and this is clearly an area which is moving very quickly. Some consideration will also be given to the limitations of structure-based drug discovery with respect to known antimalarial drugs.

Keywords: Adenylosuccinate synthetase, PfAdSS, crystallography, cyclophilin, PfCYP19, dihydrofolatereductase/thymidylate synthase, PfDHFR-TS, Enoyl-acyl carrier reductase, PfENR, formylmethionine deformylase, PfPDF, glutathione S-transferase, PfGST, hypoxanthine-guanine-xanthine phosphoribosyltransferase, PfHGXPRT, L-lactate dehydrogenase, PfLDH, malaria, medicines for malaria venture, MMV, Plasmepsin II, PfPMII, Plasmepsin IV, PfPMIV, *Plasmodium falciparum,* protein kinase 5, PfPK5, purine nucleoside phosphorylase, PfPNP, structure-based drug discovery triosphosphate isomerase, PfTPI.

Crystallography can provide an empowering rationale for chemical modification in drug discovery [1]. The ability to see how an active site is configured, particularly in the presence of an inhibitor, is a unique glimpse into the physical realities of a system which is normally understood only in the abstract. The possible analogues of even a simple lead compound define an enormous and largely inaccessible chemical space [2]; a three dimensional structure of the compound bound to its target can serve immediately to restrict that chemical space to only that which will be the most profitable to pursue. The enormous disease burden of malaria [3] and the ever-growing issue of drug resistance [4, 5] have underscored the importance of drug discovery for this disease. The recent publication of the gene sequence of *P. falciparum* [6], which causes the most virulent form of malaria, and a Structural Genomics initiative which includes the organisms among its targets [7], should provide fertile ground for structure-based drug design in the near future. This review will focus on the most advanced, structure-based drug discovery efforts in *P. falciparum* and the difficulties inherent in the heterologous expression of *P. falciparum* proteins for crystallographic study.

THE CHALLENGE OF HETEROLOGOUSLY EXPRESSING *P. falciparum* PROTEINS

P. falciparum is a challenging organism from which to clone and express proteins. It is eukaryotic, and the presence of introns in the genes requires the use of cDNA for cloning purposes. Eukaryotic proteins are often expressed in *E. coli* as insoluble inclusion bodies, and *P.falciparum* has not proven to be an exception to this. The genome of *P. falciparum* is 80% AT-rich, requiring the use of longer primers for PCR and exhibiting a codon bias with heavy emphasis on AT-rich codons [6]. In addition, the presence of low-complexity regions in proteins is relatively commonplace; the *P. falciparum* homologue of CDK-2, for example, has a stretch of 83 asparagines in a row [8]. These disordered regions may be expected to prevent the proteins from crystallizing. There is also accumulating evidence that *P. falciparum* proteins may bind the DNA and RNA which codes for them [9], a regulatory process which would hamper attempts to overexpress the protein. It is not uncommon for *P. falciparum* genes to contain cryptic start sites for *E. coli*, leading to the generation of multiple, truncated products [10]. Finally, *P. falciparum* proteins are as much as 50% longer than their yeast orthologues, making them more difficult to clone and express [8]. The Structural Genomics of Pathogenic Protozoa (SGPP) [7], which contains among its components a systematic effort to clone and crystallize *P. falciparum* proteins, has reported a soluble expression rate of about 5% for their first 368 targets, an attrition rate due mostly to protein either being insoluble or unexpressed [11]. The vast majority of these targets are under 450 amino acids in length and do not contain predicted transmembrane regions. As most of these targets lack significant homology to known proteins, some of these failures may be attributed to open reading frames which do not represent true coding regions. Fig. (**1**) shows the progress of 90 *P. falciparum* proteins selected for likely medical relevance in the SGPP. Unlike most of the targets picked for Structural Genomics,[12] many of these were quite large in size and were annotated with a known enzymatic activity. It can be seen from the figure that the large targets did not express at all, and the small targets, even when expressed, tended not to be soluble. The lack of expression in about half of these cases could be attributed to frameshift mutations, typically in long, continuous regions of adenosines or thymidines [13].

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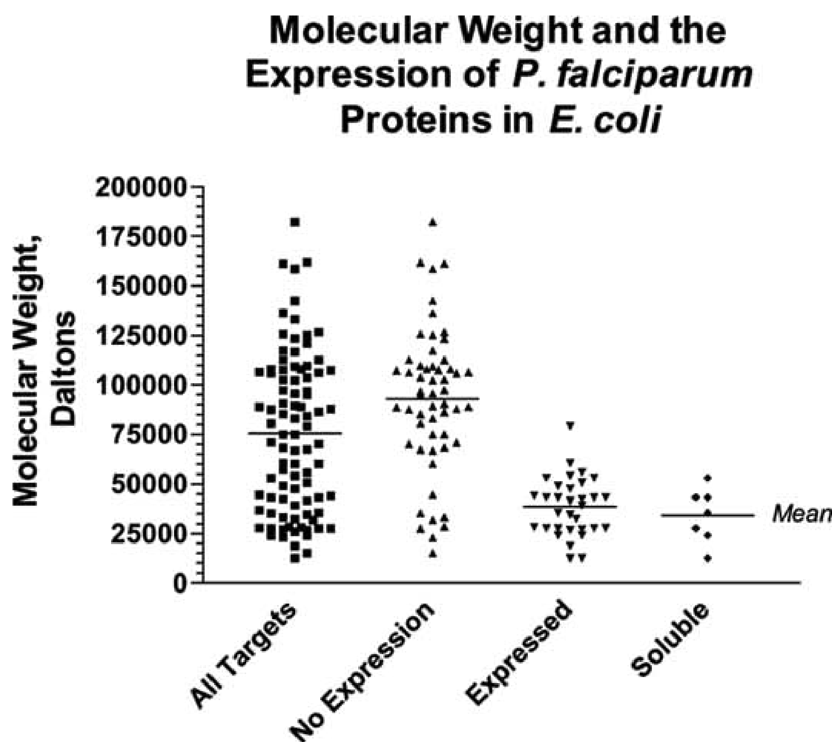

Fig. (1). 90 *P. falciparum* proteins targeted in the Structural Genomics of Pathogenic Protozoa pipeline are illustrated above. This scatter plot shows the molecular weight of all targets at each stage of the process. It can be seen that the larger proteins tend not to express at all while the smaller proteins may express but tend to do so insolubly [13].

P. falciparum proteins display unique glycosylation patterns on their proteins which are likely to prove difficult to imitate with standard protein production methodology. By far the most common motif is the GPI anchor which is found on the majority of cell surface proteins in *P. falciparum* [14, 15]. This is a GPI anchor which is distinguished from the mammalian GPI anchor both in the sugar residues and in the nature of the lipid. There is very little other glycosylation which occurs in *P.* falciparum. [14] There appears to be some N-linked glycosylation, although the evidence for O-linked glycosylation is very limited [16]. This issue is further clouded by the presence of RBC enzymatic pathways which can append carbohydrates onto *P. falciparum* proteins [17]; the nature of parasitism leaves as somewhat an open question what the “real” form of the protein is. Attempts to express malaria proteins in eukaryotic expression systems, such as baculovirus, typically lead to overglycosylation of *P. falciparum* targets; this is coupled with very low levels of GPI anchor addition [18, 19].

CURRENT STATUS OF *P. falciparum* STRUCTURE-BASED DRUG DISCOVERY

Table **1** illustrates the current contents of the Protein Data Bank with respect to *P. falciparum* soluble (non-surface) proteins [20]. There are only a few structures in the PDB from other malaria species, and all of these have PDB entries for the *P. falciparum* homologue as well, so this table of 21 proteins is representative of all known *Plasmodium* structures. Of course, many of these proteins have had several structures determined, but the low total number of structures underscores the difficulty of x-ray structure determination for this organism. Notable is the dominance of *E. coli* in protein expression, as this has been the host employed for every *Plasmodium* structure outside of membrane surface proteins. Also of note is the relatively small size of the proteins, as the average size of these is under 30kD.

Thirteen of the 21 proteins have structures with bound inhibitors, and these will be examined more fully here. A broader overview of *P. falciparum* crystal structures may be found in the recent review by R. Leo Brady and Angus Cameron [21].

L-Lactate Dehydrogenase (PfLDH)

L-lactate dehydrogenase was the first detailed structure determined from *P. falciparum* [22]. PfLDH is essential for energy production in the parasite, catalyzing the production of lactate from pyruvate, and inhibitors of PfLDH have been shown to kill parasites [23]. The dependence of *P. falcipaum* upon glycolysis has inspired a fair amount of research into inhibitors of glycolysis [21]; currently there are inhibitor bound structures of PfLDH and *P. falciparum* triosphosphate isomerase (below). Other glycolytic enzymes which have been crystallized are *P. falciparum* aldolase [24] and glutaraldehyde-3-phosphate dehydrogenase from several other protozoans [25-27]. PfLDH has been co-crystallized with a variety of inhibitors, including chloroquine [28] and oxamic acid. Although chloroquine does appear to inhibit NADH cofactor binding, this interaction is very weak and probably does not contribute meaningfully to the antimalarial action of

Table 1. *P. Falciparum* Soluble Proteins with Crystal Structures

PlasmoDB Name [127]	Function	Number of Structures		Pathway
		Total	w/inhibitor	
PF13_0141	L-lactate dehydrogenase	8	7	glycolysis
PF14_0077	Plasmepsin 2	7	5	heme digestion
PF14_0378	triose-phosphate isomerase	5	4	glycolysis
MAL6P1.275	enoyl-acyl carrier reductase	5	3	fatty acid synthesis
PFD0830w	dihydrofolate reductase-thymidylate synthase	3	3	folate synthesis
MAL13P1.279	PK5	4	2	cell cycle
PFE0660c	Purine nucleotide phosphorylase	3	2	purine salvage
PFC0975c	cyclophilin (PFCYP19)	2	2	unknown
PF14_0187	Glutathione S-transferase	3	1	detoxification
PF13_0287	adenylosuccinate synthetase	1	1	purine salvage
PFI0380c	formylmethionine deformylase	1	1	protein processing
PF10_0121	hypoxanthine phosphoribosyltransferase	1	1	purine salvage
PF14-0075	Plasmepsin IV	1	1	heme digestion
PF14_0749	acyl CoA binding protein	1	0	fatty acid metabolism
MAL13P1.95	ferredoxin	1	0	unknown
PF14_0425	fructose-bisphosphate aldolase	1	0	glycolysis
PF13_011	Gamete antigen 27/25	1	0	unknown
PF14_0192	Glutathione reductase	1	0	detoxification, redox
PFI1105w	phosphoglycerate kinase	1	0	glycolysis
PF11_0461	Rab6	1	0	vesicular transport
PF14_0545	thioredoxin	1	0	redox maintenance

chloroquine [29]. Selectivity for the parasite enzyme has been an issue with PfLDH inhibitors; humans have three LDH isoforms, the structures of two of which are known. The selectivity problem is compounded by the high concentrations of the parasite glycolytic enzymes, requiring high potency inhibitors for use as antiparasitics [30]. However, comparison of the *P. falciparum* structure to the human structures reveals that selectivity should be possible: PfLDH has a larger active site cleft due to a shift of the NADH cofactor and the use of a longer loop which closes over the active site in the PfLDH [21, 22, 28]. Gossypol is a PfLDH inhibitor with *in vitro* antiparasitic activity of about 10uM [31] but a high level of cytotoxicity, presumably due to the presence of an aldehyde [21]. A large amount of effort has been focused of PfLDH, including gossypol derivatives [32] and promising of napthoic acid-based compounds [21]. PfLDH is among the most advanced targets of antimalarial structure-based drug design, and research has been funded by the Medicines for Malalaria Venture (MMV), although funding from this source recently ended due to lack of a clinical candidate [33]. Recently, a comparative analysis of LDH enzymes from four *Plasmodium* species revealed that the high degree of structural similarity between the parasite enzymes should allow for inhibitors which are effective against multiple species; as the structural studies did not serve to explain the kinetic differences between the enzymes, though, there are likely to be factors at work which are incompletely understood [34].

Plasmepsin II (PfPMII)

The plasmepsins are aspartic proteases involved in the early steps of hemoglobin degradation by *P. falciparum*, and there are estimated to be ten different isoforms of these enzymes [35]. As aspartic protease inhibitors such as Pepstatin A kill parasites in culture, these proteases are compelling drug targets [36-38]. It remains unclear, however, which of these enzymes represents the best drug target, leading to some suggestions of adaptive inhibitors which target the entire family [39]. Plasmepsins I, II, IV, and histo-aspartic protease (HAP) are the four key plasmepsins in the *P. falciparum* food vacuole [40]. PfPMII is one of the most advanced targets of structure-based drug design, and there are currently crystal structures of the enzyme both free and bound to five different

inhibitors. The progress against plasmepsin II was recently reviewed [41]. The human enzyme most closely related to PfPMII is Cathepsin D [42], an aspartic protease which has attracted pharmaceutical interest due to its link to cancer [43]. The enormous chemical libraries generated against renin and HIV protease[44] are also likely to contain inhibitors of PfPMII, and screening these libraries against PfPMII could accelerate initial lead discovery, the so-called piggy-backing approach [45]. PfPMII inhibitors with Ki values in the low nanomolar range and lacking in Cathepsin D inhibitory activity have been reported [46]. It is of great interest that the early structure pf PfPMII bound to Pepstatin A would not have accommodated some of the later inhibitors which have followed; crystallographic analysis of EH-58, a non-peptidic inhibitor with a 100nM Ki[47], revealed that binding induced an active site shift which may allow for the use of bulkier groups in this class of compounds [48]. This underscores the importance of multiple crystal structures with multiple bound inhibitors in structure-based drug discovery.

Triosphosphate Isomerase (PfTPI)

Triosphosphate isomerase is an essential enzyme in the glycolytic pathway of intraerythrocytic *Plasmodia*, and as mentioned for PfLDH, this is a compelling pathway for drug design. PfTPI has been structurally well defined, and structures of both the uncomplexed enzyme and the enzyme bound to four different inhibitors have been determined [49-51]. The production of TPI in *E. coli* was relatively straightforward; TPI negative *E. coli* were used in order to ensure that the recombinant preparation was free of the bacterial homologue [52]. The action of PfTPI requires considerable movement of a catalytic loop, and different inhibitors have frozen this loop open or closed in the crystal structures [21]. Structural differences between *P. falciparum* TPI and the human TPI have been observed, including a mutation of a conserved mammalian serine residue near the active site to a phenylalanine in the parasite enzyme. This alteration has profound effects on the interplay between the active site catalytic loop and some inhibitors and could potentially be exploited by future therapeutics [51].

Enoyl-Acyl Carrier Reductase (PfENR)

This enzyme is involved in fatty acid biosynthesis II (FAS II) pathway of *Plasmodium*. As the FAS II pathway is completely lacking in humans, this is an attractive antimalarial target [53]. In addition to the unliganded enzyme and the enzyme bound to NADH, there are three structures with bound inhibitors [54]. These inhibitors are triclosan, a common antibiotic used in soaps and household products, and two triclosan derivatives. Triclosan has demonstrated antimalarial activity, both *in vitro* and *in vivo* in a *P. berghei* mouse model of the disease [55], and PfENR is one the most promising structure-based antimalarial targets. Triclosan has a Ki of 50nM against PfENR and the IC50 against both drug sensitive and multidrug resistant organisms is in the high nanomolar to low micromolar range [54]. PfENR is localized to the apicoplast, an organelle bounded by four membranes. The apicoplast is unique to the apicomplexan parasites and has been suggested to be a rich source of potential drug targets [56]. The difficulty inherent in delivering drugs to a target bounded by a total of seven membranes (including the parasite outer membrane, the parasitopherous vacuole membrane and the erythrocyte membrane) may well be shown by the studies with triclosan analogues. Although triclosan is more effective in inhibiting PfENR *in vitro* by an order of magnitude or more, these analogues are roughly equivalent to triclosan in killing parasites, indicating that they may have better penetration into the apicoplast [54]. These structures have formed the basis for the design of several hundred triclosan analogues, some of which exhibit considerably better *in*

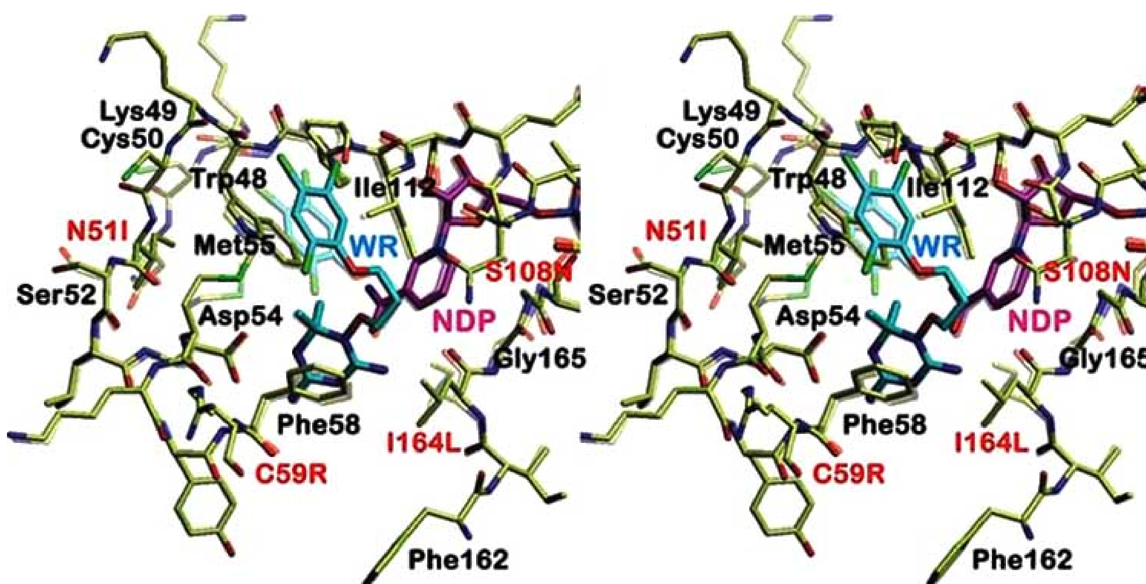

Fig. (2). This is the DHFR active site from the wild-type enzyme (lighter) with the V1/S quadruple mutant resistant DHFR superimposed on top of it. The four mutations giving rise to resistance are shown in red. Shown in cyan is the inhibitor WR99210 (WR) and in magenta is NADPH (NDP). The flexibility of the inhibitor allows it to bind even the mutant active site. [128].

vitro activity (J. Sacchettini, personal communication). Mutational studies of PfENR have helped to define the key residues for binding triclosan [57], and several kinetic studies have recently been published on triclosan PfENR inhibitors [58, 59]. This project has been picked up by the MMV [33].

Dihydrofolate Reductase/Thymidylate Synthase (PfDHFR-TS)

DHFR-TS is a bifunctional enzyme in *Plasmodium*, and there is a long history of DHFR (and not TS) inhibitors used clinically as antimalarials [60]. This enzyme is important in the folate pathway and is essential for the generation of dTMP, and, by consequence, DNA replication. Pyrimethamine and proguanil, the prodrug of cycloguanil, are antifolates which were subject to resistance very soon after their introduction [5, 61]. Active site mutations have been associated with this resistance, and the three structures of DHFR-TS, all with bound inhibitors, are especially intriguing as they include the structure of a quadruple mutant highly resistant to pyrimethamine bound to the related antifolate WR99210 [62]. Fig. (**2**) shows how the flexibility of this new antifolate allows it to bind the DHFR active site even in the presence of mutations which exclude other antifolates.

The ability to see how these mutations affect the binding of the antifolates allows for the contemplation of novel drugs which will be less affected by the mutations. Unfortunately, these structures also underscore the adaptability of the parasite and the serious difficulty inherent in finding antifolates which will not be subject to mutational resistance. The bifunctional nature of this enzyme, unique to parasitic protozoa, has been suggested to be essential for its activity in the folate pathway [62, 63], and the junction region of the enzyme is critical for TS activity [64]. Unlike dihydrofolate reductase, the absence of bound inhibitors to the TS or linker regions leaves little to go on for the discovery of novel drugs. However, TS inhibitors are still an active area of antimalarial research [65], even given very close conservation between the human enzyme and the parasite enzyme active sites [21, 62].

Protein Kinase 5 (PfPK5)

PfPK5 has about 60% sequence identity with the human cyclin dependent kinases 1 and 5 [66]. By analogy, PfPK5 is involved in cell cycle regulation, and the rigid cell cycle of *Plasmodium* makes this an attractive target [67]. The pharmaceutical industry has poured substantial effort into the development of kinase inhibitors as chemotherapeutics, and there are a large number of ATP analogues which have been made, creating the opportunity to jump-start antimalarial programs [45]. The large number of human enzymes which utilize ATP and the relatively high levels of intracellular ATP will require exceptionally high potency and selectivity in antimalarials which are active against the ATP binding site of parasite kinases – in chemotherapeutic research, kinases were thought not very long ago to be “impossible” targets [68]. Structure-based drug design can be an immense help in this regard, and with four structures for PfPK5, two with bound inhibitors, this will be an area worth watching. Cyclin dependent kinases are activated by cyclin binding and phosphorylation, which can cause a substantial conformational shift of the kinase [69, 70]. While the activation pathway of PfPK5 *in vivo* remains unclear, the crystal structure of PfPK5 bound to indirubin-5-sulphonate revealed a close similarity to activated human CDK2 [66].

Purine Nucleoside Phosphorylase (PfPNP)

Plasmodium lack synthesis pathways for purines and so rely upon salvage pathways in order to obtain purines for the for the replication of DNA and RNA [71, 72]. PNP is responsible for the conversion of either inosine or 5’-methylthioinosine to hypoxanthine, the main precursor for purines in *P. falciparum* [73, 74]. Immucillin H, a potent inhibitor of PNP, has been shown to be effective in killing parasites in blood-stage culture. As human PNP is active in erythrocytes and the parasites are able to salvage hypoxanthine directly, killing blood-stage parasites requires inhibiting both the parasite and host PNP [75]. A potent and selective inhibitor of parasite PfPNP, 5’-methylthio-Immucillin H, has been developed based upon the crystal structure of Immucillin H bound to the parasite enzyme [76].

Cyclophilin (PfCYP19)

Diffracting crystals of PfCYP19 were only obtained in the presence of cyclosporin (W.N. Hunter, personal communication 2004), and the only structures of this enzyme are in complex with the inhibitor. Cyclosporin is a potent immunosuppressant in common use today to prevent organ rejection after transplants [77]. Its mechanism of action in humans has been studied extensively and results from a complex formed between cyclosporin and cyclophilin acting upon the phosphatase calcinerurin [78, 79]. Cyclosporin is a known antimalarial [80], and were it not such a large and synthetically intractable compound it is likely that these crystal structures would embolden structure-based drug discovery against this target. Fortunately, a large number of naturally-occurring cyclosporins exist [81], and it has been demonstrated that the antiparasitic activities of the cyclosporins do not correlate with the immunosuppressant activities [80]. Although cyclosporin binding proteins, including this one, have been identified from *P. falciparum* [82], the antiparasitic mechanism of this class of compounds is likely to be complex and is certainly not well understood. It does not appear to be correlated either with affinity for the parasite cyclophilin or inhibition of the proline isomerase activities of the cyclophilin [83].

Glutathione S-Transferase (PfGST)

Glutathione S-transferases are associated with detoxification pathways in mammalian cells [84], and there is an association with GST activity and chloroquine resistance in *P. falciparum* [85, 86]. GST is typically present in several isoforms, but *P. falciparum* appears to have only one isoform. As the PfGST has an active site which is considerably more open than the closest human isoform, it is thought that the substrate specificity of the

parasite enzyme is likely to be broader than the human enzyme and that considerable opportunity for the development of selective inhibitors exists. The crystal structures which have been determined are the unliganded enzyme and PfGST bound to two peptides and a glutathione analogue [87]. It has been suggested that there is an extended hydrophobic binding pocket in PfGST which could be exploited for drug development [88].

Adenylosuccinate Synthetase (PfAdSS)

AdSS catalyses the ligation of aspartate to inosine monophosphate creating adenylosuccinate, the first committed step in the purine salvage pathway leading to adenosine monophosphate [89]. As this step is downstream from hypoxanthine, PfAdSS inhibitors would not be rendered useless by salvage of hypoxanthine [74]. The crystal structure of PfAdSS contains hadacidin, an aspartate analogue, as well as 6-phosphoryl IMP, GDP, and magnesium [90]. Although the structure of the human enzyme is not known, the mouse and *E. coli* structures have been determined, and there is a high level of conservation in the active site structures of the three enzymes. One difference which may turn out to be significant for inhibitor design is Thr307 which is unique to the *P. falciparum* enzyme and forms a hydrogen bond with the inhibitor.

Formylmethionine Deformylase (PfPDF)

Formylmethionine deformylase, or peptide deformylase, is a metalloprotease responsible for deformylating the N-terminal methionine of newly synthesized proteins in prokaryotes and has been targeted for the development of novel antibiotics [91, 92]. The enzyme is also found in eukaryotic mitochondria and organelles, and the signal sequence on the PfPDF suggests that the *P. falciparum* enzyme, like PfENR, is sequestered in the apicoplast [93]. The crystal structure with a bound inhibitor is a little puzzling due to the presence of an unexpectedly modified inhibitor in the binding site [94]. Given the considerable interest in the pharmaceutical industry in PDF inhibitors for use as antibiotics [95-98], there is great potential to leverage chemical libraries of bacterial PDF inhibitors by screening them against the *P. falciparum* enzyme. The MMV recently initiated a screening campaign with GlaxoSmithKline to find novel inhibitors of PfPDF [33].

Hypoxanthine-Guanine-Xanthine Phosphoribosyltra-nsferase (PfHGXPRT)

HGXPRT is responsible for catalyzing the transfer of a phosphoribosyl group to hypoxanthine, xanthine, or guanine and is an early step in the purine salvage pathway. This enzyme required the presence of an inhibitor in order for diffracting crystals to be harvested, and the single reported crystal structure contains Immucillin HP, a transition state analogue [72]. The structure of the human enzyme, bound to a related inhibitor, is available [99]. The crystal structure of the *P. falciparum* enzyme revealed a very close similarity to the human isozyme, with conservation of every amino acid contact between the enzymes and inhibitors. This is somewhat surprising given that the human enzyme is incapable of utilizing xanthine as a substrate [100]. Complimentary NMR experiments revealed that although the human and *P. falciparum* enzymes utilize identical hydrogen bonding patterns, the weighting of these bonds differs and may potentially be exploited for selectivity in drug design [72, 101]. Kinetic studies have suggested that hypoxanthine may be the preferred substrate for PfHGXPRT and that the enzyme may have several different activated states [102].

Plasmepsin IV (PfPMIV)

As for PfPMII, PfPMIV is involved in the early degradation steps of hemoglobin, however, much less work has been completed on PfPMIV than PfPMII. There is a single crystal structure of PfPMIV with a bound peptide inhibitor, pepstain A. Recent work suggests that plasmepsin IV may be the most important of these enzymes, as isoforms of plasmepsin IV are found in at least six other *Plasmodium* species [103]. Using a panel of chromogenic substrates, the specificities of four *Plasmodium* PMIV enzymes have been assessed, and they all show similar substrate preferences [104]. Although this substrate preference was mostly shared by human Cathepsin D, it appears that substrates with an S3' serine had selectivity for the parasite enzyme.

METHODS USED TO EXPRESS PROTEIN FOR STRUCTURAL STUDY

As was mentioned earlier, the heterologous expression of *P. falciparum* proteins is often very difficult. Several methods have been employed in order to gain access to refractory proteins including the use of added tRNAs, whole gene synthesis, and refolding.

The codon bias of *P. falciparum* has been dealt with through the use of constructs encoding tRNAs for codons which are represented at much higher levels in the parasite than in *E. coli* genes. The RIG plasmid, encoding tRNAs for arginine, isoleucine, and glycine, has been shown to enhance the expression of several *P. falciparum* proteins [105]. Also commonly employed are "Codon Plus-RIL" cells (Stratagene) which have a similar construct encoding arginine, isoleucine, and leucine tRNAs. These RIL cells were used in the expression of protein kinase 5, PfENR, and glutathione S- transferase from *P. falciparum.* Clearly, though, these added codons are not a panacea, and more recent work suggests that RNA stability and secondary structure may be more important than tRNA abundance [106]. Whole gene synthesis allows one the prospect of dealing with codon bias, RNA secondary structure, and cryptic start sites all at once. One of the best examples of this is PfDHFR-TS, which was obtained in significantly greater yield after the gene was synthesized. A large amount of effort had been spent on getting this protein from the wild-type gene, including the use of different strains of *E. coli*; this led to higher expression of the protein but was plagued by truncated products resulting from cryptic start sites in the gene [107, 108]. These problems were resolved, and protein expression increased tenfold, when a synthetic gene was synthesized [109]. Interestingly, the protein used in the final structure determinations was

obtained from a construct encoding the wild-type gene (Y. Yuthavong, Personal communication 2004). Low expression of PfENR was considerably enhanced by the introduction of four silent mutations in a 10-adenosine stretch in the native gene [54]. The *P. falciparum* vaccine candidate MSP-1 could only be made in *E. coli* after whole gene synthesis [110]. Initial attempts to express pfLDH resulted in N-terminally truncated, inactive material. Further investigation showed the presence of a Shine-Dalgarno sequence upstream of the third methionine in the protein, and although mutation of the third methionine did not result in the production of full-length protein, mutation of the Shine-Dalgarno sequence was effective in allowing for full-length expression [10].

Even when proteins are expressed well in *E. coli*, solubility is typically a limiting factor. Refolding of PfPMII from *E. coli* inclusion bodies is a universal step in the preparation of the enzyme [42, 111-113]. Plasmepsin II also requires removal of the N-terminal portion for activation; this has been accomplished by allowing the enzyme to auto-activate [113], by cloning just the active portion, or a combination of these approaches [112]. Internal mutations of PfPMII have been engineered to both enhance solubility and prevent unwanted proteolysis [112, 114] Both the N- and C- termini of PfENR were truncated in order to obtain the crystal structure [54, 115]. It is interesting to note that about half of the *P. falciparum* structures with bound inhibitors are not represented with unbound structures as well. A bound inhibitor can play an important role in getting a protein to crystallize, and in cases where the unbound protein will crystallize on its own a bound inhibitor can enhance the resolution [94].

HIGH-VALUE TARGETS IN *P. falciparum* FOR FUTURE STRUCTURE-BASED DRUG DESIGN

While some drug targets in *P. falciparum* are readily amenable to structure-based approaches to optimization, it is clear that many successful antimalarials are not likely to benefit from this kind of methodology. The 4-amino quinolines such as chloroquine, quinine, and mefloquine all likely function through inhibition of heme polymerization [60]. As these drugs lack a specific target or binding site, there is little possibility of optimizing a bound structure. The artemesinins are among the most promising antimalarials and are thought to operate through the generation of heme-activated radical species that exhibit general toxicity to the parasites, a mechanism which also lacks a specific binding site [33, 60]. Recent evidence has linked the artemesinins to a SERCA receptor [116], but as this receptor is a membrane protein and the bound species is a short-lived, reactive moiety, this does little to encourage thoughts that crystal structures with the bound inhibitor will be coming anytime soon. In point of fact, the lack of a defined binding site may be very much an asset when it comes to avoiding the emergence of drug resistance. Resistance to chloroquine took decades and is associated with multiple mutations. In contrast, dihydrofolate reductase inhibitors have seen the development of resistance within a year associated with mutations in the target enzyme [4, 117]. Structure-based drug discovery may allow for the visualization of mutations which give rise to resistance and thereby show how novel drug analogues could get around these mutations, such as was demonstrated in the case of the DHFR-TS structures. It is somewhat humbling to remember, though, that one is always playing catch-up to the parasites with this kind of approach.

High-value structures which could serve to enhance our ability to design drugs against malaria include further members of the purine salvage and pyrimidine biosynthesis pathways, structures of the falcipains, GAPDH, and protein farnesyl transferases. All of these have active antimalarial programs associated with them and have been targeted by the Medicines for Malaria Venture (MMV). It is worth noting that the farnesyl transferases were completely recalcitrant to expression in both *E. coli* and baculovirus systems, even with synthetic genes (W. VanVoorhis, personal communication). Multiple crystal structures of a target with different bound inhibitors are fundamental to defining the chemical space the inhibitors can operate within, and these data are especially useful when the binding kinetics of these inhibitors are understood as well [118]. In this sense, the proteins which we have structures of already may be some of the most valuable leads for further structural study with novel compounds.

The goal of using crystal structures to screen compounds through strictly computational means, so-called *in silico* or virtual screening, is highly attractive, but perhaps the greatest benefit of this technique is that it inspires researchers to experiment with compounds they might not otherwise try. A notable example for this is a set of benzamidines which were found by virtual screening to be likely antagonists of PfLDH. Synthesis of the compounds revealed that they had negligible activity against this enzyme but possessed potent antimalarial activity through an unknown mechanism [119]. A similar screen for compounds which inhibit PfDHFR revealed a dozen compounds with modest activity against the enzyme (low micromolar Ki) but, importantly, these compounds were completely different classes of compounds from those known to be traditional antifolates [120]. One of the most promising areas of crystallography-driven drug discovery is fragment-based drug discovery. This technique utilizes an array of small molecules which are soaked into protein crystals and serve to define parts of a final compound [121, 122]. Although this approach is fundamental to several biotechnology companies (notably Structural GenomiX[123] and Astex[124]), it has yet to be applied to *P. falciparum* targets.

Structure-based drug discovery is just one of many tools in the effort to find novel drugs against malaria, and it is a method which is clearly still in its infancy for most targets. Protein expression remains one of the fundamental challenges with broadening the scope of structure-based drug discovery for this disease, and there are, as yet, no easy solutions to this problem. A further complication which the full genome sequence of *P. falciparum* has brought into sharp relief is that we do not understand the function of about 60% of the encoded genes [6], and many proteins which have been experimentally demonstrated to be present are encoded by unknown genes [125]. It may well be that the most valuable drug targets are amongst

this set, and the contribution of structure determination may in large part be to define what some of these targets are. Clearly, though, given the large amount of protein-specific experimental modification frequently required for heterologous expression, a “low hanging fruit”[126] structural genomics approach will almost certainly miss compelling targets. Defining high-value targets at an early stage and then focusing every trick we have on obtaining protein and structures will be an important part of future structure-based drug discovery for malaria.

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