Chapter 3

Drug Target Validation Methods in Malaria - Protein Interference Assay (PIA) as a Tool for Highly Specific Drug Target Validation

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Abstract

Background: The validation of drug targets in malaria and other human diseases remains a highly difficult and laborious process. In the vast majority of cases, highly specific small molecule tools to inhibit a protein’s function in vivo are simply not available. Additionally, the use of genetic tools in the analysis of malarial pathways is challenging. These issues result in difficulties in specifically modulating a hypothetical drug target’s function in vivo.

Objective: The current “toolbox” of various methods and techniques to identify a protein’s function in vivo remains very limited and there is a pressing need for expansion. New approaches are urgently required to support target validation in the drug discovery process.

Method: Oligomerization is the natural assembly of multiple copies of a single protein into one object and this self-assembly is present in more than half of all protein structures.

Thus, oligomerization plays a central role in the generation of functional biomolecules. A key feature of oligomerization is that the oligomeric interfaces between the individual parts of the final assembly are highly specific. However, these interfaces have not yet been systematically explored or exploited to dissect biochemical pathways in vivo.

Results and Conclusion: This mini review will describe the current state of the antimalarial toolset as well as the potentially druggable malarial pathways. A specific focus is drawn to the initial efforts to exploit oligomerization surfaces in drug target validation. As alternative to the conventional methods, Protein Interference Assay (PIA) can be used for specific distortion of the target protein function and pathway assessment in vivo.

Keywords: Drug target validation, in vivo specificity, malaria, oligomerization, protein Interference, protein: protein interactions.
1. Introduction

1.1. Novel Small Molecules as Research Tools in Drug Target Validation

The generation of novel small molecules (NSMs) is a major bottleneck for both academia and the pharmaceutical industry. NSMs are primarily needed to provide specific tools to dissect and understand biological problems addressed in basic research and are required in the biological sciences for three fundamental reasons: firstly, to counter the Harlow-Knapp effect, secondly, to provide validation of a proposed drug target, and thirdly, to generate starting points (“leads”) for drug development by the pharmaceutical industry. Any successful NSMs must possess minimal cross-reactivity (or high specificity) if the effect of interference in a single protein’s function is to be established with any reasonable degree of rigorousness.

1.2. The Harlow-Knapp Effect: Searching Under the Lamp Post

In 2008, Harlow and co-workers highlighted that a small subsection of human protein kinases are the subject of the vast majority of scientific publications. This results in an imbalance in the research into this important class of proteins and the \textit{in vivo} function and/or role of the majority of kinases in human disease remains relatively unexplored [1]. Knapp and colleagues extended this observation in 2010 by noting that the same minor fraction of kinases was also statistically over-represented in patent applications [2]. The same effect is also seen in the relationship between small molecule tools available to study individual nuclear hormone receptors and publications arising from research on these receptors (Fig. 1). Thus, the Harlow-Knapp effect has been defined as “the propensity of the biomedical and pharmaceutical research communities to focus their activities, as quantified by the number of publications and patents, on a small fraction of the proteome” [3]. This can also be expressed to the layperson as scientists exploring only areas that are already well illuminated (or “under the lamp post”). The obvious solution to counter the Harlow-Knapp effect is to increase the availability of more general tools to modulate protein function in vivo. This has been previously expressed in

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the observation that “where there has been a shift in research activity, it was often spurred by the emergence of tools (Table 1) to study a particular protein, not by a change in the protein’s perceived importance” (Fig. 1; [4]). Thus, the availability of novel and specific tools generally results in a more complete understanding of biological systems of interest.

1.3. The Need for New Validated Targets for Drug Discovery for the Treatment of Malaria

In humans, malaria is caused by infection by one of six species of Plasmodium (P. falciparum, P. vivax, P. malariae, P. knowlesi, classic P. ovale curtisi and its variant type P. ovale wallikeri). The species P. falciparum, P. vivax, P. malariae and P. ovale are spread directly between human hosts by female mosquitoes of the genus Anopheles. More recently, P. knowlesi (a species previously thought to cause malaria only in primates) has been detected in humans in South-East Asia. Of these five species, P. falciparum and P. vivax pose the greatest threat to global health. While P. falciparum predominates on the African continent and causes the majority of deaths, P. vivax is found over a wider geographical area - as it can survive and develop in the Anopheles host at lower temperatures. This potentially makes P. vivax the more dangerous of the two species as, in addition to its ability to survive and spread into cooler climates, P. vivax possesses a dormant liver stage (the hypnozoite). This dormancy enables it to survive for long periods (acting as an undetected reservoir for further infection for periods of many months). Dormancy can also contribute to the development of multi-drug resistance, as parasites exiting in the hypnozoite state whilst the patient is at decreased doses of chemotherapy are not sufficiently challenged. This may lead to the development of resistance genes in individual parasites that are not cleared from the blood before producing gametocytes able to transmit the resistant genotype. In spite of significantly increased research efforts in recent years (US$ 2.7 billion in 2013) malaria remains a significant threat for global health. In 2013 almost 200 million cases of malaria were responsible for 584,000 deaths worldwide, with the majority (90%) of cases in Africa, although 3% of all cases originated from the Eastern Mediterranean region (Fig. 2). Of
**Figure 1**

TOOLS ARE TELLING
The availability of research tools influences a protein’s popularity.

![Graph showing relationship between tool availability and degree of scientific examination of a nuclear hormone receptor. Figure adapted from [4].](image)

Fig. (1). Relationship between tool availability and the degree of scientific examination of a nuclear hormone receptor. Figure adapted from [4].

**Figure 2**

![Map showing global distribution of malaria infections in 2013. Source: WHO Malaria Report [5]).](image)

**Figure 2.** Global distribution of malaria infections in 2013 (Source: WHO Malaria Report [5]).
the 584,000 reported deaths, 78% were of children under 5 years old [5]. While the gap between the current funding levels (US$ 2.7 billion) and those required to eliminate the disease (US$ 5.1 billion) has decreased, the emergence of multidrug resistant strains of the malaria-causing parasites has increased the therapeutic burden on front-line antimalarials, such as artemisinin-based combination therapies (ACTs). Unfortunately, drug resistance to artemisinin and its derivatives has recently emerged in South-East Asia [6-10].

While South-East Asia accounts for only approximately 7% of the global malarial incidence, the emergence of artemisinin resistance is a serious threat to all current gains made towards control, treatment and elimination [11-13]. The Harlow-Knapp effect is already strongly present in the development of novel antimalarials as the current crop of therapeutic targets is a limited subset of malarial proteins [14]. As stated above, one of the major challenges for the future is to develop novel drug targets to expand the repertoire of chemotherapeutics available in combination therapies. These future combination therapies should clear the parasite from infected hosts with sufficient precision to minimize the risk of further drug resistance emerging [15]. For an excellent overview of the current status in drug resistance in the malarial parasite, the reader is directed to a recent review [16]. It should be borne in mind that the ultimate control/elimination of malaria is highly likely to require an effective vaccine. Currently, much focus is on the performance of RTS, SA/AS01, which provides protection in children with an efficacy of 30–50% [17, 18]. However, this efficacy falls significantly short of that required to provide “herd immunity” for the human species (90–95%). Additionally, the rapid emergence of drug resistance in malaria is also mirrored by observations that the parasite has also developed mechanisms to evade the immune system, increasing the challenge for successful vaccine development [19]. Thus, until an effective vaccine is discovered, fully certified and ready for public use (which might take a long time), novel chemotherapeutics (and perhaps more importantly, validated targets for novel chemotherapeutics) are urgently required to support global efforts to control and eradicate this disease.
2. Genetic Approaches in Drug Target Validation in Malaria

To obtain novel drug targets and vaccine candidates it is indispensable to have a robust molecular genetic toolbox to manipulate the parasite. However, classical genetic technologies are non-trivial in *Plasmodium*, as the parasite’s nucleus is protected by four membranes and the parasite’s A/T-rich DNA is unstable in *Escherichia coli* (*E. coli*). The complete genome sequence of *P. falciparum*, which facilitates functional genomic studies, has been available since 2002 [20], although Wu and colleagues already succeeded in transfecting the parasite in 1995 [21]. In this transient transfection, circular plasmid DNA is maintained inside the parasite under drug selection as epismes, initially in an unstable replicating form (URF) that, after extended selection times, changes to an apparently stably replicating form (SRF) [22]. Both are concatameric structures with a head-to-tail orientation of at least three plasmids, which seems to be an essential modification for the transfected parasite [22]. It is believed that the various physical barriers surrounding the parasite [21, 23-25] and the requirement for concatamerization lead to a low transfection efficiency in *P. falciparum* (estimated at $1 \times 10^{-6}$ [26]). It is still not known how these plasmids are replicated or segregated during asexual division in the bloodstage, but there is an extended period they can be maintained in culture [22, 27]. Nevertheless the absence of positive selection leads to the loss of the plasmid, probably because of uneven segregation during mitosis [22, 28].

Transient transfection of *P. falciparum* makes classic reverse genetic approaches possible, such as stage-specific expression [29], expression of proteins for drug susceptibility assays compared to wild type [30] or localization and trafficking analysis with green fluorescent protein (GFP) fusion proteins [31, 32]. To date, there are four positive selectable markers available: the human dihydrofolate reductase (hdhfr) [33, 34]; Blasticidin S deaminase [35], neomycin phosphorotransferase [36] and puromycin-N-acetyltransferase [37] which enhances the number of possible constructs.
2.1. Single and Double Crossover

Stable transgene expression via homologous, single crossover recombination into the haploid genome of *P. falciparum* during the blood stage has been demonstrated [21, 37, 38]. Drug selection can isolate the few parasites with integrated DNA by taking advantage of the unstable episomally plasmids. In this example, the selection pressure on a parasite population by a drug is removed for 3–4 weeks, before reselection using the same drug. This drug cycling is repeated various times to remove all episomes and to achieve only the required integrants. This process can take around 12 weeks. Due to the haploid nature of the parasite and the high number of single copy genes, a single crossover event is typically sufficient to generate genetic modification in the parasite (knockout or single point mutations). This homologous recombination methodology has provided important insights into erythrocyte invasion [39], sexual differentiation and cyto-adherence of infected erythrocytes by providing direct *in vivo* analysis of the effects of genetic manipulation.

However, gene integration and/or knockouts remain a long and inefficient procedure. Therefore, Duraisingh and colleges established a double crossover using the Herpes simplex virus (HSV) thymidine kinase (tk) for negative selection [40]. The viral tk phosphorylates nucleoside analogues, such as gancyclovir [41], which will form nucleoside triphosphates that inhibit DNA synthesis and the enzyme thymidylate synthase [42]. After a transfection of *P. falciparum* a positive selection will isolate the successfully transfected parasites. Subsequently, negative selection via gancyclovir will remove all parasites, which have not integrated the gene of interest via double crossover. This technique made possible the first genetic deletion in *P. falciparum*, as shown by studies of the non-essential gene Pfhr3 [40]. Additionally, the potential for integration events is highly increased, while the time required to obtain the selected parasite is drastically reduced. However, this method shows a marked “bystander” effect, in which gancyclovir kills parasites even when they do not express tk. This leads to a decreased selection of low frequency double crossover recombination events [43, 44].
To improve the efficiency of gene disruption it was recently shown that zinc-finger nucleases (ZFNs) are functional in *P. falciparum* [45]. Customized ZFNs generate double-strand breaks (DSBs) of targeted DNA, which allows the generation of knockouts or allele replacements much faster than conventional methods [45]. Although this technique is promising, it is associated with high costs. For each targeted genomic region a new sequence-specific nuclease has to be established and validated on the specific target [46, 47].

An efficient alternative is based on clustered regularly interspaced short palindromic repeats (CRISPR). CRISPR-associated proteins (CRISPR-Cas) system has been used recently in *P. falciparum* [48]. In this methodology, a single guide RNA (sgRNA) is used to direct a Cas9 endonuclease causing a DSB at a target DNA site. To do so the sgRNA has an upstream 20-nucleotide sequence that is homologous to the target site and the -NGG- protospacer adjacent motif (PAM). The repair mechanism of DSBs through error-prone non-homologous end joining (NHEJ), as described for human cells [49-52], seems to be absent in the malarial parasite. Therefore, *Plasmodium* depends on homologous recombination to maintain genome integrity [20, 53, 54]. The efficiency of this method was already shown by disruption of the non-essential knob-associated histidine-rich protein (kahrp) and erythrocyte binding antigen 175 (eba-175) by integrating a selectable marker [48]. Additionally, successful gene disruption using linear DNA has been reported [48]. As linear DNA is apparently lost in *P. falciparum* after 4 days [55], this could make negative selection dispensable [56]. However, the modification of origin recognition complex 1 (orc1) by single point mutation without integrating a selectable marker has also been obtained with this technique [48]. The CRISPR-Cas system could be the method of choice for gene disruption. Nevertheless, proteins that are essential during the asexual blood stage still cannot be investigated by conventional knockout systems since loss-of-function mutants are dying or are overgrown by non-integrators. A new potential drug target needs to have an essential role in the parasites survival; therefore new tools to investigate essential proteins are urgently required.
2.2. RNA Based Genetic Tools

A powerful tool to investigate essential blood stage proteins would be the utilization of RNA interference (RNAi), causing the silencing of the corresponding transcript. However, RNAi is not functional in malaria parasites due to the lack of the complete RNAi machinery [57-59]. Although RNAi silencing is not functional in *Plasmodium*, there are several other possible genetic manipulation systems based on RNA that provide opportunities. Long double-stranded RNA (dsRNA) that interferes with the cognate messenger expression led to a growth inhibition of 40% as shown for the transcription factor *Pfmyb1* [60] is required for intra-erythrocytic growth and controls key genes for cell cycle regulation. Downregulation of gene expression can also be achieved by autocatalytic RNA (riboswitches), employing self-cleaving ribozymes N9, integrated into the transcriptional unit of different genes [61]. The use of protein-binding RNA aptamers has also been reported to be functional in *P. falciparum* [62, 63]. Recently, unique peptide-morpholino oligomer (PMO) conjugates have been designed to bind to specific mRNA that is subsequently cleaved by RNaseP, resulting in a reduction of the protein expression. This strategy has already been applied to study the plasmodial gyrase A (*Pf*GyrA) [64, 65].

2.3. Knockout and knockdown of essential genes

Another possibility to overcome the limitation in analyzing essential *P. falciparum* genes is the use of site-specific recombinases. This technique has already been applied to *P. berghei* and *P. falciparum* using different recombinases. While in *P. berghei* a flippase recombinase (FLP) recognizes a pair of FLP recombinase target sequences (FRT) that flank the genomic region of interest [66, 67], in *P. falciparum* a ligand-activated DiCre recombinase seems more promising. Cre recombinase catalyzes the recombination between two 34 bp sequences, known as LoxP. In the DiCre system Cre is split into two inactive fragments, where each is fused to either FK506-binding protein (FKBP12) [68] or FKBP12±rapamycin-associated protein (FRAP) [69]). Heterodimerization can be then induced by rapamycin, which leads to a tight regulation of recombinase activity within the parasite.
In *T. gondii*, the deletion of an essential gene has already been performed following this approach [70]. The first application of the DiCre system in *P. falciparum* was performed by Collins and colleagues targeting *Pf*sera5, which could not be disrupted using conventional homologous recombination [71]. They showed that the use of an alternative transcription termination site does not affect the targeted protein. This has also been shown for the FLP/FRT-mediated excision [72]. Establishing a new robust Tet-repressible transactivator allows control of the transcription to analyze essential *Plasmodium* genes via the Tet-off-System [73]. Thereby, an upstream of the gene of interest integrated transcription factor - consisting of the tet-repressor and its activating domain sequence (TRAD) - binds to tet-operator sequence (TetO) that controls the promoter of the respective open reading frame. Subsequently, anhydrotetracycline (ATc) is used to mediate the binding of the TRAD to TetO [73-76]. Through this strategy an efficient knockdown of genes essential in the blood stage of the murine malarial parasite *P. berghei* could be demonstrated [73].

Another inducible system in *Plasmodium* is the use of the human protein FKBP12. Fusing the FKBP12 protein destabilization domain (ddFKBP) to the gene of interest will lead to an expression of the protein with an unstructured tail, which will be targeted for protein degradation [77]. The expressed fusion protein can be stabilized through a rapamycin-derived ligand called shield (Shld-1), which specifically interacts with the ddFKBP. This tool has already been successfully used in *T. gondii* as well as for several proteins of *P. falciparum* [78-81]. However, it remains unclear whether fusion of the ddFKBP influences the conformation of the targeted protein in terms of protein-protein/ligand interaction, intracellular trafficking or protein secretion. Further, it has been proposed that long-term exposure to Shld-1 could lead to transgenic parasites that would impact the effectiveness of Shld-1 [82]. As an alternative, Muralidharan and colleagues introduced the *Escherichia coli* dihydrofolate reductase (DHFR) degradation domain (DDD). A GFP-DDD fusion protein has been generated whose degradation can be modulated by folate analogues such as trimethoprim (TMP). Recently, a new strategy using the Glucosamine-6-
phosphate activated ribozyme (GlmS ribozyme) has been investigated as inducible genetic tool in *P. falciparum*. In *Saccharomyces cerevisiae* the GlnS ribozyme has been shown to control reporter gene expression response to exogenous glucosamine (GlcN) [83]. Prommana and colleagues were able to insert the ribosome sequence into the untranslated region (UTR) of a targeted gene. This leads to the expression of a chimeric target mRNA encoding for an additional ribozyme RNA. The chimeric RNA self-cleaves upon the addition of GlcN to the parasite culture medium, resulting in the degradation of the mRNA and the knockdown of the target protein. The use of glmS system resulted in a successful knockdown of the essential *P. falciparum* dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) [83]. Although this technique seems highly promising, prolonged GlcN treatment at high dose seems toxic to parasites [84].

In summary, the molecular genetics toolbox has been greatly enhanced in the last decade, but still remains insufficient in terms of essential gene analysis (Table 1). The long periods required isolating parasites with integrated DNA modifications, combined with the low efficiency and the difficulty of essential gene analysis shows the importance to develop new methods for the function analysis of *P. falciparum* proteins.

3. **Mitochondrial electron transport proteins of *P. falciparum* as potential drug targets**

Mitochondria are membrane bound organelles found in most eukaryotic cells. The typical function of mitochondria is the production of ATP through recurrent oxidation of substrates within the TCA cycle. Oxidation of substrates within the TCA cycle generates electrons, which are used to supply the ETC at the inner mitochondrial membrane. Thereby, a proton gradient is generated across this membrane and is typically utilized by ATP synthase for the production of ATP.

There are significant differences in the behavior of parasitic mitochondria when compared to the classical behavior of mitochondria, such as those of the human host. However, the precise function of the TCA cycle of *P. falciparum* remains unclear and the role of the parasite mitochondria has been the subject of much debate. Indeed, the TCA cycle of the malarial parasite was recently suggested to be uniquely bifurcated, although this
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<th>Technique</th>
<th>System</th>
<th>Mode of action</th>
<th>(Dis-) Advantages</th>
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<td>Genomic modification (knock in/ knockout)</td>
<td>Single crossover</td>
<td>On and off drug cycling to select for integration</td>
<td>Time consuming</td>
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<td>Double crossover</td>
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<td>Customized ZFNs</td>
<td>ZFN induces the break of double-stranded DNA</td>
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<td>CRISPR-Cas</td>
<td>Double-strand breaks mediated by the Cas9 endonuclease</td>
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<td>Cre/LoxP system</td>
<td>Rapamycin induced dimerization of Cre</td>
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<td>Conditional and inducible gene expres-</td>
<td>Tet-off-System</td>
<td>ATc needed for initiation of transcription</td>
<td>Not available for <em>P. falciparum</em></td>
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| sion (knock-down)                      | FKB12 destabilization domain (DD) | DD domain leads to protein degradation which can be prevent by Shld-1 | Fast and regulative
Can influence protein function or localization |
| E. coli DHFR degradation domain (DDD)  | TMP stabilizes proteins fused to the DDD domain           | Parasite needs to express hDHFR due to the toxic effect of TMP |
| GlmS ribozyme                          | GlcN induces the GlmS ribozyme which subsequently degrades the transcript of interest | Knockdown of essential genes
GlcN is cytotoxic                                      |
| DNA/RNA based techniques               | Peptide-morpholino oligo- | PMO conjugates bind to mRNA which leads to its degradation by RNaseP | High costs                                              |
|                                        | mer                      |                                                         |                                                        |
|                                        | Aptamers                 | Selection of nucleic acid oligomers against epitopes of interest (SELEX) | Identification of novel proteins
Broad application
Time consuming                                 |
| Protein interference                    | Protein/peptide interference | Knockdown of intracellular protein activity           | Applicable on essential proteins
Structural information of the target is needed       |

Table 1: Overview of the genetic toolbox for target validation in *P. falciparum*.
assertion was subsequently retracted [85]. It has more recently been demonstrated that blood-stage parasites possess a conventional cyclic (oxidative) TCA cycle [86, 87]. However, the essential nature of the mitochondria to parasite development and survival has led to a great degree of interest in it as a target for drug development (e.g., [88, 89]). The reader is referred to recent reviews [90-92] for a detailed overview of the current state of mitochondrial inhibitors.

Early studies showed that during the intraerythrocytic stages *P. falciparum* relies primarily on anaerobic glycolysis [93-95]. In these experiments, the majority of the radiolabeled glucose fed to the parasite, was shown to be converted to lactate. Only a minor fraction of radiolabeled metabolites was found within the TCA cycle, suggesting a minimal feed of substrate into the TCA cycle and an inactive TCA cycle during the blood stages of the parasite. This is correlated with an almost 100-fold increase of glucose consumption of parasite-infected erythrocytes compared to healthy erythrocytes. This increased use of glucose as a food source leads to increased production of lactate, resulting in lactic acidosis in the human host, which, together with hypoglycaemia, is the major cause of mortality during severe malaria [96].

While *P. falciparum* encodes all the genes necessary for a conventional TCA cycle [20, 97], the pyruvate dehydrogenase (PDH) complex is localized in the apicoplast, where it may be involved in fatty-acid biosynthesis [98, 99]. Within the conventional TCA cycle the PDH complex is responsible for oxidation of pyruvate imported from cytosol into acetyl-CoA. Despite the dependence of *P. falciparum* on anaerobic glycolysis during blood stages, the presence of the functional respiratory chain and maintenance of the electrochemical potential across the inner mitochondrial membrane has been proposed to be critical for the parasite's survival (e.g., [100, 101]). This is additionally demonstrated through the parasite’s sensitivity to atovaquone, which causes a failure in mitochondrial electron transport via an inhibition of the cytochrome *bc1* complex [102-104]. This results in a collapse of the electrochemical potential (DP) across the inner mitochondrial membrane. Studies have subsequently shown that
atovaquone is a competitive inhibitor of $Q_0$ interactions [105, 106]. As a result it has been postulated that a possible function of the mitochondrial respiratory chain is the reoxidation of mitochondrial dehydrogenases through the regeneration of ubiquinone (CoQ). Currently, five proteins are thought to compose the malarial “Q-cycle” responsible for supplying electrons to the $bc_1$ complex. Two of these are soluble proteins found in the mitochondrial matrix (glycerol-3-phosphate dehydrogenase ($Pf$GPD) and malate quinone oxidoreductase ($Pf$MQO)), one spans the inner mitochondrial membrane (succinate dehydrogenase) and two are found within the mitochondrial intermembrane space (NADH-dehydrogenase ($Pf$NDH2) and dihydroorotate dehydrogenase ($Pf$DHODH)). The function of $Pf$NDH is currently thought to be to maintain the inner mitochondrial membrane potential [107, 108], although it is not an essential gene as knocking out $Pf$NDH2 is not lethal [109]. In 2007, Painter and co-workers created transgenic $P.falciparum$ parasites expressing the non-CoQ dependent dihydroorotate dehydrogenase ($y$DHODH) from $S. cerevisiae$ [110]. While completely resistant to all cytochrome $bc_1$ complex inhibitors those parasites showed hypersensitivity to proguanil. This demonstrated that collapsing the mitochondrial membrane potential with proguanil was only effective in killing parasites in combination with mitochondrial electron transport inhibition caused by atovaquone. The role of $Pf$DHODH is thought to be in supporting parasite proliferation through de novo pyrimidine biosynthesis, as the parasite depends on glycolysis to supply energy [100]. This is supported by the lack of enzymes for pyrimidine salvage identified within the parasite genome (3D7, [20]). Thus, the malarial Q-cycle enzymes (and specifically $Pf$DHODH) link the function of malarial mitochondrial electron transport proteins with pyrimidine biosynthesis [111, 112].

4. Purine and Pyrimidine Biosynthetic Pathways

The intraerythrocytic phase of $P. falciparum$ is associated with extraordinary resource uptake from the host cell. Active proliferation during this stage requires a supply of purines and pyrimidines for parasite growth to support the rapid replication of parasites within erythrocytes. Plasmodial
purine and pyrimidine metabolic pathways are promising targets for anti-malarial drug research [113-115] as they significantly differ from those in host cells. Plasmodium parasites lack the de novo purine synthesis pathway and salvage host cell purines for growth [116, 117]. Inhibition of this pathway was shown to be lethal for *P. falciparum in vitro* [118]. Early biochemical studies on Plasmodium parasites *P. berghei* [119, 120], *P. Knowlesi* [121] and *P. lophurae* [122, 123] demonstrated the inability of Plasmodium species to metabolize pyrimidines. The parasites lack thymidine kinase, an enzyme responsible for salvaging host thymidine. Therefore, *P. falciparum* does not possess active pyrimidine salvage pathways and depends entirely on de novo synthesis through a series of enzymatic reactions.

5. Generation of CoQ: The Apicoplast as a Drug Target

Apicomplexan parasites such as *Plasmodium* species possess a relict plastid-like organelle known as apicoplast [124]. It represents a promising antimalarial drug target [125-130]. The isoprenoid biosynthesis pathway located in the apicoplast was shown to be an effective source of drug targets for antimalarial chemotherapy both in vitro and in vivo [125]. Multidrug-resistant *P. falciparum* strains showed significant sensitivity to the treatment with fosmidomycin (an inhibitor of the apicoplast located DXP reductoisomerase [125] and its derivative, FR-9000098. Additionally, mice infected with the rodent malaria parasite *P. vinckei* showed full recovery after the fosmidomycin treatment.

As mentioned above, ubiquinone (CoQ) is a pivotal component of the ETC of *Plasmodium* species. CoQ is composed of a benzoquinone ring, which participates in redox reactions, and a side chain of several isoprenic units which is used to attach the molecule to the mitochondrial inner-membrane [131]. The length of the isoprenoid tail varies between organisms [132]. The biosynthesis of the isoprenoid tail occurs in the apicoplast, IPP and DMAPP are condensed via the enzyme prenyltransferase to form the isoprenoid chains of defined lengths [98, 125, 133]. The side chain of CoQ
in *P. falciparum* consists of eight or nine units and labeling experiments showed an active non-mevalonate isoprenoid pathway followed by CoQ biosynthesis [134]. Authors have also presented data showing that parasites treated with nerolidol, a structural analog of isoprenoid tail intermediate, showed reduced CoQ biosynthesis activity in all intraerythrocytic stages [135]. This study, using a natural source of nerolidol, showed clear growth inhibition of *P. falciparum* cultures - again highlights the importance of CoQ biosynthesis pathway for parasites survival.

The first stage of isoprenoid biosynthesis results in production of isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). These two isomers serve as precursors of isoprenoids, a large and diverse family of compounds that play an important role in many metabolic processes, including lipid biosynthesis, cell membrane maintenance and steroid biosynthesis [136, 137]. Human isoprenoid biosynthesis was reported as promising drug target, as it is important for diverse cellular processes involved in the rapid growth of cancer cells [138]. While the isoprenoid biosynthesis in humans is carried out through a classic mevalonate pathway, most bacteria including important pathogens and apicomplexian parasites synthesize their IPP and DMAPP through an alternative methylerythritol phosphate pathway pathway [139].

6. Bridges and Crosstalk

Studies using radioactive fumarate, a byproduct of both the purine salvage pathway and a TCA cycle intermediate, suggested a high degree of metabolic crosstalk between nucleic acid biosynthetic/salvage pathways and the mitochondrial ETC [140]. Indeed, based on series of labeling experiments, the authors showed that instead of secreting radioactive fumarate as metabolic waste *P. falciparum* converts it into aspartate through malate and oxaloacetate. The proposed fumarate to aspartate conversion pathway within *P. falciparum* involves fumarate hydratase (*PfFum*), malate dehydrogenase (*PfMDH*)[141, 142], malate-quinone oxidoreductase (*PfMQO*) and aspartate aminotransferase (*PfAspAT*) [143, 144].
PfAspAT catalyzes a reversible reaction between L-aspartate and 2-oxoglutarate (or α-ketoglutarate, α-KG) into oxaloacetate and L-glutamate (EC 2.6.1.1). It has been structurally classified as PLP-dependent enzyme, much like other aminotransferases [145]. Bulusu et al. also suggested that incorporation of fumarate into nucleic acid occurs only via incorporation into the pyrimidine backbone, as no significant fractions of labeled purines were observed during incubation of the parasite with radioactive fumarate. In addition, parasites treated with atovaquone showed no enrichment of labeled aspartate and the conversion of fumarate to malate was unaffected. These observations indicate that the fumarate-to-aspartate pathway depends on a functional ETC. The AspAT of *P. falciparum* is also believed to play a significant role in supplying intermediates into the TCA cycle [146, 147] and conversion of fumarate into aspartate [140]. Thus, PfAspAT bridges the glycolytic, amino acid biosynthesis, TCA cycle, *de-novo* pyrimidine biosynthesis and purine salvage pathways [148].

Extensive GFP-labeling experiments have shown that PfAspAT is localized in the cytosol and no traces of mitochondria-localized PfAspAT were observed [146]. Additionally, specific inhibition of PfAspAT in parasitic cytosol resulted in a complete loss of glutamate-oxaloacetate transferase activity, demonstrating that no other parasite cytosolic protein can compensate for loss of PfAspAT activity. Finally, sequence analysis does not indicate the presence of any protein homologous to AspAT encoded in the parasite’s genome [20], supporting the hypothesis that PfAspAT activity is localized only in the cytosol.

7. Proteases and Uptake Targets

In addition there are several other important pathways and processes as well as enzymes and surface proteins that can function as valid drug targets for malaria. Degradation of hemoglobin and uptake from glucose by the parasite are essential processes that are promising drug targets [149, 150]. The processes are well understood with several crucial enzyme already discovered and are a main focus of research. Within the pathway of hemoglobin degradation, plasmepsins, a class of aspartic proteases of *P.*
*falciparum*, emerged as a prominent targets due to the well-studied crystal structure of plasmepsin II [151]. Further studies showed that single plasmepsins were dispensable for parasite survival, and several plasmepsin types within the class needed to be simultaneously targeted for lethal effect [152]. Similarities in structure to other proteases such as cathepsin D, renin and HIV-1 proteases [153, 154] give hints for possible inhibitors [30, 155]. Several inhibitors also showed an effect on plasmepsin II [156-158], however low selectivity between plasmepsin II and other proteases is a major problem in research. Structure-based screening (including virtual screening) has also yielded a number of nonpeptidomimetic plasmepsin inhibitors [152]. Although, despite the excellent inhibitory potential *in vitro*, several compounds were less active *in vivo* and *vice versa* [158]. This effect could potentially be attributed to poor permeability or non-specific effects of the tested compounds.

As the parasites do not express the mitochondrial pyruvate dehydrogenase [99], glucose catabolism relies completely on glycolysis. To sustain viability, the parasite needs to import glucose from the host’s metabolism [159]. The hexose/glucose transporter *PfHT* from *P. falciparum* has been shown to be crucial for the import of glucose and the survival of the parasite [160, 161]. With just 28% amino acid identity to its closest related human ortholog GLUT1, *PfHT* is a promising target for antimalarial drugs [162-165]. While screening with different compound libraries, several promising candidates were found [150]. Nevertheless, additional modifications have to be applied before these compounds can be considered as antimalarial drugs.

Further approaches are based on the similarity of essential enzymatic pathways between the malaria parasite and plants [166]. For example, known herbicides can serve as possible drug leads. One of these pathways is the synthesis of dTMP, in which the enzyme serine hydroxymethyltransferase (SHMT) is key player [167, 168]. Screening for inhibitors using the target-based herbicide programs of BASF [169, 170] identified promising compounds against SHMT [166]. These compounds show high activity against some species of *Plasmodium* and at different stages of the parasite life cycle. This makes them interesting candidates for blood stage malarias as well as hypnozoite stage *P. vivax* malaria [171]. However, the low met-
abolic activity of the most promising compound is one major problem to be overcome.

8. Vaccination

In addition to the possibility of finding drugs against the infection with malaria, another approach is the development of a vaccine. The most prominent candidate for vaccination is the circumsporozoite protein (CSP), which is a major surface protein of sporozoites of several species of *Plasmodium* [172-180]. The protein contains random repeats of an immunodominant B cell epitope [176-179, 181-184] surrounded by N-terminal and C-terminal domains. Several antibodies against the epitope of the protein, as well as the surrounding domains have been developed [174, 175, 185-190]. Transduction of the antibody in mosquitos and mice protect the host from an infection [191-193]. Based on this findings, the vaccine RTS,S/AS01 for human has been developed and first Phase III trials have been performed in children [18, 194, 195]. However, vaccination shows a modest efficacy against clinical and severe malaria. Further problems are the high costs and technical difficulties associated with manufacturing the vaccine at a sufficient volume.

9. Oligomeric Interfaces as targets for Protein Interference Assays (PIA) in drug target validation

In the brief review of a subset of the potentially druggable malarial enzymatic pathways given above, one feature dominates: where a clear indication as to the essential nature of a gene product has been demonstrated or disproved a specific tool compound is available. While these tool compounds are frequently suggested to be lead-compound for drug discovery, perhaps their greater importance is in the ability to validate (or invalidate) any particular protein as a drug target. This is another clear example of the Harlow-Knapp effect slowing efforts in the development of novel anti-malarials. In addition, it is frequently observed, that compounds previously shown to be active in *in vitro* assays are poorly taken up *in vivo*. This can be due to host of different reasons that are difficult to predict in
Figure 3. Oligomeric state of all protein entries in the protein data bank (www.rcsb.org; August 2017). Molecules composed of one, two, three or four copies of identical protein chains are known as “monomers”, “dimers”, “trimers” or “tetramers”, respectively.

advance (e.g., poor membrane passage/subcellular localization or rapid metabolism). This non-translation of compound activity between \textit{in vitro} and \textit{in vivo} further limits the availability of novel small molecule tools and may be an additional contributing factor in the Harlow-Knapp effect. We are attempting to address these gaps by providing an alternative route to the specific enzymatic inhibition provided by small molecules by utilizing a natural property of many proteins: oligomerization.

9.1. Oligomerisation is a Common Feature of Enzymes

Oligomerisation (the assembly of two or more copies of a single protein into one object) is a prominent feature in more than one half of all protein structures currently available within the protein data bank (PDB, http://www.rcsb.org; [196]) and plays a key role in the generation of functional biomolecules. While oligomeric interfaces are highly specific, the biomechanics of self-assembly in protein function has not been systematically explored or exploited as a method to dissect biochemical pathways. Interference in the self-assembly of macromolecules represents an excellent
opportunity in the analysis of biochemical pathways \textit{in vivo}, particularly in cases where standard techniques (e.g., RNAi/knock in/out) have a low success rate [57].

Based on an examination of >100,000 structures in the PDB it is apparent that more than one half of all proteins in the PDB are present in self-assembled states/oligomers (dimers, trimers, \textit{etc.}, Figure 3). These molecules generally display extremely high affinity and specificity for their cognate partners, typically due to extended molecular surfaces between the monomers. Our recent work [142, 143, 147, 197-200] has focused on the carbon metabolism pathway of the malarial parasite. This pathway contains a number of oligomeric enzymes, making it an ideal system to test our hypothesis that oligomeric self-assembly can be used to modulate \textit{in vivo} behavior. In the case of oligomers possessing enzymatic activity, the active site can be found both at oligomeric surfaces (e.g., the dimeric \textit{PfAspAT} (aspartate aminotransferase; Figure 4; [144, 146])) and contained within a single chain of the oligomer (e.g., the tetrameric \textit{PfMDH} (malate dehydrogenase)).

\subsection*{9.2. PIA-based inhibition of \textit{PfAspAT}}

The Crystal structures of AspATs from other organisms are available, including \textit{E.coli} AspAT [201-204], \textit{S. cerevisiae} cytosolic AspAT [205], Pig heart cytosolic AspAT [206] and both cytosolic and mitochondrial AspATs from chicken [207-209]. A structural comparison between plasmodial AspAT and its homolog from the human host is in preparation and will be published elsewhere (Bosch, Batista, Lunev \textit{et al.}, in preparation)

\textit{PfAspAT} is a homodimeric enzyme (PDB code 3K7Y; [146, 210]) with a molecular weight of each subunit of 45 kDa. Each subunit consists of a large PLP (cofactor) binding domain, a smaller domain, that shifts the enzyme from “closed” to “open” form in order to provide substrate binding and N-terminal “Arm” of 13 residues, that stabilizes the interaction between the two monomeric subunits into a dimer (Figure 4a, [207, 211, 212]). Two independent active sites are positioned near the oligomeric interface and are formed by residues from both subunits (Figure 4b). The spatial arrangement of substrate recognition sites, cofactor-binding sites and catalytic machinery are highly similar between known AspATs [146].
Comparison of available AspAT structures shows a highly conserved active site architecture in terms of both sequence similarity and atomic arrangement, making the design of a specific active site inhibitor that does not interact with the host AspAT, very challenging. Berger et al. [213] had previously identified a glycine (G197) to serine mutation found only in plasmodial species. However, this mutation is highly buried from the solvent and would require significant unfolding of the protein before any access would be available to any exogenously added molecules (Figure 4b). However, the difference in calculated surface charge between PfAspAT and the AspATs of the human could be pivotal for the design of specific inhibitors [146]. It should be noted, that PLP-dependent enzymes from protozoan parasites were suggested to be promising drug targets due to high metabolic diversity [214, 215]. Moreover AspATs (EC 2.6.1.1) were found to be present in all available genomes, again underlying their importance [216].

When compared to other organisms using BLAST [218], it is clear that 73% of the 405 AspAT residues are not evolutionary conserved (Table 2). The remaining 27% are divided into slightly conserved (7%), strongly conserved (11%) and absolutely conserved residues (9%). A similar distribution is observed when the residues involved in oligomeric interactions are examined. However, while the percentage of non-conserved residues involved in oligomeric interactions (69%) remains very similar to the overall number (73%), there are more absolutely conserved residues involved (17%; Figure 4, 5). This difference is due to the fact that both active sites are located at the oligomeric surface between two subunits and include highly conserved residues [146]. The fact that 69% of the residues involved in the dimer interface are non-conserved represents a significant opportunity for specific inhibition of PfAspAT.

Although there is a high level of sequential and structural homology between AspATs from different species, the N-terminal residues represent a very important difference. Indeed, all the currently known AspAT structures possess the structural difference in N-terminal region. Further experiments showed, that not only the first 13 N-terminal residues of PfAspAT are distant from active sites, non-conserved and represent conformational difference when compared to another known AspATs, but
Figure 4. \(Pf\)AspAT. (a) \(Pf\)AspAT is a dimer (individual monomers shown in teal and yellow), with the dimeric interface stabilize by a 13-residue “Arm” (red). b) The two active sites of \(Pf\)AspAT are formed by contributions from both of the monomers (Y70 (yellow) from one monomer and R257 (teal) from the other). Both Tyr70 and Arg257 donate the hydrogen bonds to the phosphate group of the cofactor PLP and are essential for activity [217].

Figure 5. An analysis of the residues involved in oligomerisation of \(Pf\)AstAT. (a) Indicates the surface contribution of residues involved in dimerization (blue), (b) highlights the subset of these residues that are absolutely or strictly conserved (red, purple), (c) highlights those residues that are slightly conserved (green). The remaining residues of the interface show no significant conservation across the AspATs aligned.
Figure 6

Figure 6. (a) The presence of PfAspATN50 results in inhibition of *in vitro* PfAspAT activity *in vitro*. (b) The presence of PfAspATN50 inhibits AspAT activity in the parasite cytosol, but does not inhibit the activity of cytosolic human AspAT (Figures reproduced from [143]).

Table 2

<table>
<thead>
<tr>
<th>Residues</th>
<th>Overall</th>
<th>Involved in Oligomeric interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-conserved</td>
<td>73%</td>
<td>69%</td>
</tr>
<tr>
<td>Slightly conserved</td>
<td>7%</td>
<td>8%</td>
</tr>
<tr>
<td>Strongly conserved</td>
<td>11%</td>
<td>6%</td>
</tr>
<tr>
<td>Absolutely conserved</td>
<td>9%</td>
<td>17%</td>
</tr>
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</table>

A table comparing the sequence conservation of available AspATs. With the exception of the catalytic machinery found at the interface, the oligomerisation surfaces do not contain a significantly higher population of conserved residues.
Figure 7. Under the condition that a plasmid-based over expression of mutant PfAspAT produces a significant excess of protein, with respect to the native protein, a single crucial mutation of one active site would result in a 50% drop of activity in vivo with extremely high specificity.

also play an important role in catalytic activity of the enzyme [146]. Several mutant forms of PfAspAT were designed and tested in vitro. Truncation of the first 7 and 13 N-terminal residues resulted in 15–50% wild type activity loss, respectively. Importantly, static light scattering experiments have also shown that truncation mutants were still able to form dimers in vitro.

Furthermore, a peptide possessing the first 13 N-terminal residues of PfAspAT was designed and recombinantly expressed linked to a C-terminal 6xHis-tag. In order to provide enough space between the 6xHis-tag and first 13 N-terminal residues the peptide construct consisted of first 50 N-terminal residues of PfAspAT (PfAspATN50). The wild type PfAspAT was shown to be able to efficiently pull down the PfAspATN50 peptide. This pull-down assays showed that the N-terminal region is sufficient for an interaction between the monomers. Circular dichroism experiments demonstrated that the PfAspATN50 peptide was unstructured in solution, excluding the pull-down interaction was a result of a partially folded PfAspAT mimicking a portion of the interaction surfaces shown in Figure 4. The addition of PfAspATN50 to wild type PfAspAT resulted in almost complete loss of the wild type activity at a ratio of 1:10 (Figure 6).

The effect of the PfAspATN50 presence on PfAspAT activity was also assayed in vivo. The lysates were extracted from both cultured parasites and
human erythrocytes and the AspAT activity was measured in presence \( Pf\text{AspATN}50 \) polypeptide \[146\]. The results of the assay demonstrated, that the \( Pf\text{AspATN}50 \) does not affect the human erythrocyte AspAT activity, while the wild type \( Pf\text{AspAT} \) activity is clearly inhibited.

Summarizing the results obtained, we believe that oligomeric surfaces offer a unique opportunity to generate highly specific interference with protein function in vivo. This opens the door to the potential to quantitatively evaluate a protein's function in vivo without recourse to complex genetic approaches, non-specific small molecules or difficulties in siRNA approaches.

We are currently performing experiments that aim to identify a phenotype of specific inhibition of \( Pf\text{AspAT} \) based on overexpressing active site mutations of \( Pf\text{AspAT} \) in the parasite. This has the additional advantage that such overexpression systems are well characterized. As the AspAT active site is composed of contributions from both monomers, overexpression of a single mutant with one disturbed active site would potentially result in a drop of AspAT activity of 50\% (Figures 4b & 7).

9.3. The Role of Pdx1/2 in the Protection of the Parasite Against Oxidative Stress

The two enzymes Pdx1 and Pdx2, which are expressed by \textit{Plasmodium falciparum}, play an important role in the biosynthesis of pyridoxal 5-phosphate (PLP). PLP is the active form of vitamin B\(_6\), which is an essential cofactor of more than 140 enzyme-catalyzed reactions in mammalian cells. For its synthesis, 12 Pdx1 enzymes assemble into a functional dodecamer and each Pdx1 is decorated by one Pdx2 enzyme, forming a multimeric complex with two hexameric rings. The interaction of \( Pf\text{Pdx}1 \) and \( Pf\text{Pdx}2 \) in the process of vitamin B\(_6\) biosynthesis represents a potential new target to identify novel drugs against the human malarial parasite \textit{P. falciparum}.

During the intraerythrocytic stage of its life cycle, \textit{P. falciparum} relies on the digestion of human hemoglobin as the main source of amino acid for its metabolism. However, while digesting hemoglobin, elevated levels of reactive oxygen species (ROS) are also generated. Although ROS such
as singlet molecular oxygen (‘O₂), superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂) are important signaling molecules of immune systems against invading pathogens, the elevated levels of oxidative stress in *P. falciparum* must be quenched in order to avoid oxidative damage to DNA and proteins [219, 220]. Cercosporin, an O₂ producer, was used to investigate the role of vitamin B₆ in fighting oxidative stress. Northern blotting results show the expression level of vitamin B₆ biosynthesis genes *PfPdx1* and *PfPdx2* increase 2-3 fold when treated with cercosporin, with respect to the untreated control [199]. This clearly demonstrates an involvement of two enzymes in combating increased amount of O₂ in *P. falciparum*.

Figure 8

![Diagram](image)

**Fig. 8.** Parasites expressing Pdx1 and Pdx2 mutations are significantly more susceptible to O₂ oxidative stress. (a,b) Under the condition that the mutated proteins are expressed at endogenous levels, the transfection of parasites with mutant Pdx1 or Pdx2 will result in an *in vivo* loss of activity of 50%. (c) Doubly transfected parasites will retain only 25% wild type Pdx1/Pdx2 activity.

9.4. PIA-based inhibition of the vitamin B6 biosynthesizing enzymes *PfPdx1/PfPdx2*

Overexpression of *PfPdx1* and *PfPdx2* in a co-transgenic cell line results in elevated PLP levels of 36.6 μM compared with 12.5 μM in wild-type parasites, leading to a higher tolerance towards oxidative stress. In contrast,
overexpressing either enzyme PfPdx1 or PfPdx2 singly does not rescue cercosporin-treated parasites. Furthermore, overexpressing inactive PfPdx1 and PfPdx2 mutant proteins also demonstrates the key role of endogenous vitamin B₆ as an antioxidant. Through in vivo interference with the assemblies of PfPdx1 and PfPdx2 expression, we have previously validated the antioxidative effect of the endogenous vitamin B₆ biosynthesis pathway in P. falciparum [199]. As stated above, PLP generation by Pdx1/Pdx2 functions only when functional Pdx1 and Pdx2 are assembled. In in vitro experiments, we demonstrated that specific Pdx mutants (i.e., PfPdx1-K83A and PfPdx2-E53Y), are correctly inserted into the Pdx1/2 PLP synthase complex but result in a loss of catalytic function, Overexpression of mutated PfPdx1 and PfPdx2 in vivo does not effect the parasites’ proliferation. Both wild type and transfected parasites were killed in the presence of high concentration of cercosporin.

However, while wild-type parasites were unaffected by lower concentrations of cercosporin, parasites transfected with either Pdx1 or Pdx2 mutated proteins were more challenged by ROS. Importantly, parasites transfected with both mutations were highly susceptible to ROS damage. In this experiment, the mutant Pdx1 and Pdx2 proteins were under the control of native promoters, strongly suggesting that these proteins are present at equivalent levels to that of the native (unmated proteins). Under these conditions, the singly transfected parasites would retain 50% Pdx1/Pdx2 PLP synthase activity, whereas the doubly transfected parasites would possess only 25% Pdx1/2 PLP synthase activity (Figure 8). This provides further evidence that oligomerization can be used in vivo to provide a highly specific analysis of interference with a targeted pathway.

10. Self-assembled molecules may pass through intermediate folding states during assembly.

We have previously shown that the plasmodial enzyme PfPdx1 passes through a defined assembly path during its association into the final active oligomer of 12 subunits. We demonstrated that the conformational mobility of a conserved glycine residue (G155) is required to allow the molecule
to pass through a transition state before assembly into the final oligomer [198]. These data show that the overall fold of constituent monomers can be significantly different to that present in the final assembly. These structural differences (or folding states during biomechanical assembly) may also offer novel opportunities for the design of small molecules that bind to transient pockets and inhibit by disrupting oligomerization, rather than through targeting an evolutionarily conserved active site.

11. Summary and Outlook

In the literature reviewed above we believe that we have presented a case for the use of PIA to specifically target and study biological pathways in vivo. This approach has a number of advantages. Firstly, the use of oligomeric surfaces provides a mechanism for highly specific targeting. This is a feature of the extent and size of oligomeric surfaces and their power to select only the “correct” binding partner from all other binding partners available within the cytosol. Additional supporting evidence for this statement is available in any publication demonstrating the purification of an oligomeric protein. Certainly such purity is generally required for the crystallization of any of the ~33,000 oligomeric structures available in the PDB (Figure 3). To our knowledge, such purifications rarely (if ever) result in the incorporation of “foreign” monomers in the purified oligomer at any detectable level.

Secondly, transfection and control of expression levels within many cellular systems (including the malarial parasite) is well understood and can be leveraged to fine-tune the degree of in vivo inhibition required. This can be achieved through the use of native promoter sequences or induc tant concentrations. Quantification and comparison of expression levels of mutants with respect to the native proteins is also straightforward through standard Western blotting techniques. This allows a quantitative analysis based on actual protein levels, rather than a more simplistic on/off approach exemplified by genetic knock-out methods. In addition, constant expression of the mutant monomers within the parasite bypasses
the transportation and possible degradation limitations associated with transfection with isolated proteins or peptides. Thirdly, this approach allows for the design of very simple control experiments (i.e., empty vectors) that only need to be performed once for any particular choice of transfection or overexpression vector. This allows a high degree of confidence in the comparison of results. This approach is limited to pathways that contain oligomeric proteins, although the analysis above (Figure 3) indicates that most biological pathways are likely to contain at least one step catalyzed by an oligomeric protein. Our future work will concentrate on identifying further oligomeric targets in biomedically relevant systems and establishing phenotypic data on the effects of specific interference to validate novel drug targets in human diseases.

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