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REVIEW ARTICLE

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

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Abstract: The validation of drug targets in malaria and other human diseases remains a highly challenging process. In the vast majority of cases, highly specific small molecule tools to inhibit a proteins 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*. Thus, new approaches to identify a protein's function *in vivo* are required to provide target validation in the drug discovery process. Oligomerisation is the assembly of two or more copies of a single protein into one object and this self-assembly is present in approximately one third of all protein structures. Thus, oligomerisation plays a central role in the generation of functional biomolecules. A key feature of oligomerisation is that the oligomeric interfaces between the individual parts of the final assembly are highly specific. However, these interface regions of essential plasmodial proteins have not yet been systematically explored or exploited as a method to dissect biochemical pathways *in vivo*. This mini review will describe initial efforts to exploit oligomerisation surfaces in drug target validation.

Keywords: Drug target validation, *in vivo* specificity, malaria, oligomerisation, protein Interference, protein: protein interactions.

INTRODUCTION

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.

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 *in vivo* function and/or role of the majority of kinases in human disease remains relatively unexplored [2]. 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 [3]. 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" [4]. 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*

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vivo. This has been previously expressed in 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; [1]). Thus, the availability of novel and specific tools generally results in a more complete understanding of biological systems of interest.

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

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 hypno-

zoite 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 the 584,000 reported deaths, 78% were of children under 5 years old. (WHO. 2014. World Malaria Report. World Health Organization: Geneva). While the gap between the current funding levels (US\$ 2.7 billion) and those required to eliminate the disease (US\$ 5.1 billion) has reduced, the emergence of multi-drug 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 [5-9].

While South-East Asia accounts for only approximately 7% of the global malarial incidence, the emergence of artemisin resistance is a serious threat to all current gains made towards control, treatment and elimination [10-12]. 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 [13]. 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 [14]. For an excellent overview of the current status in drug resistance in the malarial parasite, the reader is directed to a recent review [15].

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% [16, 17]. 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 [18]. Thus, until an effective vaccine is discovered, fully certified and ready for the 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.

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

Table 1. Overview of the genetic toolbox for target validation in *P. falciparum*.

Technique	System	Mode of Action	(Dis-) Advantages
Genomic modification (knock in/knockout)	Single crossover	On and off drug cycling to select for integration	Time consuming
	Double crossover	Genetic deletion of non-essential gene	Time consuming
	Customized ZFNs	ZFN induces the break of double-stranded DNA	High costs
	CRISPR-Cas	Double-strand breaks mediated by the Cas9 endonuclease	Highly efficient but not for essential genes
	Cre/LoxP system	Rapamycin induced dimerization of Cre	Requires co-transfection solely for small fragments
Conditional and inducible gene expression (knock-down)	Tet-off-System	ATc needed for initiation of transcription	Not available for <i>P. falciparum</i>
	FKBP12 destabilization domain (DD)	DD domain leads to protein degradation which can be prevented by Shld-1	Fast and regulative Can influence protein function or localization
	<i>E. coli</i> 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 oligomer	PMO conjugates bind to mRNA which leads to its degradation by RNaseP	High costs
	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
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Fig. (2). Global distribution of malaria infections in 2013 (Source: WHO World Malaria Report).

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 [19], although Wu and colleagues already succeeded in transfecting the parasite in 1995 [20]. In this transient transfection, circular plasmid DNA is maintained inside the parasite under drug selection as episomes, initially in an unstable replicating form (URF) that, after extended selection times, changes to an apparently stably replicating form (SRF) [21]. 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 [21]. It is believed that the various physical barriers surrounding the parasite [20, 22-24] and the requirement for concatamerisation lead to a low transfection efficiency in *P. falciparum* (estimated at 1.10^{-6} [25]). It is still not known how these plasmids are replicated or segregated during asexual division in the blood-stage, but there is an extended period they can be maintained in culture [21, 26]. Nevertheless the absence of positive selection leads to the loss of the plasmid, probably because of uneven segregation during mitosis [21, 27].

Transient transfection of *P. falciparum* makes classic reverse genetic approaches possible, such as stage-specific expression [28], expression of proteins for drug susceptibility assays compared to wild type [29] or localization and trafficking analysis with green fluorescent protein (GFP) fusion proteins [30, 31]. To date, there are four positive selectable markers available: the human dihydrofolate reductase (hdhfr) [32, 33]; Blastidicin S deaminase [34], neomycin phosphotransferase [34] and puromycin-N- acetyltransferase [35] which enhances the number of possible constructs.

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 [20, 36, 37]. 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 amount 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 [38], 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 colleagues established a double crossover using the Herpes simplex virus (HSV) thymidine kinase (tk) for negative selection [39]. The viral tk phosphorylates nucleoside analogues, such as gancyclovir [40], which will form nucleoside

triphosphates that inhibit DNA synthesis and the enzyme thymidylate synthase [41]. 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 Pfrh3 [39]. Additionally, the potential for integration events is highly increased, while the required time 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 [42, 43].

To improve the efficiency of gene disruption it was recently shown that zinc-finger nucleases (ZFNs) are functional in *P. falciparum* [44]. Customized ZFNs generate double-strand breaks (DSBs) of targeted DNA, which allows the generation of knockouts or allele replacements much faster than conventional methods [44]. 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 [45, 46].

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* [47]. 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 [48-51], seems to be absent in the malarial parasite. Therefore, *Plasmodium* depends on homologous recombination to maintain genome integrity [19, 52, 53]. 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 [47]. Additionally, successful gene disruption using linear DNA has been reported [47]. As linear DNA is apparently lost in *P. falciparum* after 4 days [54], this could make negative selection dispensable [55]. 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 [47]. 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.

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 [56-58]. 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* [59]. 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 [60]. The use of protein-binding RNA aptamers has also been reported to be functional in *P. falciparum* [61, 62]. 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 (*PfGyrA*) [63, 64].

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 [65, 66], 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) [67] or FKBP12± rapamycin-associated protein (FRAP)[68]. Heterodimerisation 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 [69]. The first application of the DiCre system in *P. falciparum* was performed by Collins and colleagues targeting *Pfsera5*, which could not be disrupted using conventional homologous recombination [70]. 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 [71].

Establishing of a new robust Tet-repressible transactivator allows control of the transcription to analyse essential *Plasmodium* genes via the Tet-off-System [72]. 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 promotor of the respective open reading frame. Subsequently, anhydrotetracycline (ATc) is used to mediate the binding of the TRAD to TetO [72-75]. Through this strategy an efficient knockdown of genes essential in the blood stage of the murine malaria parasite *P. berghei* could be demonstrated [72].

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 [76]. The expressed fusion protein can be stabilized through a rapamycin-derived ligand called shield (ShId-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* [77-80]. 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 ShId-1 could lead to transgenic parasites that would impact the effectiveness of ShId-1 [81]. As an alternative, Muralidharan and colleagues introduced the *Escherichia coli* dihydrofolate reductase (DHFR) degradation domain (DDD). A GFP-DDD fusion protein has been generated which 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 GlmS ribozyme has been shown to control reporter gene expression response to exogenous glucosamine (GlcN) [82]. 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*) [82]. Although this technique seems highly promising, prolonged GlcN treatment at high dose seems toxic to parasites [83].

In summary, the molecular genetics toolbox has been greatly enhanced in the last decade, but still remains insufficient in the terms of essential gene analysis. 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.

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 tricarboxylic acid (TCA) cycle. Oxidation of substrates within the TCA cycle generates electrons, which are used to supply the electron transport chain 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 behaviour of parasitic mitochondria when compared to the classical behaviour of mitochondria, such as those of the human host. However, the precise function of the TCA cycle of *P. falciparum* re-

mains 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 assertion was subsequently retracted [84]. It has more recently been demonstrated that blood stage parasites possess a conventional cyclic (oxidative) TCA cycle [85, 86]. 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. [87, 88]). The reader is referred to recent reviews [89-91] 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 [92-94]. In these experiments, the majority of the radiolabelled glucose fed to the parasite, was shown to be converted to lactate. Only a minor fraction of radiolabelled 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 [95].

While *P. falciparum* encodes all the genes necessary for a conventional TCA cycle [19, 96], the pyruvate dehydrogenase (PDH) complex is localized in the apicoplast, where it may be involved in fatty acid biosynthesis [97, 98]. 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. [99, 100]). 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 *bcl* complex [101-103]. This results in a collapse of the electrochemical potential (ΔP) across the inner mitochondrial membrane. Studies have subsequently shown that atovaquone is a competitive inhibitor of Q_0 interactions [104, 105]. As a result it has been postulated that a possible function of mitochondrial respiratory chain is the reoxidation of mitochondrial dehydrogenases through the regeneration of ubiquinone (CoQ). Currently, 5 proteins are thought to compose the malarial "Q-cycle" responsible for supplying electrons to the *bcl* complex. Two of these are soluble proteins found in the mitochondrial matrix (glycerol-3-phosphate dehydrogenase (*PfGPD*) and malate quinone oxidoreductase (*PfMQO*)), one spans the inner mitochondrial membrane (succinate dehydrogenase) and two are found within the mitochondrial intermembrane space (NADH-dehydrogenase (*PfNDH2*) and dihydroorotate dehydrogenase (*PfDHODH*)). The function of *PfNDH2* is currently thought to be to maintain the inner mitochondrial membrane potential [106, 107], although it is not an essential gene as knocking out *PfNDH2* is not lethal [108]. In 2007, Painter and co-workers created transgenic *P. falciparum* parasites expressing the non-CoQ

dependent dihydroorotate dehydrogenase (γ DHODH) from *S. cerevisiae* [109]. While completely resistant to all cytochrome *bcl* 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 *PfDHODH* is thought to be in supporting parasite proliferation through *de novo* pyrimidine biosynthesis, as the parasite depends on glycolysis to supply energy [99]. This is supported by the lack of enzymes for pyrimidine salvage identified within the parasite genome (3D7, [19]). Thus, the malarial Q-cycle enzymes (and specifically *PfDHODH*) link the function of malarial mitochondrial electron transport proteins with pyrimidine biosynthesis [110, 111].

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 [112-114] 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 [115, 116]. Inhibition of this pathway was shown to be lethal for *P. falciparum* *in vitro* [117]. Early biochemical studies on *Plasmodium* parasites *P. berghei* [118, 119], *P. Knowlesi* [120] and *P. lophurae* [121, 122] 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.

Generation of CoQ: The Apicoplast as a Drug Target

Apicomplexan parasites such as *Plasmodium* species possess a relict plastid-like organelle known as apicoplast [123]. It represents a promising antimalarial drug target [124-129]. The isoprenoid biosynthesis pathway located in the apicoplast was shown to be an effective drug target for antimalarial chemotherapy both *in vitro* and *in vivo* [124]. Multi-drug resistant *P. falciparum* strains showed significant sensitivity to the treatment with fosmidomycin (an inhibitor of the apicoplast located DXP reductoisomerase [124]) and its derivative, FR-900098. 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 electron transport chain 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 [130]. The length of the isoprenoid tail varies between organisms [131].

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 [97, 124, 132]. 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 [133]. 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 [134]. 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 [135, 136]. 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 [137]. While the isoprenoid biosynthesis in humans is carried out through a classic mevalonate pathway, most bacteria including important pathogens and apicomplexan parasites synthesize their IPP and DMAPP through an alternative non-mevalonate pathway [138].

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 electron transport chain [139]. 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*), malate-quinone oxidoreductase (*PfMQO*) and aspartate aminotransferase (*PfAspAT*) [140, 141].

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 [142]. 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 electron transport chain. The *AspAT* of *P. falciparum* is also believed to play a significant role in supplying intermediates into the TCA cycle [143, 144] and conversion of fumarate into aspartate [139]. Thus, *PfAspAT* bridges the glycolytic, amino acid biosynthesis, TCA cycle, *de novo* pyrimidine biosynthesis and purine salvage pathways [145].

Extensive GFP-labeling experiments have shown that *PfAspAT* is localized in the cytosol and no traces of mitochondria-localized *PfAspAT* were observed [143]. Additionally, specific inhibition of *PfAspAT* in parasitic cytosol resulted in a complete loss of glutamate-oxaloacetate transference 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 [19], supporting the hypothesis that *PfAspAT* activity is localized only in the cytosol.

PROTEASE 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 candidates for malaria. Degradation of hemoglobin and uptake from glucose by the parasite are essential processes that are promising drug targets [146, 147]. The processes are well understood with several crucial enzyme already discovered and are a main focus of research. Within the pathway of the hemoglobin degradation, plasmepsin II, an aspartic proteases of *P. falciparum*, emerged as a prominent target due to its well-known crystal structure [148]. Similarities in structure to other proteases such as cathepsin D, renin and HIV-1 proteases [149, 150] give hints for possible inhibitors [151, 152]. Several inhibitors also showed an effect on plasmepsin II [153-155], however the low selectivity between plasmepsin II and other proteases is a major problem in research. As the parasites do not express the mitochondrial pyruvate dehydrogenase [98], glucose catabolism relies completely on glycolysis. To sustain viability, the parasite needs to import glucose from the host's metabolism [156]. 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 [157, 158]. With just 28 % amino acid identity to its closest related human ortholog GLUT1, *PfHT* is a promising target for antimalarial drugs [159-162]. While screening with different compound libraries, several promising candidates were found [147]. 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 [163]. 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 [164, 165]. Screening for inhibitors using the target-based herbicide programs of BASF [166, 167] identified promising compounds against SHMT [163]. 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 [168]. However, the low metabolic activity of the most promising compound is one major problem to be overcome.

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) that is a major surface protein of sporozoites of several species of *Plasmodium* [169-177]. The protein contains random repeats of an immunodominant B cell epitope [173-176, 178-181] 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 [171, 172, 182-187]. Transduction of the antibody in mosquitos and mice protect the host from an infection [188-190]. Based on this findings, the vaccine RTS,S/AS01 for human has been developed and first Phase III trials have been performed in children [191-193]. 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.

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 number of different reasons that are difficult to predict in advance (*e.g.* poor membrane passage/subcellular localization or rapid metabolism). This non-translation of compound activity between *in vitro* and *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: oligomerisation.

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 one third of all protein structures currently available within the protein data bank (<http://www.rcsb.org>; [194]) 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 have 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 *in vivo*, particularly in cases where standard techniques (*e.g.* RNAi/knock in/out) have a low success rate [56].

Based on an examination of >100,000 structures in the Protein Data Bank it is apparent that approximately one third

(36.4%) of all proteins in the PDB are present in self-assembled states/oligomers (dimers, trimers, *etc.*, Fig. 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 [140, 144, 195-199] 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 *in vivo* behaviour. In the case of oligomers possessing enzymatic activity, the active site can be found both at oligomeric surfaces (*e.g.* the dimeric PfAspAT (aspartate aminotransferase; (Fig. 4); [141, 143]) and contained within a single chain of the oligomer (*e.g.* the tetrameric PfMDH (malate dehydrogenase)).

Fig. (3). Oligomeric state of all protein entries in the protein data bank (www.rcsb.org; August 2014). Molecules composed of one, two, three or four copies of identical protein chains are known as “monomers”, “dimers”, “trimers” or “tetramers”, respectively.

PIA-BASED INHIBITION OF PfAspAT

The Crystal structures of AspATs from other organisms are available, including *E. coli* AspAT [200-203], *S. cerevisiae* cytosolic AspAT [204], Pig heart cytosolic AspAT [205] and both cytosolic and mitochondrial AspATs from chicken [206-208]. A structural comparison between plasmodial AspAT and its homolog from human host would be a significant contribution to antimalarial research, however neither the structure of human cytosolic, nor mitochondrial AspAT is yet available.

PfAspAT is a homodimeric enzyme (PDB code 3K7Y; [143, 209]) 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 (Fig. 4a, [206, 210, 211]). Two independent active sites are positioned near the oligomeric interface and are formed by residues from both subunits (Fig. 4b). The spatial arrangement of substrate recognition sites, cofactor-binding sites and catalytic machinery are highly similar between known AspATs [143]. 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.* [212] 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 (Fig. 4b). However, the difference in calculated surface charge between *Pf*AspAT and the AspATs of the human could be pivotal for the design of specific inhibitors [143]. It should be noted, that PLP-dependent enzymes from protozoan parasites were suggested to be promising drug targets due to high metabolic diversity [213, 214]. Moreover, AspATs (EC 2.6.1.1) were found to be present in all available genomes, again underlying their importance [215].

Fig. (4). *Pf*AspAT. **a)** *Pf*AspAT is a dimer (individual monomers shown in dark and light grey), with the dimeric interface stabilize by a 13-residue “Arm” (black). **b)** The two active sites of *Pf*AspAT are formed by contributions from both of the monomers (Y72 from one monomer and R257 from the other).

When compared to other organisms using BLAST [216], 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%; Fig. 4). 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 [143]. The fact that 69% of the residues involved in the dimer interface are non-conserved represents a significant opportunity for specific inhibition of *Pf*AspAT.

Table 2. A table comparing the sequence conservation of AspATs for which structural information is available. With the exception of the catalytic machinery found at the interface, the oligomerisation surfaces do not contain a significantly higher population of conserved residues.

Residues	Overall	Involved in Oligomeric Interactions
Non-conserved	73 %	69 %
Slightly conserved	7 %	8 %
Strongly conserved	11 %	6 %
Absolutely conserved	9 %	17 %

Although there is a high level of sequential and structural homology between AspATs from different species, the N-terminal residues present the 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 *Pf*AspAT are distant from active sites, non-conserved and represent conformational difference when compared to another known AspATs, but also play an important role in catalytic activity of the enzyme [143]. Several mutant forms of *Pf*AspAT were designed and tested *in vitro*. Truncation of 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 first 13 N-terminal residues of *Pf*AspAT 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 *Pf*AspAT (*Pf*AspATN50). The wild type *Pf*AspAT was shown to be able to efficiently pull down the *Pf*AspATN50 peptide. These pull-down assays showed, that the N-terminal region is sufficient for an interaction between

Fig. (5). An analysis of the residues involved in oligomerisation of *PfAspAT*. **a)** Indicates the surface contribution of residues involved in dimerization, **b)** highlights the subset of these residues that are absolutely or strictly conserved, **c)** highlights those residues that are slightly conserved. The remaining residues of the interface show no significant conservation across the AspATs aligned.

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 Fig. (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 (Fig. 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 *PfAspATN50* polypeptide [143]. The results of the assay demonstrated, that the *PfAspATN50* does not affect the human erythrocyte AspAT activity, while the wild type *PfAspAT* 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 proteins 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 *PfAspAT* based on overexpressing active site mutations of *PfAspAT* 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 (Y72F) will result in a drop of AspAT activity of 50% (Figs. 4b and 7).

THE ROLE OF Pdx1/2 IN THE PROTECTION OF THE PARASITE AGAINST OXIDATIVE STRESS

The two enzymes Pdx1 and Pdx2, which are expressed by *Plasmodium falciparum*, play an important role in the biosynthesis of pyridoxal 5-phosphate (PLP). PLP is the active form of vitamin B₆, 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 *PfPdx1* and *PfPdx2* in the process of vitamin B6 biosynthesis displays a potential new target to identify novel drugs against the human malaria parasite *P. falciparum*.

During the intraerythrocytic stage of its life cycle, *P. falciparum* relies on the digestion of human hemoglobin as the main source of amino acid for its metabolism. However,

The presence of *PfAspATN50* results in *in vitro*.

The presence of *PfAspATN50* inhibits AspAT activity in the parasite cytosol, but does not inhibit the activity of cytosolic human AspAT.

(Figures reproduced from Wrenger *et al.*, 2011)

Fig. (6). Loss of *PfAspAT* activity upon addition of *PfAspATN50*.

Under the condition that a plasmid-based overexpression of mutant *Pf*AspAT produces a significant excess of protein, with respect to the native protein, a single mutation will result in a 50% drop of activity *in vivo*, with extremely high specificity. A double mutation will result in a complete loss of *Pf*AspAT activity.

Fig. (7). Concept of AspAT *in vivo* Protein Interference Assays.

while digesting hemoglobin, elevated levels of reactive oxygen species (ROS) are also generated. Although ROS such as singlet molecular oxygen ($^1\text{O}_2$), superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) 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 [217, 218]. Cercosporin, an O_2 producer, was used to investigate the role of vitamin B_6 in fighting oxidative stress. Northern blotting results show the expression level of vitamin B_6 biosynthesis genes *Pf*Pdx1 and *Pf*Pdx2 increase 2-3 fold when treated with cercosporin, with respect to the untreated control [197]. This clearly demonstrates an involvement of these two enzymes in combating increased amount of O_2 in *P. falciparum*.

PIA-BASED INHIBITION OF THE VITAMIN B_6 BIOSYNTHESIZING ENZYMES *Pf*Pdx1/*Pf*Pdx2

Overexpression of *Pf*Pdx1 and *Pf*Pdx2 in a co-transgenic cell line results in elevated PLP levels of $36.6 \mu\text{M}$ compared with $12.5 \mu\text{M}$ in wild-type parasites, leading to a higher tolerance towards oxidative stress. In contrast, overexpressing either enzyme *Pf*Pdx1 or *Pf*Pdx2 singly does not rescue cercosporin treated parasites. Furthermore, overexpressing inactive *Pf*Pdx1 and *Pf*Pdx2 mutant proteins also demonstrates the key role of endogenous vitamin B_6 as an antioxidant. Through *in vivo* interference with the assemblies of *Pf*Pdx1 and *Pf*Pdx2 expression, we have previously validated the antioxidative effect of the endogenous vitamin B_6 biosynthesis pathway in *P. falciparum* [197]. 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 (ie. *Pf*Pdx1-K83A

and *Pf*Pdx2-E53Y), are correctly inserted into the Pdx1/2 PLP synthase complex but result in a loss of catalytic function. Overexpression of mutated *Pf*Pdx1 and *Pf*Pdx2 *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 on 25% Pdx1/2 PLP synthase activity (Fig. 8). This provides further evidence that oligomerisation can be used *in vivo* to provide a highly specific analysis of interference with a targeted pathway.

Self-assembled Molecules May Pass Through Intermediate Folding States During Assembly

We have previously shown that the plasmodial enzyme *Pf*Pdx1 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 [196]. This data shows that the overall fold of constituent monomers can be significantly different to that present in the final assembly. These structural differ-

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%. Double transfected parasites will retain only 25% wild type Pdx1/Pdx2 activity.

Fig. (8). Parasites expressing Pdx1 and Pdx2 mutations are significantly more susceptible to $^1\text{O}_2$ oxidative stress.

ences (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 oligomerisation, rather than through targeting an evolutionarily conserved active site.

SUMMARY AND OUTLOOK

In the literature reviewed above we believe that we have presented a case for the use of protein interference assays 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 generally required for the crystallisation of any of the ~33,000 oligomeric structures available in the PDB (Fig. 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 inducant 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.

Finally, this approach is limited to pathways that contain oligomeric proteins, although the analysis above (Fig. 3) indicates that most biological pathways are likely to contain at least one step catalyzed by an oligomeric protein. The PIA method described above requires the production of a complete protein in the parasite using a plasmid-based expression system. As the proteins are produced directly within the parasite cytosol, there are no issues in the transportation of the (mutated) protein or tool compound into the parasite. Degradation of the protein produced within the parasite will remain a concern, but as there is a constant production of new protein from the inserted plasmid we feel that degradation of the protein is unlikely to represent a major concern in the analysis of data from the PIA approach.

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.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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