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Oligomeric protein interference validates druggability of aspartate interconversion in *Plasmodium falciparum*

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**Abstract**
The appearance of multi-drug resistant strains of malaria poses a major challenge to human health and validated drug targets are urgently required. To define a protein's function in vivo and thereby validate it as a drug target, highly specific tools are required that modify protein function with minimal cross-reactivity. While modern genetic approaches often offer the desired level of target specificity, applying these techniques is frequently challenging—particularly in the most dangerous malaria parasite, *Plasmodium falciparum*. Our hypothesis is that such challenges can be addressed by incorporating mutant proteins within oligomeric protein complexes of the target organism in vivo. In this manuscript, we provide data to support our hypothesis by demonstrating that recombinant expression of mutant proteins within *P. falciparum* leverages the native protein oligomeric state to influence protein function in vivo, thereby providing a rapid validation of potential drug targets. Our data show that interference with aspartate metabolism in vivo leads to a significant hindrance in parasite survival and strongly suggest that enzymes integral to aspartate metabolism are promising targets for the discovery of novel antimalarials.

**KEYWORDS**
drug target validation, oligomeric state, phenotypic mapping, *Plasmodium falciparum*, structural biology
1 | INTRODUCTION

The parasite *Plasmodium falciparum* is responsible for the most lethal form of human malaria (World Health Organization, 2017). The spreading of *P. falciparum* in the human host depends on the availability of specific metabolites during its blood stage (Kirk & Saliba, 2007; Lindner, Meissner, Schettert, & Wrenger, 2013). The metabolism of these external nutrients represents a key-step for parasite proliferation, and it is believed to be essential for its survivability. Although these metabolic steps would open new avenues for drug discovering targeting *P. falciparum*, the validation of these metabolic steps remains challenging due to limitations of applicability of probe techniques in *P. falciparum* and dependence upon reverse genetics (Meissner et al., 2016). This highlights the necessity of development of novel validation techniques capable of simplifying the validation of potential targets in *P. falciparum*—as well as other parasitic organisms. The examination of interaction surfaces between subunits of oligomeric proteins might offer a relatively straightforward alternative to this process (Meissner et al., 2016).

Protein oligomerization, the assembly of two or more copies of a single protein into one object, is a feature shared by all organisms and is present in more than 60% of all protein structures currently available within the Protein Data Bank (PDB; Hashimoto, Nishi, Bryant, & Panchenko, 2011). The biological importance, physicochemical properties, and evolutionary aspects of protein oligomerization have been recently summarized (Hashimoto & Panchenko, 2010; Hashimoto et al., 2011; Nishi, Hashimoto, Madej, & Panchenko, 2013). Furthermore, lower degree of evolutionary conservation of the oligomeric interfaces (Caffrey, Somaroo, Hughes, Mintseris, & Huang, 2004; Valdar & Thornton, 2001), as well as high specificity and binding affinity between the cognate partners, could successfully be utilized in drug target validation (Lunev et al., 2018). Based on these features, we hypothesized that the introduction of functionally incompetent forms of an enzyme into the native oligomeric assembly could be exploited 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.

The aspartate metabolism pathway within *P. falciparum* 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 behavior. Aspartate interconversion is essential for nitrogen metabolism of all organisms. In *Plasmodium* species, it was also proposed to play a key role in de novo pyrimidine biosynthesis (Wrenger et al., 2011). Plasmodial aspartate aminotransferase (*PfAspAT*) and malate dehydrogenase (*PfMDH*) catalyzes the reversible reaction from aspartate + 2-oxoglutarate to oxaloacetate + glutamate and malate + NAD to oxaloacetate + NADH, respectively. The crystal structure of *PfAspAT* has been previously solved (Wrenger et al., 2011). As reported, *PfAspAT* is a homo-dimer with a molecular weight of 48.42 kDa per monomer (Wrenger et al., 2011). Similarly to the previously described AspAT of *E. coli* (Jäger, Moser, Sauder, & Jansonius, 1994), each subunit consists of three major domains: an N-terminal arm (residues 1–14), a large coenzyme-binding domain (residues 36–321) and a smaller domain (residues 15–36 and 322–404). The N-terminal arm domain (residues 1–14) distal from either active site is thought to stabilize the *PfAspAT* dimer and is necessary for activity, as truncated species lacking the N-terminal extension showed significantly reduced activity while retaining dimeric structure (Wrenger et al., 2011). The two active sites of *PfAspAT* are formed in a cleft between the big and small domains near the oligomeric interface and each active site pocket is composed of residues contributed from both subunits. Previous experiments where *PfAspAT* was selectively inhibited in vitro using a polypeptide chain consisting of first 50 *PfAspAT* amino acids (Wrenger et al., 2011) confirm the hypothesis that oligomeric interfaces show significantly higher sequence divergence amongst homologs and thus offer potential in specific interference with a target protein.

The structure *PfMDH* has also been recently solved, and we have provided an insight into the role of oligomeric assembly in the regulation of *PfMDH* activity (Lunev et al., 2018). *PfMDH* possesses a tetrameric conformation where each monomer is comprised of 326 residues and is composed of two major domains: an N-terminal co-factor-binding domain containing a parallel structure of six beta-sheets (Rossmann-fold) and C-terminal substrate-binding domain. Our previous results suggested that a correctly formed tetrameric assembly of *PfMDH* is essential for activity (Lunev et al., 2018). Indeed, the introduction of a cryptoprotein residue at one of the interfaces facilitating oligomerization (V190W) results in disruption of the tetramer, breaking it down into two dimers, and a significant reduction in activity. Co-purification and western blot experiments with mixed lysates of recombinantly expressed wild-type *PfMDH* (Strep-tagged) and *PfMDH*-V190W (His$_{6}$-tagged) mutant, with a predicted molecular mass of 35.28 and 36.74 kDa for each monomer, respectively, demonstrated that *PfMDH*-V190W was able to insert itself into a pre-formed wild-type *PfMDH* assembly (Lunev et al., 2018). As shown by subsequent activity assays, the isolated wild-type-V190W chimera possessed no detectable activity in either direction, while recombinant wild-type *PfMDH* displayed both reductive and oxidative activity. These data demonstrate that recombinant mutants can be used as specific modifiers of wild-type *PfMDH* activity in vitro, offering the potential to validate it as a drug target, without recourse to complex genetics or initial tool compounds that may display significant off-target effects (Lunev et al., 2018). However, neither *PfAspAT* nor *PfMDH* has as yet been validated as a drug target in vivo.

In this study, structural information of the enzymes *PfMDH* and *PfAspAT* was used to generate mutants for use in in vivo protein interference experiments following two different approaches. In the first approach, we designed mutants that would incorporate within the native oligomer and disrupt the native oligomeric state, thereby inhibiting the function of the native assembly. In a second complementary approach, a mutant was designed to incorporate within the native oligomer and inhibit its function, without disruption to the native oligomeric state. In both approaches, the activity of the target enzymes was determined within the lysate
of transgenic parasites, suggesting successful incorporation of the mutants within the targeted assemblies. The resulting data clearly indicate a significant dependence of the parasite on functional aspartate metabolism. Our data also provide proof-of-principle for protein interference assay (PIA) as a general approach to the use of oligomeric assemblies to obtain functional data in vivo.

## 2 | RESULTS

### 2.1 | The oligomeric interface of PfAspAT shows higher sequence diversity than its active site

BLAST (Altschul et al., 1990) analysis of the close homologs of PfAspAT showed overall 38.7% sequence conservation, while the residues comprising the active site of PfAspAT showed a sequence conservation of 100% (Table 1; Figure 1). PISA (Krissinel & Henrick, 2007) analysis of the structural assembly of PfAspAT identified 98 residues involved in the inter-oligomeric contact, showing sequence conservation of 34.7% (Table 1), where 10.2% accounts for the active site residues. As previously mentioned (Wrenger et al., 2011), the N-terminal arm domain shows 100% sequence diversity (Figure 2). Only six residues involved in the contact with the N-terminal arm domain (Asn277, Phe116, Ile263, Leu117, Val209, and Phe241) are somewhat conserved (Figures 1 and 2).

### 2.2 | Point mutations of the key active site residues abolish catalytic activity of PfAspAT in vitro while not disturbing the dimerization and overall fold

Based on the crystal structure of PfAspAT (3K7Y; Wrenger et al., 2011), point mutations were designed in order to interfere with the catalytic 

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**TABLE 1** Sequence conservation across the different oligomeric interfaces of PfAspAT. Sequence conservation amongst close homologs (identity above 28%) was analyzed using BLAST (Altschul et al., 1990). Analysis of the residues supporting the oligomeric contact was performed using PISA (Krissinel & Henrick, 2007)

| No. of residues | Conserved residues (% of total No.) | Absolutely | Strongly | Weakly |
|-----------------|-------------------------------------|------------|----------|--------|
| PfAspAT (PDB 3K7Y) |                                      |            |          |        |
| 405             | 157 (38.7%)                          | 49 (12.1%) | 66 (16.2%)| 42 (10.4%)|
| Active site residues (% of active site residues) | 19 |
| Absolutely | 14 (73.7%) |
| Strongly | 3 (15.8%) |
| Weakly | 2 (10.5%) |
| Oligomeric interface | 98 |
| Interface residues (% of interface residues) | 34 (34.7%) |
| Absolutely | 11 (11.2%) |
| Strongly | 12 (12.3%) |
| Weakly | 11 (11.2) |
| Total ASA per monomer (Å²) | 19,890 (100%) |
| Buried ASA (Å²) | 2,436 (12.3%) |

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**FIGURE 1** (a) PfAspAT homodimer (3K7Y), where the N-terminal arm domains are clearly visible. (b) and (d) (side view) Residues (blue) supporting oligomerization, as predicted by PISA (Krissinel & Henrick, 2007). (c) and (e) (side view) Evolutional diversity of the residues involved in the oligomeric contact: absolutely conserved (red), strongly conserved (orange) and slightly conserved (pale yellow). Sequence conservation amongst close homologs (identity over 28%) was analyzed using BLAST (Altschul et al., 1990). Figures were prepared using PyMol (Delano, 2018)
FIGURE 2  Sequence alignment of PfAspAT against the closest homologs
activity of PfAspAT. Arginine 257 and Tyrosine 68 of a single PfAspAT monomer contribute to distinct catalytic sites and are both required for cofactor binding and catalytic functions (Figure 3). As previously described for E. coli, homologs of both residues (Arg266 and Tyr70; Jäger et al., 1994) are involved in hydrogen bonds to the phosphate group of the cofactor PLP. As a result, we hypothesized that mutations of these residues would significantly affect the catalytic activity of PfAspAT. Furthermore, as Tyr68 and Arg257 belong to different subunits, the double mutation Y68A/R257A in a single monomer would affect both active sites of the dimer. The PfAspAT-Y68A/R257A mutant was recombinantly expressed and purified. Static light scattering (SLS) measurements confirmed that the introduction of both active site mutations did not impact the oligomeric assembly, as both wild type and mutant versions had a molecular weight of approx. 94 kDa, consistent with a dimeric assembly (Figure 4). The specific activity of the double mutant of PfAspAT-Y68A/R257A was measured, showing loss of activity (0.01 ± 0.0005 U/mg) compared to the wild type (1.71 ± 0.12 U/mg). This represents an approx. 170-fold reduced catalytic activity of mutant (Figure 5). These data suggest that the introduction of two-point mutations Y68A and R257A would have the desired inactivation effect on PfAspAT while not affecting its folding or ability to form dimers.

2.3 | Inactivated PfAspAT mutant copies can be incorporated into the native assembly during recombinant expression in E. coli

Assuming that double mutation Y68A/R257A affects both active sites of one PfAspAT dimer, we further hypothesized that formation of PfAspAT wild-type/mutant chimera would also affect both active sites and result in a significantly less active enzyme.

The PfAspAT-Y68A/R257A gene was sub-cloned into the pBM1 vector using sequence-specific primers (Appendix 1) and the resulting pBM1-PfAspAT-Y68A/R257A plasmid encoded the full-length PfAspAT gene with both mutations and a His$_6$-tag fused at the C-terminus. The wild-type PfAspAT with C-terminal Strep-tag was expressed according to a previously reported protocol (Jain, Jordanova, Müller, Wrenger, & Groves, 2010).

Recombinant co-expression of both wild-type PfAspAT (Strep-tagged) and PfAspAT-Y68A/R257A (His$_6$-tagged) mutant was performed in E. coli and the lysate from the co-expression was purified by Strep tacitin-affinity and Ni-affinity chromatography (Figure 5a). A western blot analysis of both eluates clearly demonstrated the presence of His$_6$-tagged double PfAspAT mutant in a Strep-purified sample (Figure 5b, lane 2) as well as the Strep-tagged wild type in the His$_6$-purified sample (Figure 5b, lane 3). These results were interpreted as the formation of a dimer consisting of both wild type and mutant species of 2.4 PfAspAT during co-expression.

Further activity measurements indicated that Strep-purified sample showed reduced activity compared to the wild type; while no activity could be detected from the His$_6$-purified sample (Figure 5c). These data indicate that single His$_6$-purification of the wild-type:mutant PfAspAT co-expression product is able to isolate a chimeric oligomer consisting of the His$_6$-tagged PfAspAT-Y68A/R257A mutant and Strep-tagged wild type. The lack of detectable activity of the purified chimera, confirms the hypothesis that a mutant copy (Y68A/R257A) can be introduced into the native PfAspAT dimeric assembly through co-expression resulting in an inactivated chimeric protein.

2.4 | Introduction of PfAspAT and PfMDH mutants results in a significant reduction in parasitaemia in aspartate-limited culture media

The cytosolic localization of PfAspAT within the parasite has previously been reported (Wrenger et al., 2011). Similarly, the
cytosolic localization of PfMDH has been visualized by expression of a PfMDH-GFP chimera (Figure 6). In order to assess the effect of the presence of mutated PfAspAT and PfMDH in vivo, expression plasmids for PfAspAT-Y68A/R257A and PfMDH-V190W were created for transfection into P. falciparum. As the expression from the introduced plasmids is likely to result in an excess of the mutant protein with respect to the endogenous protein, it is not unreasonable to hypothesize that a significant proportion of the wild-type oligomeric PfMDH or PfAspAT would contain at least a single copy of the mutant protein. Based upon our co-expression and in vitro activity assays, the assembly of an endogenous PfMDH with PfMDH-V190W or PfAspAT with PfAspAT-Y68A/R257A would be expected to yield a dimeric assembly with no activity. Thus, the overexpression of PfMDH-V190W or PfAspAT-Y68A/R257A in vivo could be anticipated to result in inhibition of PfMDH or PfAspAT activity with little or no off-target effects. As one of the major functions of these two proteins is to support the synthesis of aspartate this also allows an examination of the role of aspartate biosynthesis in blood-stage cultures.

For this reason, proliferation curves of the parasites were analyzed in different media. The growth of wild-type parasites (control experiments using MOCK plasmids) was not significantly affected by the absence of aspartate in the culture media (Figure 7), providing a further demonstration that P. falciparum possesses a fully functional aspartate biosynthetic pathway. The introduction of PfAspAT-Y68A/R257A or PfMDH-V190W alone resulted in no detectable effects on blood-stage parasite growth in aspartate rich or aspartate limiting media (Figure 7a, b). However, this demonstrates that there are no significant negative effects of introducing either the plasmids or the mutant proteins in vivo of effects on parasite growth. While no effect is observed when both mutant proteins are introduced simultaneously in aspartate-rich media, a significant effect (an almost 100-fold reduction in parasitaemia) is seen on parasite proliferation in aspartate-limiting cultures (Figure 7c). As neither mutant alone showed any negative effect on parasite growth in aspartate-limiting media, we interpret this data to mean that we have successfully inhibited both PfMDH and PfAspAT in vivo. As indicated above, our approach is unlikely to result in complete inhibition of either PfAspAT or PfMDH so the residual growth seen in the mutant-inhibited cultures is most probably due to residual activity of uninhibited PfAspAT and PfMDH. Subsequently, to prove that the double-transfect cell line expresses the transgenic proteins the overexpression of both transcripts was determined by qRT-PCR. Compared to the wild-type strain the mdh- and aspat-genes were 1.64-fold ±0.32 and 4.10-fold ±0.59 higher at the transcriptional level, respectively (Figure 8). Furthermore, the protein production within the transgenic parasites was visualized by western blotting from parasite lysate (Figure 9).

### 2.5 Activity measurements of PfMDH and PfAspAT in parasite lysates confirm the formation of the heterocomplexes

In order to confirm the activity profile of both enzymes in the respective transfected parasites, we performed specific activity assays using parasite extracts (Figure 10). As expected, proteins extracted from parasites transfected with plasmids encoding additional copies of wild-type PfMDH or PfAspAT genes presented a statistically higher enzymatic activity (100.41 ± 1.05 and 8.83 ± 0.28 mU/mg, respectively) compared to non-transfected parasites (3D7), confirming that both enzymes were not only being overexpressed (Figure 10a) but were also functional. The proteins extracted from parasites transfected with the mutant gene PfMDH-V190W showed a mild reduction in specific activity (68.78 ± 8.65 mU/mg) compared to the control (76.06 ± 0.68 mU/mg, Figure 10a). However, this difference was not statistically significant. The effect of the transfection with PfAspAT-Y68A/R257A mutant was more pronounced (3.32 ± 0.06...
compared to the control of 5.03 ± 0.46 mU/mg, Figure 10b). These results confirm that the presence of mutant or extra wild-type copies of proteins within the parasite could form complexes with the native enzymes and, as a consequence, decrease or increase the activity of the targeted oligomers.

3 | DISCUSSION

In order to show the use of oligomeric surfaces in establishing protein interference studies in an in vivo setting, we have characterized the assembly surfaces of two plasmodial enzymes: aspartate aminotransferase (PfAspAT) and malate dehydrogenase (PfMDH), that are both localized in the cytosol of the parasite (Figure 6a; Wrenger et al., 2011). In order to validate the druggability of the targeted malate-aspartate pathway, structure-based mutagenesis experiments of the two involved genes were performed. We have recently demonstrated that a surface mutation in PfMDH, where valine 190 was mutated into tryptophan, disrupted the A-C interface as anticipated, causing significant activity loss (Lunev et al., 2018). Our previous data suggested that the introduction of the V190W mutation at the oligomeric interface (A-C) not only caused the splitting
of the tetramer into a pair of dimers but it also made the re-formation of the tetramer highly unlikely due to the introduction of molecular clashes. It was also previously reported that the mutated \( \text{PfMDH-V190W} \) was unable to incorporate into the native \( \text{PfMDH-WT} \) assembly in vitro, disturbing the native oligomeric state of the target protein as well as inhibiting its activity (Lunev et al., 2018). In this work, we also demonstrate that the hypothesized \( \text{PfMDH} \) chimeras are formed inside the parasite in vivo, through measurement of \( \text{PfMDH} \) activity in the lysate of transgenic parasites.

We performed similar experiments with \( \text{PfAspAT} \). In contrast with \( \text{PfMDH} \) where the oligomeric state is disturbed, in our \( \text{PfAspAT} \) mutant, the native oligomeric state is maintained. Incorporation of mutant \( \text{PfAspAT} \) showed that the enzyme’s native oligomeric assembly could also be utilized in order to target the wild-type protein and show an effect on activity in vitro (Figure 5c) and in vivo (Figure 10). The inactive \( \text{PfAspAT-Y68A/R257A} \) mutant was shown to be able to incorporate into native \( \text{PfAspAT-WT} \) dimeric assembly during recombinant co-expression, as confirmed by western blot (Figure 5b), resulting in complete loss of activity (Figure 5c). The mutated \( \text{PfAspAT} \) protein was then expressed in \( \text{P. falciparum} \) blood-stage cultures, and we have demonstrated that the hypothesized \( \text{PfAspAT} \) chimeras are formed inside the parasite in vivo, through measurement of \( \text{PfAspAT} \) activity in the lysate of transgenic parasites. While no effect is seen in aspartate-rich media, the introduction of both mutant proteins in aspartate-limited media results in a clear phenotype (Figure 7), without recourse to complex genetic manipulations. We have termed this approach the oligomeric protein interference assay (PIA; Meissner et al., 2016).

Our data show that oligomeric surfaces can be used to specifically inhibit protein activity in vivo, especially in cases when opportunities for genetic manipulation are limited. The introduction of \( \text{PfAspAT-Y68A/R257A} \) or \( \text{PfMDH-V190W} \) alone did not result in any significant effect on parasite proliferation in blood-stage cultures (Figure 7a,b). Nonetheless, we believe that these findings demonstrate that the expression of mutant proteins in cultured parasites has overall no negative effects on parasite growth. This also indicates that, while the expression levels of the mutant proteins are significantly higher (up to fourfold) than their endogenous counterparts, they are likely not overexpressed at a level that would induce metabolic stress on \( \text{P. falciparum} \) through depletion of amino acids. As the mutant proteins introduced are designed and demonstrated to be inactive, it is also highly unlikely that we have significantly altered the metabolic balance within the parasite. In addition, our experiments utilized well-characterized expression plasmids to introduce the mutant proteins and numerous previous publications have not reported significant effects of these plasmids on parasite growth (Butzloff, 2013; Knöckel et al., 2012; Meissner et al., 2016;
Müller et al., 2009). The lack of negative effect on the proliferation of culture parasites is supported by the measurements of specific activity of both enzymes in lysates of parasites transfected with the mutant genes, which show that these parasites retain a partial activity of both enzymes (Figure 10a,b). Additionally, continuous expression of the inserted mutant reduces the risk of possible degradation of the mutant protein by cellular proteases before it reaches the intended targets. Further, our mutations were designed to result in inactive proteins (confirmed by our in vitro activity assays (Lunev et al., 2018), Figure 3c). In fact, the activity assay in lysates of parasites supports not only the hypothesis that the introduction of mutated proteins will cause a decrease in the activity, but also indicates that the formation of heterocomplexes occurs in vivo, thereby influencing the function of the native protein.

Finally, in contrast to current small molecule inhibition approaches, no limitations regarding drug or compound delivery to the cytosol are encountered as the mutant proteins are expressed directly within the parasite. Based on this analysis, we believe that the introduction of inactive proteins specific for oligomeric targets represents a minimally perturbing method to specifically inhibit metabolic pathways of interest in the human malaria parasite P. falciparum that will have a minimal off-target effect, and, in this manner, offers a possible tool to be used in the validation of target candidates.

While the insertion of the individual mutations of PfMDH or PfAspAT results in no negative effect on parasitemia, the transfection of parasites with both plasmids results in a significant reduction in parasite proliferation in aspartate-limited media (Figure 7c). This is a clear change in phenotypic behavior that has been generated without recourse to complex genetic approaches. These data strongly suggest that while the correct function of either PfMDH or PfAspAT is sufficient to support parasite proliferation during the blood stage, simultaneous inhibition of both results in a significant reduction in parasite growth. While our data support the concept of oligomeric protein interference assays for cytosolic proteins, in principle the use of the native targeting sequence will also allow the approach to be used for proteins present in other cellular compartments (mitochondrial, apicoplast, membrane inserted, etc.).

A number of recent manuscripts have focused on elucidating the role of the mitochondria of the malarial parasite (Ke et al., 2015; Nixon et al., 2013). Amongst the pathways supported by the mitochondria are those involved in the biosynthesis of aspartate. While aspartate is essential in the formation of new proteins, it is also a key precursor in pyrimidine biosynthesis (Cassera, Zhang, Hazleton, & Schramm, 2011; Hyde, 2007), another promising pathway for drug discovery, as confirmed by validation of dihydroorotate dehydrogenase (DHODH) as a drug target (Vyas & Ghate, 2011). During proliferation, the malaria parasite catabolizes hemoglobin as an amino acid source. Although aspartate is available in hemoglobin, the host cell protein does not sufficiently suit the need of P. falciparum for the rapid proliferation within blood stage as demonstrated by the presented growth experiments of blood-stage cultures in aspartate-limited media. Thus, aspartate biosynthesis or uptake is highly likely to be a key element in supporting the rapid proliferation of P. falciparum in human red blood cells. It has long been known that aspartate is the least common of all the amino acids available within
the human serum, with recent measurements suggesting that the concentration of L-aspartate in human sera is <20 µM (Psychogios et al., 2011). This strongly suggests that biosynthetic pathways would be the main source of the aspartate required by *P. falciparum* in the human host (Figure 11).

The significant drop in proliferation upon oligomeric-based inhibition of both PfMDH and PfAspAT in aspartate-limited cell culture media suggest that aspartate biosynthesis in the malarial parasite depends upon the function of both of these enzymes and validates this metabolic pathway as drug target in *P. falciparum*. This is in accord with recent data that suggest the products of glycolysis (both pre- and post-mitochondrial) are used in biosynthesis in the malarial parasite (Ke et al., 2015). In another recent study (Zhang et al., 2018), mutagenic index scores (MISs) calculations have predicted PfMDH as an essential enzyme. In contrast, the PIA experiments revealed no effect on the proliferation of MDH mutant overexpressing parasites. However, this down-regulation of intra-cellular MDH activity will not warrant a total “knock-down” of the plasmoidal MDH due to the presence of residual endogenous wild-type MDH. On the other hand, the MIS approach suggested PfAspAT as non-essential, corroborating with our data, which shows no effect on the knock-down of PfAspAT alone although an intra-cellular simultaneous over-expression of PfAspAT and PfMDH mutated proteins clearly causing a severe growth defect. While the simultaneous inhibition of two enzymes may be highly challenging for the development of novel antimalarials, our data strongly suggest that future drug targets to treat malaria infection may be found within downstream components of the aspartate metabolism pathway (Figure 11). Taken together, our data also show that oligomeric surfaces offer a highly promising opportunity to specifically influence protein behavior in vivo and offer a novel avenue in the validation of pathways for downstream drug development, particularly in the field of infectious diseases.

**FIGURE 10** Specific activity values of PfMDH (a) and PfAspAT, respectively, measured from *Plasmodium falciparum* cell lysates. The activity of the wild-type MDH (a) was significantly higher compared to the V190W mutant (p < 0.05). The activity of AspAT-WT (b) was significantly higher compared to both control (3D7) and double mutant (p < 0.05). Mutant PfAspAT transfectant showed lower activity compared to the control (3D7; p < 0.05). The activity was measured in triplicates, in two independent experiments. GraphPad Prism 5.0 was used for one-way ANOVA analysis.

**FIGURE 11** Importance of the aspartate metabolism in *Plasmodium falciparum*. Our experiments showed that inhibition of the de-novo aspartate biosynthesis via PfMDH and PfAspAT is a viable target for future antimalarial drug design.

### 4 | MATERIAL AND METHODS

#### 4.1 | Expression and purification of recombinant PfAspAT

The purification of PfAspAT has been previously reported (Jain et al., 2010). Wild-type PfAspAT open reading frame (ORF) was cloned into pASK-IBA3 expression plasmid with additional C-terminal His_6-tag to facilitate purification via Ni-NTA chromatography. The generated pASK-IBA3-PfAspAT plasmid was transformed into the commercially
available E. coli expression strain BLR (DE3; Novagen) for expression. After expression, the cells were lysed using sonication and centrifuged to separate the lysate. Soluble His-tagged PfAspAT was purified using Ni-NTA agarose (Qiagen) according to the manufacturer’s recommendations. PfAspAT was further purified via size-exclusion chromatography using HiLoad 16/60 Superdex S75 column (GE Healthcare).

4.2 Site-directed mutagenesis

The single mutants PfAspAT-Y68A and PfAspAT-R257A, and the double mutant PfAspAT-Y68A/R257A were generated via site-directed mutagenesis using specific oligonucleotides containing the altered codons (Appendix 1) and the pASK-IBA3-PfAspAT plasmid (Jain et al., 2010) as a template. All constructs were verified by Sanger sequencing.

4.3 Determination of oligomeric state

The analysis of the oligomeric state of recombinant WT PfAspAT and mutants was performed according to the previously described protocol (Wrenger et al., 2011). Briefly, PfAspAT-WT, PfAspAT-Y68A, PfAspAT-R257A, and PfAspAT-Y68A/R257A samples were applied onto Superdex S75 10/300 (GE Healthcare) size exclusion column. The wild-type PfAspAT sample eluted as a single peak with at approx. 60 ml, while all three mutant samples eluted somewhat later at approx. 62 ml (Figure 8). Further SLS analysis (MiniDAWN TREOS [Wyatt]) confirmed the dimeric state of the wild-type PfAspAT as well as mutants, with an approximate weight of 97.5 kDa (including the purification tags).

4.4 Recombinant co-expression and co-purification of PfAspAT-WT and PfAspAT-Y68A/R257A double mutant

The wild-type PfAspAT ORF was re-cloned into pASK-IBA3 and the resulting plasmid-encoded full-length PfAspAT-WT with C-terminal Strep-tag. The His₆-tagged PfAspAT-Y68A/R257A double mutant was sub-cloned into pACYC184 vector (NEB) containing the expression cassette of pJC40 to allow co-expression of the WT and mutant version in E. coli. The co-expression of Strep-tagged PfAspAT-WT and His₆-tagged PfAspAT-Y68A/R257A was performed using cotransformed BLR (DE3) competent cells induced with both IPTG and AHT. The co-purification was performed via the Strep-tactin as well as via Ni-NTA agarose (Qiagen). The co-purified proteins were visualized by western blot using a monoclonal Strep-tag II antibody (IBA) or anti-His antibody (Pierce, USA) and a secondary anti-mouse horseradish peroxidase-labeled goat antibody (Bio-Rad, Germany).

4.5 In vitro activity assays

The specific activity of the double mutant of PfAspAT-Y68A/R257A was measured following the same procedure as previously reported (Wrenger et al., 2011). The effect of incorporation of mutant His₆-tagged PfAspAT-Y68A/R257A into the native Strep-tagged wild-type assembly was also analyzed using the samples of the co-expression experiments (described above).

4.6 Cloning and transfection of PfMDH V190W and PfAspAT Y68A, R257A

In order to obtain transgenic parasites, the ORFs of WT-PfAspAT and PfAspAT-Y68A/R257A were amplified via PCR using sequence-specific primers (Appendix 1) and subsequently cloned into pARL 1a- with the hDHFR (human dihydrofolate reductase) resistance cassette (Wrenger & Müller, 2004). The resulting plasmids encoded for the full-length WT-PfAspAT and PfAspAT-Y68A/R257A mutant with an additional C-terminal Strep-tag followed by the stop-codon before the GFP gene encoded on pARL 1a-. Similarly, WT-PfMDH and the PfMDH-V190W mutant with C-terminal His₆-tag (cloning procedure described in (Lunev et al., 2018)) were cloned into pARL 1a- with BSD (Blasticidin S) resistance cassette in order to facilitate double transfection (Knöckel et al., 2012).

In order to determine WR992010 and Blasticidin S drug-selection effects, two MOCK plasmids were generated (pARL-MOCK-hDHFR, pARL-MOCK-BSD) as described in (Knöckel et al., 2012). All constructs were confirmed by automatic sequencing (Sanger) before transfection into P. falciparum. The transfection of the resulting constructs pARL-PfAspAT-Y68A/R257A-Strep-hDHFR, pARL-PfMDH-V190W-his-BSD, pARL-MOCK-hDHFR and pARL-MOCK-BSD was performed into ring stage P. falciparum 3D7 as described in (Knöckel et al., 2012; Müller et al., 2010). The co-transfected cell lines were generated using the stabilized parasites previously transfected with pARL-PfAspAT-Y68A/R257A-Strep-hDHFR or pARL-MOCK-hDHFR, which were then electroporated with pARL-PfMDH-V190W-his-BSD or pARL-MOCK-BSD plasmids, respectively. The selection of transgenic parasites was performed using 5 nM of WR99210 and 1 μg/ml Blasticidin S. Transfected parasites were maintained in continuous culture using the conditions of Trager and Jensen modified as described in (Das et al., 2005).

4.7 qRT and western blot

An asynchronous culture of transgenic 3D7 parasites was isolated via saponin lysis. The total RNA of these parasites was extracted using TRIZOL following the manufacturer’s instruction. The cDNA synthesis was performed as described in (Butzloff, 2013; Chan et al., 2013; Knöckel et al., 2012; Müller et al., 2010). The quantitative real-time PCR was performed using specific primers (Appendix 1), that identify products from 150–190 bp. Briefly, 2 μl of each primer (5 pmol/μl) were used together with 9 μl of 2.5× Real Master Mix SYBR (20×; 5Prime), 6 μl RNase-free dH₂O and 1 μl cDNA (50 ng/μl). The reaction was performed in a thermocycler (Corbett Cycler) with the following program: 2 min 95°C followed by 35 cycles of 95°C for 15 s, 49°C for 20 s and 69°C for 20 s, and a final step of 95°C for 2 min. Normalization and calibration were performed using
the aldolase gene (Salanti et al., 2003) and the respective MOCK cell line like (Chan et al., 2013). The data were analyzed using the Corbett Rotor-Gene 6.1.81 software and the 2−ΔΔCt method (Livak & Schmittgen, 2001).

The protein expression of the transgenic cell lines was verified via western blot analysis as described in (Knöckel et al., 2012). Briefly, isolated parasites were resuspended in 5x SDS-PAGE sample buffer, boiled for 5 min at 95°C and centrifuged for 5 min at 14,000 xg. The supernatant was separated by 10% SDS-PAGE and transferred on a nitrocellulose membrane (Bio-Rad). The expressed proteins were detected via their Strep- or His-tag by using a monoclonal anti Strep- or anti-His antibody (1:5,000 dilution; IBA; Pierce) and a secondary anti-mouse HRP-labeled antibody (1:10,000 dilution; Pierce) and visualized on X-ray films using the SuperSignal West Pico detection system (Thermo Scientific).

4.8 Activity assays from parasites lysate

In order to analyze the specific activity of PfAspAT and PfMDH, we performed activity assays in parasites lysates. For this analysis, cultures of transgenic cell lines pARL-PfAspAT-WT, pARL-PfAspAT-Y68A/R257A, pARL-PfMDH-WT, and pARL-PfMDH-V190W, as well as the WT 3D7 culture used as a control, were isolated via saponin lysis.

The specific activity of PfMDH was measured with the Malate Dehydrogenase Assay Kit (Sigma Aldrich). The reaction was carried out at 37°C in a final volume of 150 µl, according to the manufacturer’s protocol. The absorbance was monitored at 450 nm.

The specific activity of PfAspAT was measured with the Aspartate Aminotransferase (AST) Activity Assay Kit (Sigma Aldrich). The reaction was carried out at 37°C in a final volume of 100 µl, according to the manufacturer’s protocol. The absorbance was also monitored at 450 nm. The amount of total protein in the lysates was quantified by Bradford assay (Bradford, 1976).

4.9 Proliferation assays

In order to analyze the long-term influence of the overexpressing cell lines pARL-PfAspAT-Y68A/R257A-Strep-hDHFR, pARL-PfMDH-V190W-his-BSD and the double transgenic cell line pARL-PfAspAT-Y68A/R257A-strep-hDHFR + pARL-PfMDH-V190W-his-BSD in comparison with their respective MOCK line, parasites growth was monitored over several days. The parasites were synchronized using sorbitol and a starting parasitaemia of 1% of ring-stage iRBC (infected red blood cells) was adjusted. Giemsa-stained thin smears were analyzed daily and the parasitaemia was determined by light microscopy in percentage of iRBC to total RBC. Cultures with more than 8% of iRBC were diluted and cumulative parasitaemias were calculated as described in (Knöckel et al., 2012). Triple repetition of the proliferation assay was performed and the growth curves were generated with GraphPad Prism 4.0. The slope of the respective curves was calculated through an exponential equation (Müller et al., 2009).

4.10 Localization of PfMDH

The ORF of PfMDH-WT with no stop-codon at C-terminus was amplified by PCR and subsequently cloned into pARL 1a- using KpnI/AvrII restriction enzymes (Appendix 1). The resulting plasmid encoded for the wild-type PfMDH fused in front of the GFP gene and was transfected into P. falciparum 3D7 parasites as described above. The localization of the MDH-GFP chimera was analyzed via live cell fluorescent microscopy (Müller et al., 2010) using an Axio Imager M2 microscope (Zeiss) equipped with an AxioCam HRC digital camera (Zeiss). In order to visualize the nucleus, the parasites were incubated with 10 µg/ml HOECHST 33342 dye (Invitrogen). The images were analyzed with the AxioVision 4.8 software.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

FAB, SL, SB, CW, and ARR and performed protein expression and purification experiments. SB, ML, IBM, and KAM performed mutagenesis experiments. FAB, SSB, and SB performed proliferation/activity assays. FAB, ARR, and SL performed SLS analysis. ASSD, MRG, and SL designed and coordinated the research. FAB, SSB, CW, and MRG wrote the manuscript, with contribution from all authors. All authors reviewed the results and approved the final version of the manuscript.

ETHICS STATEMENT

None required.

DATA ACCESSIBILITY

All data supporting this study are provided in full in the results section of this paper.
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### APPENDIX

Primer sequences used in the study. The recognition sites for restriction enzymes used (specified in the primer name) are highlighted in bold. Mutations sites are underlined.

| Primer | Sequence |
|--------|----------|
| PfAspAT cloning for recombinant expression (pASK-IBA3, His<sub>6</sub>-tag) | |
| IBA3-AspAT-s (BsaI, His<sub>6</sub>) | 5′-GCGCGGCGTCTCAATGGATAAAGTTATTAGCAGCTTAT-3′ |
| IBA3-AspAT-as (BsaI, His<sub>6</sub>) | 5′-GCCGCGGCGTCTCAACAGCGTTTTAATGATGATGATGATGGCCCTTGAAAATAAGATCTTATTTGAACGGAATAAAATTTGGCT-3′ |
| PfAspAT cloning for recombinant expression (pASK-IBA3, Strep-tag) | |
| IBA3-AspAT-s (BsaI, Strep) | 5′-GCGCGGCGTCTCAATGGATAAAGTTATTAGCAGCTTAT-3′ |
| IBA3-AspAT-as (BsaI, Strep) | 5′-GCCGCGGCGTCTCAACAGCGTTTTAATGATGATGATGATGGCCCTTGAAAATAAGATCTTATTTGAACGGAATAAAATTTGGCT-3′ |
| PfAspAT Site-directed mutagenesis primers (pASK-IBA3) | |
| AspAT-R257A-s | 5′-ATGTCGCTTTATGGAGAAGCAGCAGGTGCTCTTCATATTG-3′ |
| AspAT-R257A-as | 5′-CAATATGAAGAGCACCCTGCTCTCTCATCAAAGGACAT-3′ |
| AspAT-Y68A-s | 5′-GAAAATTATAAAGAGAAACCAGCATTGTTAGGTAACGGTACAGAA-3′ |
| AspAT-Y68A-as | 5′-TTCTGTACCGTACCTAACAAATACTGCTTCTCTTTAATTTTC-3′ |
| PfAspAT sub-cloning primers for recombinant co-expression in *E. coli* (pACYC184) | |
| pACYC184-AspT-s (Ndel) | 5′-AGAGCATATTGATAAGTTATTAGCAGCTTAT-3′ |
| pACYC184-AspT-as (XmaI) | 5′-ATATCCCGGCTCATATTGACTTACGGAAGA-3′ |
| PfAspAT double mutant sub-cloning primers for in vivo overexpression (pARL 1a<sup>−</sup>) | |
| pARL-AspAT-s (KpnI) | 5′-GAGAGGATCCATGATAAGTTATTAGCAGCTTAT-3′ |
| pARL-AspAT-Strep-as (XhoI) | 5′-GCCGCTCGAGTTATATTCTTTGCAAACGCGGTTGGC-3′ |
| PfAspAT qPCR primers | |
| qPCR-AspAT-s | 5′-CGCCTTTTGCGTATGCAATCC-3′ |
| qPCR-AspAT-as | 5′-AGTTGGCATAGAATACGATTCGT-3′ |
| PfMDH double mutant sub-cloning primers for in vivo overexpression (pARL 1a<sup>−</sup>) | |
| pARL-MDH-s (SmaI) | 5′-AGAGCCCGGGAATGTCGCTCTCGAGCCCTAAGGACTAAATTGCCCTTAATTGGAAG-3′ |
| pARL-MDH-His-as (XhoI) | 5′-GAGACTCGAGTTATATTGATGATGATGATG-3′ |
| PfMDH qPCR primers | |
| qPCR-MDH-s | 5′-CCACGCTGCGCTGCAATCC-3′ |
| qPCR-MDH-as | 5′-CTGAGTGGCCCTTCTAAT-3′ |
| PfAldolase qPCR control gene | |
| qPCR-PfAldolase-s | 5′-TGTACCACCAAGGCTTACAG-3′ |
| qPCR-PfAldolase-as | 5′-TTCTTGGCCATGTTTCAAT-3′ |