Review Article

Trafficked Proteins—Druggable in *Plasmodium falciparum*?

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Malaria is an infectious disease that results in serious health problems in the countries in which it is endemic. Annually this parasitic disease leads to more than half a million deaths; most of these are children in Africa. An effective vaccine is not available, and the treatment of the disease is solely dependent on chemotherapy. However, drug resistance is spreading, and the identification of new drug targets as well as the development of new antimalarials is urgently required. Attention has been drawn to a variety of essential plasmodial proteins, which are targeted to intra- or extracellular destinations, such as the digestive vacuole, the apicoplast, or into the host cell. Interfering with the action or the transport of these proteins will impede proliferation of the parasite. In this mini review, we will shed light on the present discovery of chemotherapeutics and potential drug targets involved in protein trafficking processes in the malaria parasite.

1. Introduction

Malaria is a disease of global importance resulting in morbidity and mortality worldwide [1, 2]. Although, since 2000 a reduction in the mortality rate of about 25% has been globally observed, nearly half of the world's population is still living at risk of infection [1, 3] leading to more than 0.5 million deaths annually [4].

Five species of the genus *Plasmodium* are known to infect humans; however, the most severe form of malaria, Malaria tropica, is caused by *Plasmodium falciparum* [5, 6].

Due to the spreading drug resistance against the currently used chemotherapeutics as well as environmental changes, the treatment of the disease is becoming more difficult. Resistance to therapeutics such as chloroquine and sulfadoxine-pyrimethamine is already widely distributed [28–31]. Furthermore resistance to Malarone, a combination of atovaquone and proguanil, became also apparent [32]. Currently only ACTs (artemisin-based combination therapies) are highly effective against malaria [33]. However, resistances to artemisinin and its derivatives have already been confirmed at the border of Cambodia-Thailand [34–36], and therefore the discovery of novel therapeutic agents against malaria is urgently required.

Proliferation of *P. falciparum* within the asexual blood stage depends on haemoglobin digestion in the food vacuole [37]. This degradation pathway was already taken into consideration as a new drug target by focussing on the proteases plasmepsin I–IV [25]. However, since haemoglobin catabolism occurs in the food vacuole, not only inhibition of the catalytic properties of the digestive enzymes is of interest but also the mode of protein trafficking. The latter was also considered to be druggable for proteins directed to other parasite-specific organelles such as the chloroplast-like organelle, the apicoplast, where several essential metabolic processes take place. Additionally to the specific intraparasitic trafficking the malaria pathogen secretes also a variety of proteins to the erythrocytic host cell surface for nutrient transport processes as well as for cytoadherence mediated by proteins for example of the *Plasmodium falciparum* erythrocyte membrane protein (*Pf*EMP)-family. Interestingly, the number of exported proteins is—in the case of *P. falciparum*—about 5–10 times larger compared to other malaria parasites [38]. Therefore, protein trafficking in

P. falciparum represents an attractive drug target; however, detailed information of its mode of action is required to interfere with this mechanism. The aim of this mini review is to summarise the current knowledge of novel promising drug targets, which are involved in protein trafficking processes in *P. falciparum*.

2. Protein Trafficking in *P. falciparum*

Like other eukaryotic cells, *P. falciparum* possesses intracellular compartments such as a nucleus, endoplasmic reticulum (ER) and a mitochondrion [39]. However, the pathogen also possesses an apicoplast.

Although the apicoplast has its own circular genome encoding for about 30 proteins, the majority of the apicoplast located proteins are nuclear encoded in *P. falciparum* [40, 41]. Around 500 proteins are predicted to be trafficked to this organelle, like the enzymes of the isoprenoid fatty acid and iron-sulfur-cluster biosynthesis pathways [42, 43]. These proteins possess target information for the transport to the apicoplast, which is encoded at the N-terminal region [44]. First, the signal peptide, a hydrophobic N-terminal sequence, directs the proteins to the ER lumen [45–47], where subsequently a signal peptidase cleaves off the signal peptide [48]. Within the N-terminal region the signal peptide is followed by a second domain, the plant-like plastid transit peptide, which contains the information for trafficking the protein to the apicoplast [49].

The vesicular transport to the food vacuole is not yet completely understood. While some proteins like the chloroquine resistance transporter (*Pf*CRT), which modulates resistance to drugs like chloroquine, are transported directly via the classical ER and Golgi secretory pathway to the food vacuole [50–53], other proteins such as plasmepsin I, plasmepsin II or dipeptidyl aminopeptidase 1 (DPAP1), and the secreted acid phosphatase (SAP) are suggested to be targeted via the parasite plasma membrane (PPM) [20, 54] or the parasitophorous vacuole to the food vacuole [55]. The occurrence of different trafficking routes is also emphasised by the sensitivity to Brefeldin A, an inhibitor of the Golgi apparatus [56, 57]. Whereas the routing mechanisms of some proteins are affected by Brefeldin A, other proteins can still be found in the food vacuole after treatment with the inhibitor [58] leading to the suggestion of at least two different transport pathways, ER/Golgi dependent and independent trafficking routes.

Since transport to the food vacuole differs, consequently the signal sequences are very divergent. While for plasmepsin IV the first 70 amino acid residues are necessary for proper translocation, a mislocalisation of plasmepsin II occurs when different positions in the first 120 amino acid residues are deleted or mutated [59, 60]. Moreover, signal peptides of other hemoglobinases like DPAP1 are not yet analysed in detail.

Additionally to the intraparasitic protein trafficking, *P. falciparum* secretes proteins to extracellular compartments like the parasitophorous vacuole, the Maurer's clefts, and into the host cell cytosol. Therefore, proteins have to enter the classical secretory pathway by a conventional signal peptide. Since the signal peptide is unable to transport a protein through the parasitophorous vacuole membrane a second signal sequence of around 25–30 amino acid residues after the ER signal (RxLxE/Q/D) called the *Plasmodium* export element (PEXEL), or vacuolar translocation signal (VTS), is necessary [61, 62]. These transport signals are conserved in *Plasmodium* spp. [62]. As about 8% of the parasitic proteome contains this targeting sequence, it is expected that most them have an important role in remodelling the host cell [61–63]. A knockout screen of 85 out of the predicted 200–300 exported proteins suggests that about 25% are essential in the blood stage of *P. falciparum* [38]. After cleaving off the PEXEL motif via the aspartic protease plasmepsin V, the proteins are transported to the parasitophorous vacuole [64]. Within the parasitophorous vacuole membrane the ATP-driven *Plasmodium* translocon of exported proteins (PTEX)-complex transports the released proteins across this barrier [65, 66].

However, *P. falciparum* possesses also a PEXEL-independent trafficking pathway consisting of the PEXEL-negative exported proteins (PNEPs) [67]. So far no consensus sequence for the export of PNEPs has precisely been identified, therefore it is hard to predict how many unidentified PNEPs are encoded by *P. falciparum*.

3. Proteins of Interest within the Apicoplast

One of the most interesting compartments in *P. falciparum* is the four membrane-bound, nonphotosynthetic organelle (the apicoplast), which has been proposed to be derived from secondary endosymbiosis. Briefly, while the uptake of a prokaryote by an eukaryote is termed primary endosymbiosis resulting in a plastid surrounded by two membranes, secondary endosymbiosis, the engulfment of an eukaryote possessing a primary endosymbiont, is leading to a four membrane-bound plastid [68].

As already outlined above, most of the apicoplast proteins are encoded in the nuclear genome and are trafficked to the organelle [12, 69–71]. Import into the plasmodial plastid is—in contrast to plants—a two-step process requiring a signal peptide and subsequently a transit peptide [43, 49]. Whereas the signal peptide mediates entry into the endomembrane system, the transit peptide directs the protein into the apicoplast [49, 72]. Plasmodial transit peptides are highly enriched in lysine and asparagine, and acidic residues are depleted suggesting that these characteristics are essential for plastid targeting in *P. falciparum*, as has been observed in mitochondrial transit peptides [73, 74]. As apicoplast transit peptides do not possess a highly conserved sequence motif [75] (they are varying widely in their length and their composition of positively charged amino acid residues near the N-terminus [42, 76]), it remains unclear how apicoplast proteins are distinguished from proteins which are intended to be translocated to other compartments. The translocation across the apicoplast periplastid membrane (second membrane from the cytosol direction) is assumed to be initiated by

a novel ER-associated degradation (ERAD) complex [77]. The luminal chaperone binding protein (BiP) and protein disulphide isomerase (PDI) differentiate misfolded proteins from folding intermediates and correctly folded proteins [78–80]. Subsequently, misfolded proteins which are recognised by ERAD are redirected to the cytosol across the ER membrane through a channel which consists of either Sec61 [81] or Der1 [82, 83].

Another factor suggested to play a role in protein targeting to plant plastids is the binding of HSP70 chaperones to plastid transit peptides [84, 85]. A specific HSP70 binding pattern is present, and putative binding sites are abundant in apicoplast transit peptides. Therefore, an important role of HSP70 in apicoplast targeting has been suggested [85]. 15-Deoxyspergualin (DSG) is an immunosuppressant with antimalarial activity acting for example on HSP70 molecules within the malaria parasite [7, 8] (Table 1). The mode of action is to date unknown, but it is assumed that DSG inhibits chaperone-transit peptide interactions, and thereby preventing apicoplast targeting [9]. Another class of drugs interfering with apicoplast targeting is the group of the tetracyclines such as doxycycline (Table 1). Tetracyclines are broad-spectrum antibiotics working as protein synthesis inhibitors by binding to microbial ribosomes and blocking the translation process. The inhibition by tetracycline and related drugs occurs probably indirectly by inhibiting the translation of apicoplast-encoded genes such as the ClpC gene which is required for protein import. Further drugs beside tetracycline acting via this mechanism are clindamycin and azithromycin [86].

Currently no clinical resistance to doxycycline has been reported [10]. Whereas doxycycline presumably targets the translation within the apicoplast, rifampicin has been proposed to attack transcription; both drugs result in delayed death (Table 1). Unfortunately the versatile use of the latter supports the development of drug resistance against these antibiotics [87]. The synthesis of isoprenoid precursors is an essential metabolic function of the apicoplast. In *P. falciparum,* isoprenoids are *de novo* generated via the DOXP (1-deoxy-D-xylulose-5-phosphate) pathway. As the canonical mevalonate pathway is apparently absent in *P. falciparum,* the apicoplast represents the only place within the parasite where isoprenoid precursor synthesis occurs [88, 89]. This biosynthetic pathway consists of seven enzymes, and all are apparently encoded in the nucleus and their deriving proteins are targeted to the apicoplast [88]. The inhibition of the DOXP reductoisomerase/IspC by fosmidomycin results in death of the *P. falciparum* intraerythrocytic stages which underlines the important role of this pathway for the malaria parasite [12] (Table 1). Moreover, the inhibition of another enzyme of the DOXP pathway, IspF, also leads to the death of the parasite's intraerythrocytic stages [90]. Therefore the apicoplast isoprenoid pathway represents a promising target for the development of novel antimalarials. However, the discovery and validation of the organelle pathways are hindered either by limitations to apply reverse genetics of essential genes or to characterize plasmodial proteins *in vitro* or to purify the organelle [91–94].

4. Interfering with Proteins Trafficked to the Food Vacuole

The digestion of haemoglobin is carried out by the malaria parasite within the food vacuole, an organelle that has an acidic pH [95, 96]. Haemoglobin is taken up via endocytosis and is suggested to be subsequently transported by lysosomal vesicles to the digestive vacuole [95, 97]. Haemoglobin degradation is substantially carried out by four different protease families. The initial degradation of the haemoglobin into globin fragments is mediated by aspartic proteases, such as the plasmepsins I,II,IV, and the histoaspartic protease (HAP) [20, 98–100]. In the next step, the cysteine proteases falcipain 2, 2b, and 3 [101, 102] digest these globin fragments into peptides which are subsequently degraded into smaller peptides by the metalloprotease falcilysin [103]. These polypeptides are cleaved into dipeptides by a dipeptidyl aminopeptidase and finally aminopeptidases convert the dipeptides into amino acids [104]. The proliferation of the parasite is repressed by protease inhibitors that block the haemoglobin catabolism. Therefore, these endo- and exopeptidases catalyzing this process are attractive targets for the development of novel antimalarial drugs [97, 105–107]. Recent analysis identified the fungal metabolite bestatin as an inhibitor of neutral aminopeptidases such as the M1 aminopeptidase (*Pf*A-M1) [18] and the aminopeptidase P (*Pf*APP) [95, 96]. Furthermore, it has been reported that this inhibitor, a natural Phe-Leu dipeptide analogue derived from the fungus *Streptomyces olivoretticuli*, prevents growth of the parasite *in vitro* and *in vivo*. [15–17]. Two phosphinate dipeptide analogues (compound **4** and **5**) were also effective against *P. falciparum* in cell culture [17] (Table 1). Both compounds inhibit the M17-family leucine aminopeptidase (*Pf*LAP) with IC_{50} values of 20–40 and 12–23 μM, respectively (Table 1) [17]. Additionally, *in vivo* studies using *P. chabaudi* resulted in an about 90% reduction of parasitaemia in mice treated with compound **4** [17]. These results clearly confirm that neutral aminopeptidases are serving as promising new antimalarial drug targets [17].

In the food vacuole, haemoglobin degradation is initiated by the aspartate proteases, plasmepsin I-IV (III has been renamed to HAP), which can be inhibited by pepstatin A at the picomolar range [108]. However, at the cellular level, this molecule is only a weak inhibitor. Recently, it has been shown that knock outs of plasmepsins result in delayed growth but is not leading—even in the presence of specific protease inhibitors—to the death of the parasite [109, 110]. Currently the aim of a new initiative between the Johns Hopkins University and Kyoto Pharmaceutical University is focussing on designing "adaptive inhibitors" (KNI) which were able to inhibit all four digestive vacuole plasmepsins simultaneously (Table 1). The function of these peptidomimetic inhibitors is based on the strongest and most specific interactions of the inhibitor against the conserved regions of the binding site and at the same time maintaining flexibility of the inhibitor allowing docking to the less conserved regions [22–24]. KNI-10006 for example is very potent against plasmepsin II but has only poor influence on parasite growth. In contrast, compound KNI-10283, which does not

TABLE 1: List of inhibitors known to interfere with protein trafficking or trafficked proteins in *P. falciparum*.
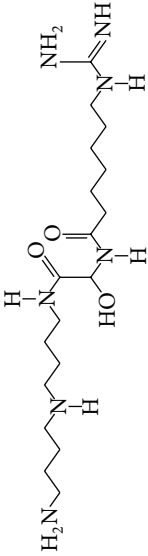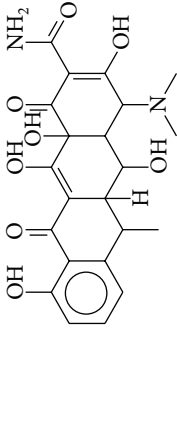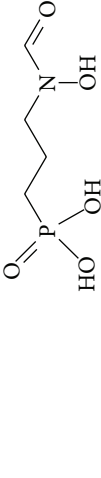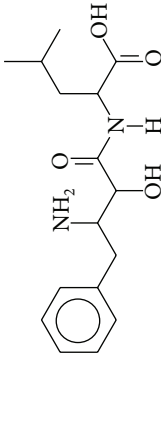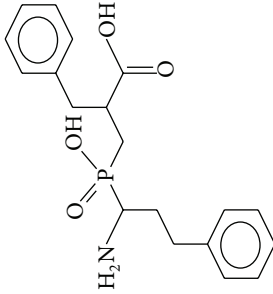
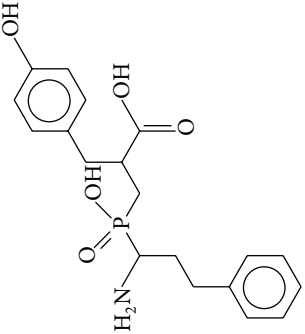| Chemical compound Structure	Chemical family	Target	Mode of action	IC$_{50}$ (μM)	Ki (nM)	Antimalarial effect	Origin	References
15-Deoxyspergualin (DSG)	Spergualin derivate	Apicoplast protein synthesis	Suggested inhibition of chaperone-transit peptide interactions	0.15		Delayed death	*Bacillus laterosporus*	[7–9]
Doxycycline	Tetracycline derivate	Apicoplast protein synthesis	Binding to microbial ribosomes and blocking the attachment of charged aminoacyl-tRNA	10		Delayed death	*Streptomyces* spp	[10, 11]
Fosmidomycin	Phosphonic acid derivative	Apicoplast Isoprenoid biosynthesis	Inhibits specifically the DOXP reductoisomerase (DXR or Isp C)	0.35		Immediate	*Streptomyces lavendulae*	[12–14]
Bestatin	Dipeptide	Aminopeptidase for example, PfM17LAP	Chelating of metal binding centres	8–21	25		*Streptomyces olivoreticuli*	[15–17]
hPheP[CH$_2$]Phe (compound 4)	Phosphinate dipeptide analogue	Aminopeptidase for example, PfM17LAP	Proposed chelation of active site metal ions	20–75	13		Synthetic	[18, 19]

TABLE 1: Continued.

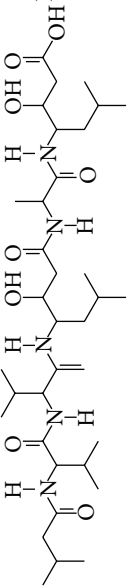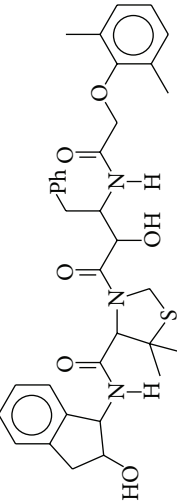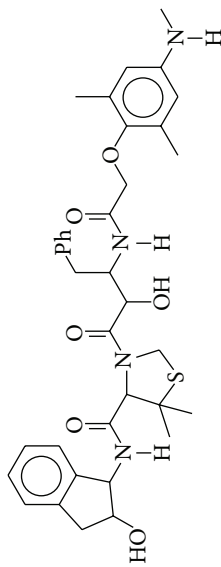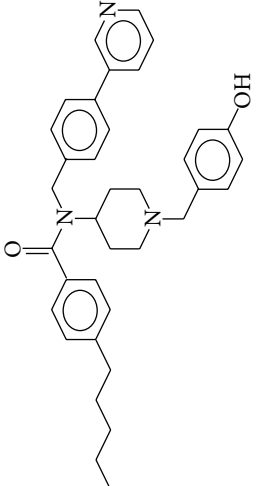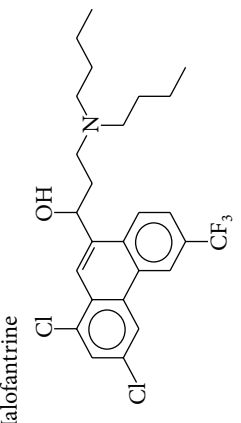| Chemical compound Structure	Chemical family	Target	Mode of action	IC$_{50}$ (μM)	Ki (nM)	Antimalarial effect	Origin	References
hPheP[CH$_2$]Tyr (compound 5)	Phosphinate dipeptide analogue	Aminopeptidase for example, PfM17LAP	Proposed chelation of active site metal ions	13–62	10		Synthetic	[18, 19]
Pepstatin A	Hexapeptide	PM I–IV	No direct effect on haemoglobin degradation	4	PMI: 0.4 PMII: 0.03 PMIII: 0.1 PMIV: 0.3		Actinomyces	[20, 21]
KNI-10006	Peptidomimetic	PM II	Mimic Phe-Leu found in haemoglobin alpha chain	0.04	0.5		Synthetic	[22–24]
KNI-10283	Peptidomimetic	PM I, II, IV		n.d.	I: 21 ii: 25 IV: 46	Expanded lifespan of mice (up to 171%)	Synthetic	[25]

TABLE 1: Continued.

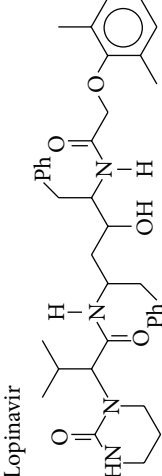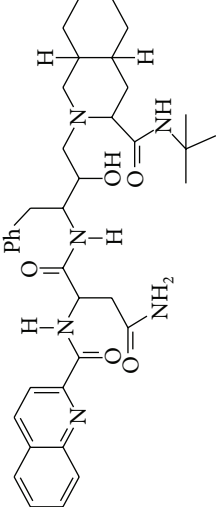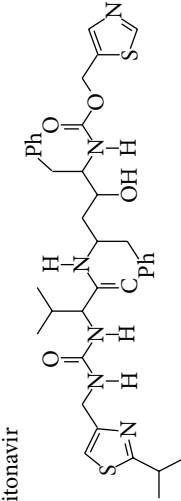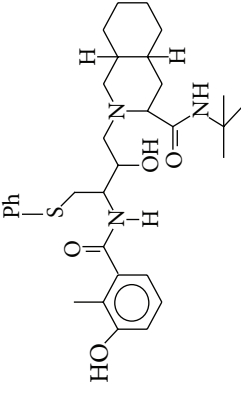| Chemical compound / Structure	Chemical family	Target	Mode of action	IC$_{50}$ (μM)	Ki (nM)	Antimalarial effect	Origin	References
ACT-056822	Peptidomimetic	PMII		0.1		Expanded lifespan of mice (up to 161%)	Synthetic	[25]
Halofantrine	Aryl amino alcohol	Not precisely known, but effective against plasmepsin I–III		0.001–0.006			Classical antimalarial drug with unknown mode of action, developed as synthetic quinine analogue	[25–27]
Lopinavir	Peptidomimetic	PMV	Inhibits the PMV cleavage of a PEXEL peptide	70			HIV protease inhibitor	[25]
Saquinavir	Peptidomimetic	PMV	Inhibits the PMV cleavage of a PEXEL peptide	n.d.			HIV protease inhibitor	[25]

TABLE 1: Continued.

Chemical compound Structure	Chemical family	Target	Mode of action	IC$_{50}$ (μM)	Ki (nM)	Antimalarial effect	Origin	References
Ritonavir								
	Peptidomimetic	PMV	Inhibits the PMV cleavage of a PEXEL peptide	15			HIV protease inhibitor	[25]
Nelfinavir								
	Peptidomimetic	PMV	Inhibits the PMV cleavage of a PEXEL peptide	n.d.			HIV protease inhibitor	[25]

The given IC$_{50}$ values refer to the cellular level. Not yet determined: n.d.

contain a basic amine, has been tested for *in vivo* efficacy in the *P. berghei*-infected mouse model revealing an expanded increase in lifespan of mice of up to 170% [25, 111, 112]. These results support the development of broad-spectrum plasmepsin inhibitors as antimalarial agents. Another group of plasmepsin inhibitors are the nonpeptidomimetic inhibitors containing a number of different structural classes such as N-alkoxyamides, guanidines, amides, ureas, thioureas, hydrazides, and amino alcohols [112–117]. Whereas many of them are only poor inhibitors, such as halofantrine, some of them including ACT-056822, which extended the survival time of mice of about 160% in the *P. berghei* mouse model, seem to be promising plasmepsin inhibitors (Table 1).

5. Inhibition of the Export System

The export of proteins which are located at the surface of the host cell like *Pf*EMP1 is responsible for the virulence of *P. falciparum*. *Pf*EMP1 is involved in the knob assembly and is therefore important for mediating cytoadherence of the infected erythrocyte to the endothelial cell of the blood vessels by binding on DC36, ICAM-1, thrombospondin, chondroitin sulfate, and other surface molecules [118]. The inhibition of the export machinery seems to be an auspicious new druggable pathway. As already outlined above, a variety of these exported proteins are trafficked via the PEXEL motif to the surface of the host cell [38]. Indeed, the proteolytic enzyme plasmepsin V seems to be a promising drug target candidate, as it is conserved in *Plasmodium* spp. and is absent from higher eukaryotes. The fundamental role of this protease for the malaria parasite has been verified by the attempt to disrupt a plasmepsin V orthologous gene (*Pb*PMV) in the rodent malaria parasite *P. berghei* [64]. Plasmepsin V is localised in the ER and cleaves the PEXEL motif between position 3 and 4 of the PEXEL sequence [119]. This protease is therefore responsible for proper processing of PEXEL mediated transport processes to the host cell. Due to the different function of plasmepsin V compared to the other plasmepsins (e.g. involved in the digestion of haemoglobin inhibitors), inhibitors designed to target these aspartic proteases might be therefore not effective against plasmepsin V. Fortunately, the HIV-1 protease cleaves a similar motif to that of plasmepsin V, emphasising the suggestion that HIV-1 protease-inhibitors could also be potent against the plasmodial protein [64]. Four promising inhibitors, lopinavir, saquinavir, ritonavir and nelfinavir, have already been tested. Unfortunately they are only revealing a moderate inhibitory profile [64, 120] (Table 1). However, in the future by applying medical chemistry these compounds could lead to a complete inhibition of plasmepsin V, and thereby result in the death of the malaria parasite.

Recently, Kulzer et al. identified a unique parasite-encoded HSP70 (*Pf*HSP70-x), which is exported into the host cytosol forming a complex with *Pf*HSP40s [121]. While in other organisms HSP70/40 complexes are important for protein folding and trafficking [122, 123], Kulzer et al. proposed that *Pf*HSP70-x is involved in refolding of other exported proteins and chaperoning these proteins to their final destination. One possible target might be *Pf*EMP1, which (i) colocalises with *Pf*HSP70-x [121] and (ii) is suggested to be transported via a multiprotein complex containing chaperones into the host cell [124]. Therefore *Pf*HSP70-x could serve as a promising novel target to interfere with transportation of the major virulence factor of *P. falciparum*.

6. Conclusion

As outlined here, an approach for identifying novel drug targets is represented by proteins which are trafficked within the parasite or its host cell as well as the mechanism of trafficking. For the survival *Plasmodium* is carrying out metabolic processes in intracellular compartments, such as the food vacuole or the apicoplast. Among the targets within the food vacuole, several proteases can be inhibited by different compounds which selectively interfere with the proteolytic digestion of the imported haemoglobin at low micromolar level (Table 1). The apicoplast is an organelle which exhibits metabolic pathways, such as the essential isoprenoid biosynthesis [125]. This pathway has already been exploited as drug target using fosmidomycin (Table 1) [12].

A novel mode to impede proliferation of the malaria pathogen is to prevent proper processing of transport sequences of the trafficked proteins. Plasmepsin V has been found to participate in the posttranslational cleavage of exportation sequences [119], and some promising inhibitors were designed to inhibit plasmepsin V, unfortunately—so far—with only a moderate effect on the parasite's growth. In the future, these molecules might lead to novel inhibitors that prevent host cell modification by *P. falciparum* [64, 120]. However, further discoveries and adaptations of the current drugs are required to hamper proliferation of the deadly human malaria parasite.

Authors' Contribution

J. Lindner and K. A. Meissner contributed equally to this paper.

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