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Review article

REVIEW ARTICLE

Targeting malaria parasite invasion of red blood cells as an antimalarial strategy

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One sentence summary: Malaria invasion of red blood cells is an essential step in parasite replication and this review discusses targets and drug chemotypes being developed to stop invasion and growth.

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ABSTRACT

Plasmodium spp. parasites that cause malaria disease remain a significant global-health burden. With the spread of parasites resistant to artemisinin combination therapies in Southeast Asia, there is a growing need to develop new antimalarials with novel targets. Invasion of the red blood cell by *Plasmodium* merozoites is essential for parasite survival and proliferation, thus representing an attractive target for therapeutic development. Red blood cell invasion requires a co-ordinated series of protein/protein interactions, protease cleavage events, intracellular signals, organelle release and engagement of an actin-myosin motor, which provide many potential targets for drug development. As these steps occur in the bloodstream, they are directly susceptible and exposed to drugs. A number of invasion inhibitors against a diverse range of parasite proteins involved in these different processes of invasion have been identified, with several showing potential to be optimised for improved drug-like properties. In this review, we discuss red blood cell invasion as a drug target and highlight a number of approaches for developing antimalarials with invasion inhibitory activity to use in future combination therapies.

Keywords: malaria; merozoites; invasion; antimalarial(s); *P. falciparum*; *P. vivax*

INTRODUCTION

Malaria is a mosquito borne disease caused by parasites of the genus *Plasmodium*. The majority of the ~445 000 malaria related deaths in 2016 were caused by *P. falciparum* and occurred in sub-Saharan Africa (Murray *et al.* 2014; WHO 2017). In addition, *P. vivax, P. malariae, P. ovale* (comprised of two different subspecies; *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland

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et al. 2010)) and two zoonotic species, *P. knowlesi* and *P. simium* (Singh *et al*. 2004; Brasil *et al*. 2017), are recognised as significant contributors to global malaria disease burden. While intervention against *Anopheles* mosquito vectors and the success of artemisinin-based combination therapies have contributed to marked decreases in disease burden since the year 2000, there is growing concern regarding the spread of *P. falciparum* strains throughout Southeast Asia which are resistant to artemisinin-based drugs and their partner drugs utilized in combination therapies (Dondorp *et al*. 2009; Ariey *et al*. 2014; Ashley *et al*. 2014; Tun *et al*. 2015; Das *et al*. 2018). Resistance to other clinically used antimalarials, such as chloroquine and sulfadoxine-pyrimethamine, is also widespread globally (Plowe *et al*. 1997; Trape *et al*. 1998; Mehlotra *et al*. 2001). Thus, there is an urgent need to bring to market new antimalarials with novel mechanisms of action which are active against all drug-resistant strains, effective against all human pathogenic *Plasmodium* spp. and can clear parasitemia rapidly for improved clinical outcomes (Burrows *et al*. 2017). Targeting multiple lifecycle stages would improve the effectiveness of combination therapies across endemic areas and help slow the development of drug resistance.

Human infections begin with the bite of a mosquito vector and release of malaria sporozoites, with the sporozoite then traveling to the liver and invading hepatocytes (reviewed in Aly, Vaughan and Kappe 2009). After rapid multiplication of liver stage parasites, the mature hepatic schizont ruptures and releases red blood cell (RBC) invading merozoites into the blood stream. In the case of *P. falciparum*, after merozoite invasion of a RBC a 48 hour cycle of growth, multiplication, RBC rupture and release of 16–32 new merozoites ensues (reviewed in White *et al*. 2014). The number of merozoites produced per cycle of growth and the length of the blood stage lifecycle varies between human malaria species. A small portion of blood stage parasites (<1%) differentiate into sexual stage gametocytes, which are transmitted to *Anopheles* vectors during blood meal feeding (reviewed in Liu, Miao and Cui 2011).

As all malaria pathology is caused by blood stage parasites, and this is when infection is diagnosed and clinical symptoms occur, antimalarials used for treatment of clinical disease or clearance of parasitemia predominantly target this stage of the lifecycle. One emerging strategy to kill blood stage parasites is to target merozoite invasion of the RBC with antimalarials. RBC invasion is an extracellular step in the blood stage lifecycle which is essential for parasite proliferation. A model for the sequential process of invasion, from late stage merozoite development, priming of invasion ligands, merozoite release from the schizont (Fig. 1), through RBC contact and invasion (Fig. 2), is described: briefly (i) merozoites attach to the RBC, (ii) the apical tip of the egg-shaped merozoite contacts the RBC, (iii) invasion ligands from organelles situated at the apical tip (the rhoptry and micronemes) are secreted upon calcium signals and an irreversible interaction known as the tight junction is formed, (iv) the actin-myosin invasion motor engages, protease cleavage events are triggered as the RBC membrane is pulled around the parasite to form the parasitophorous vacuole and (v) the invasion pore is fused behind the invaded parasite (Dvorak *et al*. 1975; Gilson and Crabb 2009; Weiss *et al*. 2015). An important consideration in terms of drug-development is that RBC invasion requires a series of co-ordinated and often irreversible events to occur in sequence, with even small perturbations of this complex process likely to limit parasite survival *in vivo*.

RBC invasion is the essential first step in the disease causing blood stage of the lifecycle and the extracellular merozoite is exposed directly to the bloodstream. Vaccine development targeting merozoite invasion is well advanced with vaccines against merozoite surface protein 1 (MSP1, Phase 2b; Genton *et al*. 2003; Ogutu *et al*. 2009), MSP2 (Phase 2b; Genton *et al*. 2003), apical membrane antigen 1 (AMA1, Phase 2b; Thera *et al*. 2011), reticulocyte binding homologue 5 (Rh5, Phase 1a; Payne *et al*. 2017), erythrocyte binding antigen (EBA-175, Phase 1a; Koram *et al*. 2016) and others reaching clinical trials, but with limited efficacy demonstrated to date (reviewed in Draper *et al*. 2015; Beeson *et al*. 2016). However, many essential proteins and protein/protein interactions required for invasion are unique to malaria parasites and are highly conserved between isolates, making them strong targets for antimalarial development. Given this, there are several broad approaches that could be used therapeutically to block merozoite invasion. The importance of key parasite-parasite and parasite-RBC protein interactions on the merozoite surface highlights the possibility of blocking protein-protein interactions directly; potentially by targeting RBC receptor(s). In addition, protease cleavage events, calcium signalling, the action of the invasion motor and structural changes are also key processes in RBC invasion which could be targeted by drugs. Importantly, a drug that blocks a merozoite's ability to invade immediately and permanently ends the parasite's lifecycle, with this cidal activity potentially having benefits in terms of reducing the risk of tolerance leading to resistance and removing the parasites ability to transition to mosquito transmissible gametocytes. However, it has only been in recent years that protocols have been developed to test invasion-inhibitory compounds against *P. falciparum* merozoites directly *in vitro*), with the availability of these techniques now allowing improved screening and characterisation of new invasion-inhibitory chemotypes.

This review will give an overview on compounds that inhibit invasion, either through inhibition of an upstream invasion priming event in the developing schizont (Fig. 1) or during the merozoite invasion process (Fig. 2), that have shown potential to be developed into antimalarial drugs. Targeting RBC invasion has been established as a proof-of-concept through the demonstration of potent inhibitory activity of numerous compounds *in vitro* (summarised in Supplementary Table S1) and through several *in vivo* studies using animal models (Xiao *et al*. 1996; Zenonos *et al*. 2015; Nasamu *et al*. 2017; Pino *et al*. 2017). Numerous chemical starting points and targets have been identified that could be the basis for the development of potent inhibitors for therapeutic use, as described below. We compare this concept to the clinical use of inhibitors which block HIV entry into host cells (Barre-Sinoussi *et al*. 1983; Gallo *et al*. 1983) to demonstrate that RBC invasion is a viable therapeutic target. In addition, we consider studies from the related apicomplexan parasite *Toxoplasma gondii*, for which a number of optimisable inhibitors have been developed against targets analogous to proteins in malaria parasites. As resistance to frontline artemisinin-based combination therapies continues to spread (Dondorp *et al*. 2009; Ariey *et al*. 2014; Ashley *et al*. 2014), this review is a timely reminder that malaria invasion of RBCs provides an 'Achilles heel' within the parasite's lifecycle that is of increasing interest for antimalarial development.

VIRAL ENTRY INHIBITORS AS A MODEL FOR DEVELOPMENT OF RBC INVASION-INHIBITORY ANTIMALARIALS

Viruses are highly successful and widespread lifeforms that require establishment of an infection inside a host cell in order

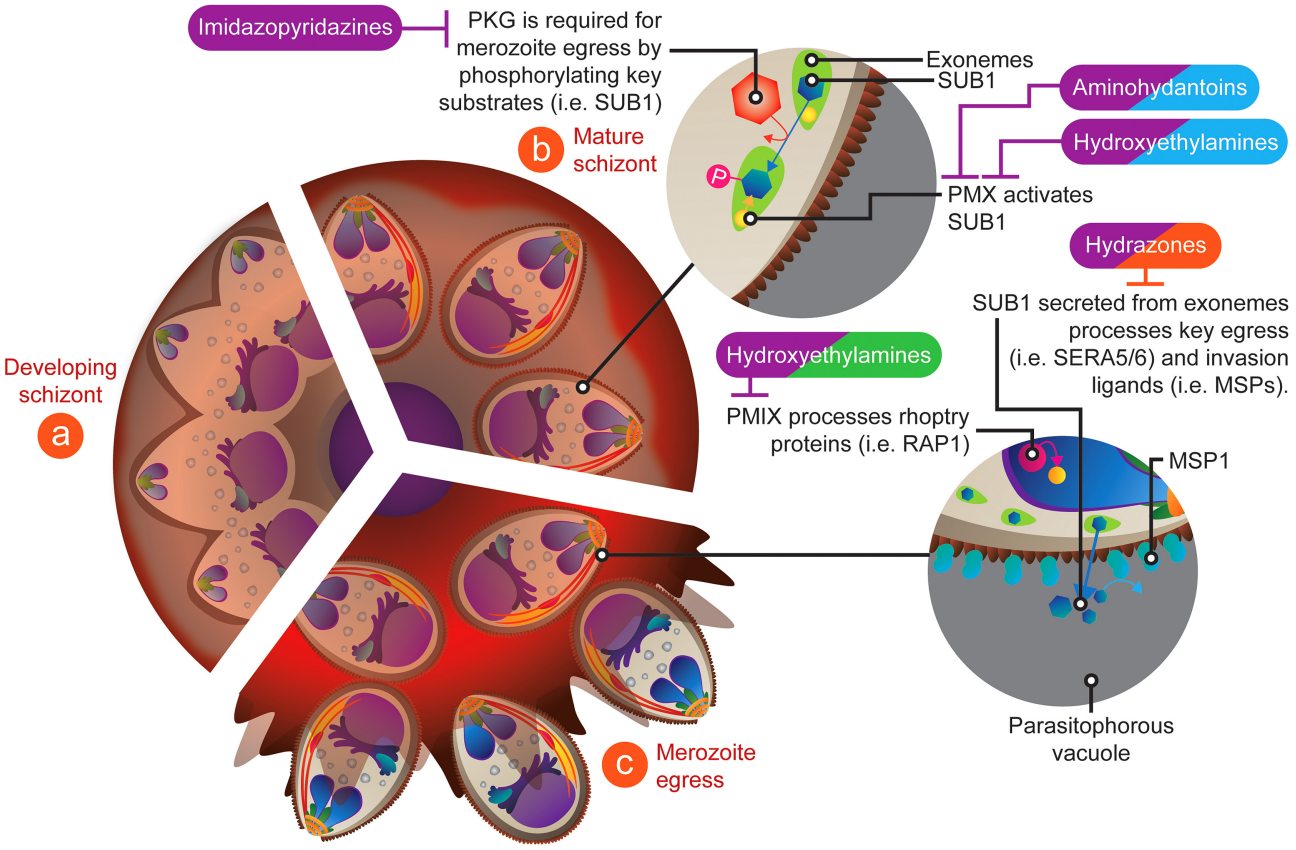

Figure 1. Druggable targets during merozoite development where inhibitors block downstream invasion of the RBC. **(a)** Late stage merozoite development showing partial formation of merozoite membranes and invasion organelles. **(b)** Merozoite formation is completed in mature schizonts. The PVM becomes permeable and PfPKG is activated, leading to activation and discharge of subtilisin-like protease 1 (PfSUB1) from the exonemes. The protease Plasmepsin X (PMX) also resides in the exonemes and is required to process PfSUB1 into an active form. **(c)** Cleavage of PfSUB1 by exoneme resident Plasmepsin X leads to activation of the egress regulating papain-like proteases SERA5 and SERA6, with loss of SERA 5/6 activity preventing merozoite egress from schizonts. Release and activation of PfSUB1 also leads to the cleavage of a number of merozoite invasion ligands including MSP1, MSP6, MSP7, AMA1, with the rhoptry antigen RAP1 processed by Plasmepsin IX (PMIX). Whilst these ligands are largely not required until merozoite contact with the RBC and invasion commences (see Fig. 2), inhibition of these cleavage events around schizont egress is associated with loss of invasion. Inhibitors have been labelled using a dual-colour system that allows their activity against merozoite development/egress (this figure) and their latter effects against invasion (Fig. 2) to be highlighted for inhibitors of: PMIX (purple/green), PMX (purple/blue), PfSUB1 (purple/orange).

to replicate. As a consequence, the essential step of viral entry into the host cell has been targeted by peptide/protein mimics and small molecule entry inhibitors for a diverse range of viruses including HIV (Kilby *et al.* 1998; Dorr *et al.* 2005), orthomyxoviruses (Malakhov *et al.* 2006), flaviviruses (Wang *et al.* 2009), paramyxoviruses (Lambert *et al.* 1996; Rapaport *et al.* 1995), filoviruses (Watanabe *et al.* 2000) and coronaviruses (Bosch *et al.* 2003). The three-step process of viral entry, consisting of attachment, co-receptor binding, and fusion, precedes release of the viral genome into the host's cytoplasm where new virions are assembled before budding and release of the virions from the cell.

HIV entry into memory CD4 + T-lymphocytes (Klatzmann *et al.* 1984; Ho *et al.* 1995) is reliant on a very small number of viral-protein/host-protein interactions and protease cleavage events. Similar to malaria RBC invasion, HIV cell-entry is orchestrated by a multi-step process and is completed within a fraction of the total viral generation time (defined as time between virion release, infection of a new host cell and generation of daughter viral particles (excluding viral latency), estimated between 48 and 63 hours (2.0–2.6 days) (Dixit *et al.* 2004; Murray, Kelleher and Cooper 2011; Puller, Neher and Albert 2017)). Host cell entry begins with virion attachment, binding to the host's co-receptor and entry; with inhibitors, both clinically approved and in development, identified to target each step (reviewed in Kuritzkes 2009; Henrich and Kuritzkes 2013). Maraviroc, the first licenced chemokine receptor antagonist and first host targeting antiretroviral drug, specifically inhibits entry of HIV isolates by binding to the host cell receptor, C-C chemokine receptor type 5 (CCR5), one of two chemokine receptors that HIV viruses use for entry into the cell (Dorr *et al.* 2005; Wood and Armour 2005). Maraviroc specifically inhibits the entry of CCR5-tropic (R5) viruses and is routinely used in second-line antiretroviral combination therapies against R5 HIV viruses (reviewed in Perry 2010).

After binding of the host cell receptors CCR5 or CXCR4 (CXC chemokine receptor type 4), a conformational change occurs within the virion membrane which exposes the heptad repeat domains on viral envelope glycoprotein gp41 (heptad repeat 1 (HR1) and HR2) (reviewed in Klasse 2012). Enfuvirtide, the first antiretroviral fusion-inhibitor approved for HIV treatment, is a 36-amino acid synthetic peptide that mimics the HR2 region of gp41 that binds to HR1, preventing the formation of the six-helix gp41 bundle that is critical for viral fusion and entry (Kilby *et al.* 1998). Enfuvirtide is typically active against HIV-1 isolates that are resistant to other classes of antiretroviral drugs and is reserved for second-line combination therapies of advanced stage HIV infections (Kitchen *et al.* 2008).

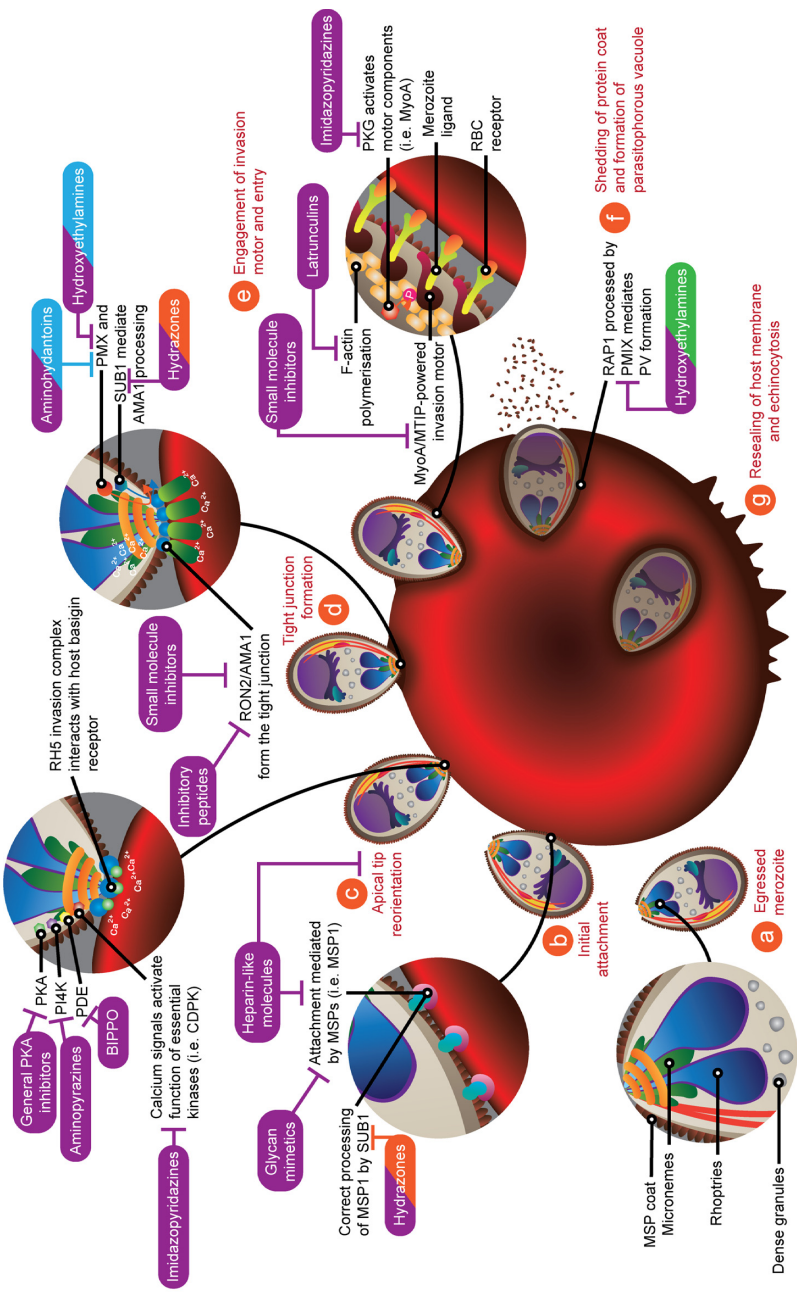

Figure 2. Malaria merozoite invasion of the RBC and invasion inhibitors. **(a)** Merozoites are released into the blood stream after rupture of schizonts (mature blood stage parasites), ready to invade new RBCs. **(b)** Initial attachment requires low-affinity interactions between the surface coat of MSPs, and host receptors on the surface of the RBC. **(c)** Merozoites reorientate such that the apical tip binds to the surface of the RBC and invasion ligands are secreted from the apical tip organelles; the rhoptries and micronemes. The rhoptry antigen PfRH5 binds to its RBC receptor basigin in a key early interaction required for merozoite invasion. **(d)** An irreversible tight-junction is formed when the microneme-secreted protein AMA1 binds to the rhoptry neck protein complex that is embedded on the RBC membrane. **(e)** Entry of the parasite is powered by an actin-myosin motor that pulls the RBC around the invading merozoite while **(f)** the surface coat of MSPs is simultaneously shed. Calcium signalling and phosphorylation by kinases are thought to play a key role in controlling the sequence of events required for invasion during this period. The vacuole membrane fuses behind the invading parasite forming a parasitophorous vacuole. **(g)** Shortly after internalization, a large proportion of RBCs temporarily distort in a process known as echinocytosis. It has been postulated that echinocytosis is caused by rhoptry secretion and rapid entry of Ca^{2+} discharged from the rhoptries into the RBC during invasion, but a more recent explanation suggests that it is incorporation of parasite rhoptry contents into the RBC membrane which leads to RBC membrane ruffling (Dvorak *et al.* 1975, Gilson and Crabb 2009). Examples of drug inhibitors which act at certain stages of the invasion process are labelled in purple. Labels with two colours indicate the inhibitor also has activity around merozoite egress (see Fig. 1).

Although the cellular targets and kinetics of HIV entry into CD4 + T-lymphocytes differ to the requirements of *Plasmodium* invasion into RBCs, these examples of clinically used HIV entry inhibitors for treatment of disease provide an informative comparison for considering the development of antimalarial drugs that target RBC invasion.

PLASMODIUM INVASION INHIBITORS AND PROSPECTS FOR DEVELOPMENT

The majority of current antimalarials target the blood stage of the lifecycle and work through various targets such as; (i) the parasites intracellular food vacuole (chloroquine, artemisinin)(Fidock *et al.* 2000; Klonis *et al.* 2011), (ii) DNA replication (pyrimethamine)(Cowman *et al.* 1988), (iii) mitochondrion function (atovaquone (Fry and Pudney 1992) and proguanil (Dickerman *et al.* 2016)) or, (iv) the apicoplast, the parasite's remnant plastid organelle (doxycycline, azithromycin, clindamycin) (Dahl and Rosenthal 2007; Goodman, Su and McFadden 2007). Currently, no clinically used antimalarial has activity against RBC invasion (Wilson *et al.* 2013), except azithromycin when used at higher concentrations (Wilson *et al.* 2015).

In vitro live-cell filming of *P. falciparum* has shown that RBC invasion, from formation of the tight junction to completion of RBC entry, generally takes less than 1 min (Gilson and Crabb 2009). However, the time taken for a merozoite to commence invasion after egress from a schizont is variable, with one study finding that it took 10 min for 80% of invasion events to be completed *in vitro*. Depending on the drug target, key processes required for RBC invasion could be susceptible to an antimalarial during merozoite development and schizont egress (Fig. 1a–c) or during the process of invasion itself (Fig. 2a–g). To be clinically useful, invasion-inhibitory drugs would need to have a long half-life so that the drug can be maintained in the blood at a high enough level to inhibit invasion as it occurs. A drug with a very short half-life (i.e. artesunate has a half-life of < 30 minutes; Dondorp *et al.* 2009) would not be suitable. Of interest, the half-life of the HIV entry inhibitor maraviroc is ~16 hours (Perry 2010). The myriad of essential and unique targets required to work in a coordinated fashion to enable the rapid process of invasion, combined with the sensitivity of the invasion process to perturbation, provides a promising avenue for new antimalarial development. In this review, we highlight several essential processes targeted in invasion-inhibitory drug development (Figs 1 and 2) and outline some of the compounds that have been tested to date (Supplementary Table S1).

Inhibition of MSPs and RBC receptors using glycan derivatives

Heparin like molecules as invasion inhibitors

Heparin, a member of the glycosaminoglycan family, is a known inhibitor of RBC invasion (Butcher, Parish and Cowden 1988; Boyle *et al.* 2017). A diversity of other sulfated carbohydrates and heparin-like-molecules (HLMs) have also been identified to inhibit invasion of *P. falciparum* merozoites, including curdlan sulfate (Havlik, Rovelli and Kaneko 1994; Evans *et al.* 1998), polyvinyl-sulfonate sodium salt (Kisilevsky *et al.* 2002), suramin (Fleck *et al.* 2003), carrageenans (Adams *et al.* 2005), sulfated cyclodextrins (Crandall *et al.* 2007), fucosylated chondroitin sulfate (Bastos *et al.* 2014), K5 polysaccharides (Boyle *et al.* 2010), inulin sulfate, xylan sulfate, tragacanth sulfate and scleroglucan sulfate (Boyle *et al.* 2017). Furthermore, HLM invasion-inhibitory activity has also been reported for the zoonotic malaria parasite *P. knowlesi* (Lyth *et al.* 2018) and *P. berghei* (Xiao *et al.* 1996), indicating that pan-species invasion inhibition of human malaria parasites is achievable with these molecules. Although precise mechanisms of action for sulfated carbohydrates in inhibiting invasion are not fully understood, HLMs have been reported to inhibit the earliest step in invasion, initial RBC attachment, and to bind MSP1 (Boyle *et al.* 2010), as well as to rhoptry and microneme proteins involved in reorientation and signalling steps of invasion (Fig. 2a-c) (Baum *et al.* 2009; Kobayashi *et al.* 2010; Kobayashi *et al.* 2013). Therefore, it is likely that these compounds target multiple essential ligands in the invasion process, thus reducing the potential for developing drug resistance. Indeed, attempts to generate heparin resistant parasite strains *in vitro* have been unsuccessful (Boyle *et al.* 2010). Although limited studies have been performed to evaluate the activity of HLMs *in vivo*, there is data from animal models (Xiao *et al.* 1996) and human clinical trials supporting their potential development (Havlik *et al.* 2005; Leitgeb *et al.* 2017).

Heparin has been historically used as an adjunct treatment for disseminated intravascular coagulation that can occur in severe malaria (Smitskamp and Wolthuis 1971; Munir *et al.* 1980; Rampengan 1991), but its use was stopped because its anticoagulative properties led to an increased risk of bleeding. Recently, heparins with periodate oxidation of non-sulfated uronic acid residues that greatly reduced anticoagulation activity of heparin (Pisano *et al.* 2005) were shown to be highly inhibitory to RBC invasion (Boyle *et al.* 2017). Similar HLMs have been tested for inhibition of lung cancer growth in mice with no anticoagulation activity reported across a range of tissue (Yu *et al.* 2010). Of further promise, curdlan sulfate (Boyle *et al.* 2010) has a 10-fold reduced anticoagulative activity and testing in a small human trial suggested that treatment reduced malaria disease severity (Havlik *et al.* 2005). A recent phase I clinical trial of the non-anticoagulant HLM sevuparin, a negatively charged polysaccharide manufactured from heparin, limited parasite replication by blocking invasion (Leitgeb *et al.* 2017). HLMs such as sevuparin also disrupt pathogenic mediators such as rosetting and sequestration of infected RBCs, (Udomsangpetch *et al.* 1989; Carlson *et al.* 1992; Rowe *et al.* 1994; Barragan *et al.* 1999; Vogt *et al.* 2006; Kyriacou *et al.* 2007; Skidmore *et al.* 2008; Bastos *et al.* 2014; Saiwaew *et al.* 2017), potentially enabling HLMs to provide dual protective mechanisms of action against severe malaria. Current HLMs that have been identified with antimalarial activity have relatively low potency (Boyle *et al.* 2017) and they have also been reported to have relatively short half-lives when used clinically (i.e. heparin < 1 hour (Perry, Herron and King 1974), sevuparin ~1 hour (Leitgeb *et al.* 2017), curdlan sulfate ~2–3 hours (Gordon *et al.* 1994)). However, the clinically used HLM fondaparinux has a longer half-life (17–21 hours)(Donat *et al.* 2002). Work on improving oral availability of heparin derivatives (reviewed in Neves *et al.* 2016), as well as prolonging HLM drug activity and potency (Hoffart *et al.* 2006; Boyle *et al.* 2017) are ongoing and offer an avenue for HLMs to be developed as antimalarials with invasion-inhibitory activity.

Targeting MSP 1 using glycan mimetics

Initial interactions between the merozoite and the RBC membrane are low affinity, reversible and can occur irrespective of the parasite's orientation. These interactions are mediated by glycosylphosphatidyl inositol (GPI) anchored proteins present on the merozoite's surface (Holder *et al.* 1992; Gilson *et al.* 2006). MSP1 is the most abundant GPI anchored protein on the merozoite surface (Gilson *et al.* 2006) and the proteolytic cleavage of MSP1 to

83 kDa, 30 kDa, 38 kDa and 42 kDa fragments is essential for RBC invasion (Blackman and Holder 1992). The N-terminal MSP1–83 fragment binds to the RBC receptor glycophorin A and the C-terminal MSP1–42 fragment has a role in binding to band-3 on the RBC surface. (Baldwin *et al.* 2015).

The 19 kDa C-terminal cysteine rich epidermal growth factor (EGF)-like domain of MSP-1 is formed after secondary cleavage of MSP1–42 and this essential proteolytic event has been investigated as a potential vaccine and drug target (Goel *et al.* 2003). Testing of EGF domain inhibitors with anticancer properties against MSP1–19 identified a small-molecule glycan mimetic, 2-butyl-5-chloro-3-(4-nitro-benzyl)-3H-imidazole-4-carbaldehyde (NIC), as a specific inhibitor of MSP1–19 function and parasite invasion (Fig. 2b, c) (Chandramohanadas *et al.* 2014). The invasion-inhibitory activity of NIC was confirmed using live filming of invasion and through the use of purified merozoites. NIC not only inhibited the growth of *P. falciparum* isolates, it also inhibited *P. falciparum* expressing *P. chabaudi* rodent malaria MSP1–19 and *P. vivax* field isolates, with IC_{50}s ~20 μM; indicating the pan-species potential of these molecules against malaria parasite invasion. The authors highlight the possibility of targeting the EGF domain of MSP1–19 using small molecule glycans that are being developed as anti-cancer agents (Fig. 2b) (Sugahara *et al.* 2012), but to date no examples of this have been published and further development of this strategy would be required before clinical applications could be assessed. A potential advantage of developing inhibitors that target MSPs, such as HLMs and glycan mimetics, is that they can target merozoites throughout their extracellular phase; post release from schizonts through to resealing of the parasitophorous vacuole membrane.

Small molecule inhibitors of the tight junction that forms between AMA 1 and the RON protein complex

After binding to the RBC and apical re-orientation, the invading merozoite releases proteins residing within specialised apical secretory organelles, the micronemes and rhoptries, to establish an irreversible zone of attachment called the tight junction (Aikawa *et al.* 1978; Bannister and Mitchell 1989). This tight junction is formed as a result of AMA1 (secreted from the micronemes) binding to the RBC bound rhoptry neck (RON) 2/4/5 (secreted from the rhoptries) protein complex (Alexander *et al.* 2006; Collins *et al.* 2009; Richard *et al.* 2010; Tonkin *et al.* 2011), with a known high affinity interaction demonstrated between AMA1 and RON2 (Srinivasan *et al.* 2011; Tonkin *et al.* 2011). The essential interaction between AMA1 and RON2 has been targeted by vaccine induced antibodies (Hodder, Crewther and Anders 2001; Kennedy *et al.* 2002), inhibitory peptides (Harris *et al.* 2005) and drug development (reviewed in Devine *et al.* 2017) (Fig. 2d). A phase 2b vaccine trial of children in Mali demonstrated high anti-AMA1 antibody titres and protection against clinical malaria caused by parasites harbouring vaccine-like alleles after 6 months, but there was minimal efficacy against clinical malaria overall, highlighting the difficulties with targeting a polymorphic antigen such as AMA1 (Thera *et al.* 2011). However, recent studies report substantial conservation of AMA1 function between species, providing evidence that generating cross-species inhibitory activity may be possible (Drew *et al.* 2018).

Several invasion-inhibitory peptides that target AMA1/RON2 binding have been developed. The 20-amino acid R1 peptide, identified from a random phage display library (Harris *et al.* 2005), exhibits high binding affinity for the 3D7 parasite line AMA1/RON2 complex (KD_{50} ~0.2 μM) (Harris *et al.* 2005). RON2L mimics a conserved peptide region of RON2 and competes with native RON2 for the hydrophobic binding pocket of AMA1, blocking formation of the tight junction and inhibiting RBC invasion (Srinivasan *et al.* 2011). Making use of the high binding affinity of peptides that block AMA1/RON2 interactions, a RON2L(peptide)/AMA1 binding inhibition assay was used to screen 21 733 small-molecule inhibitors for activity against AMA1/RON2 (Srinivasan *et al.* 2013), with three hits suggested to directly inhibit RBC invasion. Modification of the lead compound, NCHC00015280 (a pyrrolopyrimidine; IC_{50} 30 μM), yielded two analogues that showed a three (9.8 μM) and five (6 μM) fold improvement in invasion inhibition (Srinivasan *et al.* 2013). However, subsequent studies failed to show binding of these compounds to AMA1 with an affinity commensurate with their reported growth inhibitory activity (Devine *et al.* 2014; Pihan *et al.* 2015), leading Devine *et al.* (2014) to conclude that these compounds inhibited invasion through an AMA1/RON2 independent manner. Nevertheless, the essential role of the AMA1/RON2 complex for invasion, the availability of complete protein structures for *in silico* screening and optimisation makes inhibitors of AMA1/RON2 complex function an attractive target for further development.

The actin-myosin invasion motor as an invasion-inhibitory target

After formation of the tight junction, the actin-myosin motor is engaged and the RBC membrane is pulled around the merozoite via treadmilling of short actin filaments (F-actin) which are pulled unidirectionally. Invasion is powered by a myosin motor complex embedded in the merozoite's pellicle (inner membrane complex; Fig. 2e) (Soldati, Foth and Cowman 2004) (reviewed in Tardieux and Baum 2016). Given the importance and complexity of the actin-myosin motor, a number of targets have been investigated for antimalarial development.

Inhibitors of actin dynamics as invasion-inhibitory drugs

A number of natural agents, such as cytochalasins (a fungal alkaloid) and latrunculins (from marine sponges) have been reported to disrupt actin polymerisation dynamics and ultimately arrest RBC invasion (Fig. 2e) (Miller *et al.* 1979; Cooper 1987; Johnson *et al.* 2016). Latrunculins bind to actin's monomeric form (G-actin) near the Adenosine Triphosphate (ATP) binding site and prevent polymerisation to filamentous actin (F-actin). A recent study identified key amino acid differences between human and *Plasmodium* spp. actin within the ATP binding pocket and sought to synthesise truncated latrunculin B analogues with improved activity against *P. falciparum* malaria and reduced toxicity against mammalian cells (Johnson *et al.* 2016). Truncated latrunculin analogues achieved a 6-fold improved potency against *in vitro* parasite growth (to 7 μM) and 17-fold higher selectivity over mammalian cell cytotoxicity (Johnson *et al.* 2016). To address whether these analogues had activity directly against parasite invasion, the authors used *T. gondii* invasion inhibition assays since this related apicomplexan parasite shares an identical amino acid sequence around the actin ATP binding pocket as *P. falciparum* (Johnson *et al.* 2016). Lead latrunculin analogues showed a >5-fold improvement in invasion-inhibitory activity against *T. gondii* (to 16 μM), indicating that latrunculin analogues target parasite actin during invasion and that activity against apicomplexan parasites is likely to be conserved (Johnson *et al.*

2016). But the high IC_{50}s of these compounds against both parasites highlights that further development is needed.

Inhibitors of the myosin A/MTIP complex

Myosin A (MyoA) is the F-actin bound motor that powers apicomplexan gliding motility during invasion (Meissner, Schluter and Soldati 2002)(Fig. 2e). The ATP-powered protomotive movement of MyoA is dependent on a conserved complex between MyoA's C-terminal domain and the conserved N-terminal domain of MyoA tail interacting protein (MTIP, called myosin light chain 1 (MLC1) in *T. gondii*) (Bosch *et al.* 2006, 2007). Not only is the interaction between MyoA and MTIP essential for parasite invasion of the RBC, but structural characterisation has also identified distinct differences between *Plasmodium* spp. and human homologs, thus presenting a viable target for drug development (Bosch *et al.* 2006).

Modelling of the interaction between MTIP and a growth inhibitory 15-amino acid C-terminal MyoA peptide (Bosch *et al.* 2006) was used to identify small molecule MTIP/MyoA binding inhibitors in a library of 300 000 compounds (Kortagere 2010). A pyrazole-urea based compound (C416) demonstrated the best growth inhibitory activity (IC_{50} of 0.145 μM) (Kortagere 2010) and further structure-based screening identified several analogues with improved activity over the original peptide (C2–1 IC_{50} 0.047 μM; C3–21 IC_{50} 0.385 μM). C3–21 was investigated further and was found to inhibit gliding motility of mosquito stage sporozoites, a marker assay for actin-myosin based motor function that is shared between sporozoites and RBC invading merozoites (Kortagere 2010). Comparative analysis identified that some compounds inhibited growth of both *P. falciparum* and *T. gondii*, thus suggesting a conserved target between the two divergent parasites. However, many analogues also showed variation in efficacy between *P. falciparum* and *T. gondii*, suggesting structural differences between the MyoA/MTIP interaction can be sufficient to reduce efficacy against different apicomplexan parasites (Kortagere 2010; Kortagere *et al.* 2011).

Another high-throughput screen of 12,160 non-cytotoxic compounds against *T. gondii* tachyzoite host cell invasion identified 21 compounds that inhibited parasite motility (Carey *et al.* 2004). One of these hits, tachyplegin A, was found to covalently bind to TgMLC1 (MTIP in *Plasmodium* spp.) and the resulting post-translational modifications caused loss of MyoA function and inability to drive the invasion motor (Carey *et al.* 2004; Heaslip 2010; Leung *et al.* 2014). These studies have identified a diverse range of chemical scaffolds that inhibit function of the MTIP/MyoA driven invasion motor, a conserved complex that is essential for RBC invasion of apicomplexan parasites.

Inhibitors of protease cleavage events required for RBC invasion

Invasion requires a co-ordinated series of proteolytic cleavage events to enable the correct function of essential proteins. The essential role of serine proteases in schizont rupture and RBC invasion have seen them become a significant target of invasion inhibitor drug development (reviewed in O'Donnell and Blackman 2005). The *P. falciparum* subtilisin proteases PfSUB1 (Blackman *et al.* 1998; Yeoh *et al.* 2007) and PfSUB2 are bacterial-like enzymes that have received significant interest because of the key role they play in the essential processing of proteins required for RBC invasion (Figs. 1 and 2) (Supplementary Table S1). PfSUB1 has been shown to cleave MSPs (MSP1, MSP6 and MSP7), invasion ligands released from the micronemes (AMA1) and rhoptry (RAP1) (Yeoh *et al.* 2007; Koussis *et al.* 2009; Silmon de Monerri *et al.* 2011) and is involved in priming the proteolytic cascade that leads to schizont rupture and merozoite egress (Fig. 1c)(Yeoh *et al.* 2007). Comparison of the stage-specific efficacy of the PfSUB1 inhibitor MRT12113 indicates that the IC_{50} against *P. falciparum in vitro* invasion inhibition (~25 μM) was lower than that for schizont rupture inhibition (~180 μM) (Yeoh *et al.* 2007), highlighting the potential sensitivity of the invasion process to chemical inhibition compared to other stages of blood stage development. Interestingly, a follow-up study identified that even partial inhibition of MSP1 processing at invasion inhibitory concentrations of MRT12113 was associated with invasion inhibition, indicating the sensitivity of the invasion process to chemical inhibition (Koussis *et al.* 2009). More recent studies have begun to optimise inhibitors of PfSUB1 from a range of chemical scaffolds (Gemma *et al.* 2012; Bouillon *et al.* 2013; Giovani *et al.* 2014; Kher *et al.* 2014). *Plasmodium* spp. SUB1 are highly conserved and trials using recombinant proteins suggest that 'pan-species' inhibitors can be developed that target the SUB1 of multiple species (Withers-Martinez *et al.* 2012). Indeed, an *in silico* screen using a 3D homology model of PvSUB1 led to the discovery of Cpd2, a compound that inhibits the activity of both recombinant PvSUB1 and PfSUB1 (Bouillon *et al.* 2013). Furthermore, Cpd2 had an *in vitro* IC_{50} of 0.37 μM against *P. falciparum* parasites and inhibited growth of *P. berghei* rodent malaria parasites in a dose-dependent manner, highlighting the pan-species potential of SUB1 inhibitors (Bouillon *et al.* 2013).

Recently its been demonstrated that the aspartic proteases Plasmepsin IX and X (Nasamu *et al.* 2017; Pino *et al.* 2017) have key roles in RBC invasion (Fig. 2d, f) and invasion/egress (Fig. 1b, c), respectively. Plasmepsin IX is located in the rhoptry organelle in merozoites and loss of this protease causes aberrant rhoptry formation and prevents cleavage of key invasion ligands (Nasamu *et al.* 2017; Pino *et al.* 2017). Plasmepsin X is located in merozoite exonemes (secreted from the merozoite prior to rupture) and is involved in activating SUB1 (essential for invasion and schizont rupture), as well as directly processing ligands excreted from the microneme (Nasamu *et al.* 2017; Pino *et al.* 2017). The activity of Plasmepsin IX and X was effectively inhibited by the hydroxylethylamine aspartic protease inhibitor 49c (Ciana *et al.* 2013) at low nanomolar concentrations, providing evidence that both essential proteases can be targeted by one drug (Pino *et al.* 2017). Furthermore, 49c was effective in a *P. berghei* rodent model of malaria against multiple life-cycle stages, including liver stage parasites and gametocytes (Pino *et al.* 2017). Recombinant Plasmepsin X was found to be inhibited by the orally bioavailable aminohydantoins (Meyers *et al.* 2014; Nasamu *et al.* 2017). Further investigation revealed the aminohydantoins inhibited *P. falciparum* growth *in vitro* at submicromolar concentrations and growth of *P. chabaudi* rodent malaria parasites *in vivo*, providing a second starting point for drug development against Plasmepsin X (Meyers *et al.* 2014; Nasamu *et al.* 2017). Since 49c is a potent inhibitor of Plasmepsin IX (rhoptry biogenesis and invasion ligand processing) and both 49c/aminohydantoins inhibit Plasmepsin X function (activation of the egress/invasion priming PfSUB1, invasion ligand processing), both chemical starting points offer activity against merozoite egress and invasion (Nasamu *et al.* 2017; Pino *et al.* 2017).

Inhibitors of malaria merozoite intracellular signalling

The targeting of cellular signal transduction pathways has successfully been used to treat non-infectious diseases such as cancer and autoimmune diseases (Aggarwal *et al.* 2007; Croce

et al. 2016; Marciano and Holland 2017) and is now of increasing interest for antimalarial development. One leading drug target involved in signalling during RBC invasion is calcium dependent protein kinase 1 (CDPK1), a parasite kinase not present in the human host (Harper and Harmon 2005) that has key roles in microneme secretion, activation of the actin-myosin motor and other processes required for RBC invasion (Fig. 2b-e) (Green *et al.* 2008; Bansal *et al.* 2013; Bansal *et al.* 2018). PfCDPK1 has been targeted in several high throughput screens (HTS) of compound libraries (Green *et al.* 2008; Kato *et al.* 2008; Lemercier *et al.* 2009; Chapman *et al.* 2013; Ansell *et al.* 2014; Chapman *et al.* 2014). 2,6,9 trisubstituted purines such as purfalcamine inhibited *P. falciparum* parasite growth (IC_{50} of 230 nM) as well as host cell invasion of related *T. gondii* tachyzoites, consistent with a CDPK in *T. gondii* having a key role in invasion (Kato *et al.* 2008; Lourido *et al.* 2010; Kumar *et al.* 2017). However, purfalcamine was unsuccessful in clearing *P. yoelii* rodent malaria parasites *in vivo*, possibly due to poor pharmacokinetics and reduced efficacy against PyCDPK1 (Kato *et al.* 2008). A second screen identified 3,6-disubstituted imidazopyridazines as compounds of interest, with modification of early leads reducing the growth inhibitory IC_{50} to < 100 nM and providing superior pharmacokinetics to the initial hit compounds (Chapman *et al.* 2013; Chapman *et al.* 2014). However, *in vivo* efficacy against *P. berghei* rodent malaria parasites was again limited (Chapman *et al.* 2013; Chapman *et al.* 2014). Further investigation revealed that a number of optimised imidazopyridazine PfCDPK1 inhibitors were more likely to be targeting *P. falciparum* cGMP dependent protein kinase G (PfPKG; Green *et al.* 2015). Based on conflicting evidence for whether PfCDPK1 is essential for blood stage parasite growth, the limited efficacy *in vivo* and variable specificity of inhibitors, it has been suggested that PfCDPK1 may not be suitable for blood stage drug development (Green *et al.* 2015; Bansal *et al.* 2018)(reviewed in Cabrera *et al.* 2018).

PfPKG has also been a focus for antimalarial development since it is expressed in multiple stages of the lifecycle and has different activation properties to mammalian kinases (McRobert *et al.* 2008; Alam *et al.* 2015; Govindasamy *et al.* 2016). Inhibitors of PfPKG are potent inhibitors of merozoite egress from the developed schizont and are being developed as antimalarials (Taylor *et al.* 2010). Studies have also identified that inhibition of PfPKG blocks invasion of mechanically released merozoites, with speculation that this invasion-inhibitory activity is due to preventing discharge of the invasion priming protease PfSUB1 (Fig. 1b, c) and interfering with phosphorylation of proteins thought to have a role in invasion including PfCDPK1 and invasion motor components (Fig. 2b-e) (Collins *et al.* 2013; Alam *et al.* 2015; Das *et al.* 2015). Recently, a highly potent series of compounds were developed based on an imidazopyridine inhibitor of PKG used for treatment of the apicomplexan parasite *Eimeria tenalla* in chickens. The most active of these, ML10, had an IC_{50} of 2 nM against *P. falciparum* parasite growth *in vitro*. ML10 was also highly efficacious in a *P. chabaudi* rodent model of malaria, with twice daily doses of 100 mg/kg for 4 days reducing parasitemia to undetectable levels in a *P. falciparum* humanized mouse model of malaria (a promising outcome for development of a PKG inhibitor for inclusion in combination therapies.

Development of inhibitors against cAMP-dependent protein kinase A (PKA), which has key roles in microneme secretion (Dawn *et al.* 2014), phosphorylation of the functional domain of AMA1 (Leykauf, 2010) and activation of the actin-myosin motor (Lasonder *et al.* 2012), has been less successful. General inhibitors of PKA, such as H89 and KT5720, and its messenger molecule cAMP have been used as biological tools to block PfPKA and to study its function, but these compounds have low potency (IC_{50} typically > 1 μM) (Syin *et al.* 2001; Beraldo *et al.* 2005; Leykauf *et al.* 2010; Salazar *et al.* 2012) and we are currently unaware of any compounds that have been optimized for activity against PfPKA and cAMP (Buskes *et al.* 2016; Cabrera *et al.* 2018).

An alternative strategy to inhibit invasion is to target 3',5'-cyclic nucleotide phosphodiesterases (PDEs) which regulate degradation of cAMP and cGMP into AMP and GMP, respectively. Increased cAMP and cGMP leads to activation of PKA and PKG, respectively, making PDEs significant regulators of signalling during egress and invasion (Fig. 2c) (Collins *et al.* 2013; Baker *et al.* 2017). Screening of human PDE inhibitors identified zaprinast (growth inhibitory IC_{50} of 35 μM) (Yuasa *et al.* 2005) and a pyrazolopyrimidinone, termed BIPPO, (growth IC_{50} of 0.4 μM) (Howard *et al.* 2015) as having activity against PfPDEα, an isoform that specifically inhibits cGMP. Since PfPDEα has been demonstrated to be dispensable to blood stage parasite growth (Wentzinger *et al.* 2008) and treatment with BIPPO causes activation of cAMP (PfPDEβ) and cGMP (PfPDEα) dependent pathways, it has been suggested that BIPPO may inhibit multiple PfPDE isoforms due to conservation in active sites (Wentzinger *et al.* 2008; Howard *et al.* 2015). Modelling suggests that there are key similarities between human, *Plasmodium* and *Toxoplasma* PDE orthologues that would support this cross-reactivity (Howard *et al.* 2015). Indeed, BIPPO retains activity against isoforms of human PDEs, including PDE9 ($IC_{50} = 30$ nM), and selectivity for *Plasmodium* PDEs would need to be greatly improved before this PDE inhibitor could be developed as an antimalarial (Howard *et al.* 2015).

Although a number of kinases with key roles in RBC invasion have been identified, the development of effective inhibitors against these signalling molecules is still a work in progress with improvements in specificity and potency required for many early leads. However, the feasibility of targeting signalling effector molecules can be demonstrated by recent efforts to develop inhibitors against phosphatidylinositol 4-kinase (PI4K), a key enzyme in protein trafficking required across multiple lifecycle stages, including merozoite development (McNamara *et al.* 2013). This has led to the 2-aminopyrazine compound UCT943 being taken forward into pre-clinical development (Brunschwig *et al.* 2018).

Invasion inhibitory starting points originating from diverse or focussed drug libraries

HTS of small molecule or compound libraries have been used extensively to identify growth inhibitory compounds of the asexual blood stages of *P. falciparum* malaria. However, relatively few screens have been directly designed to identify inhibitors of RBC invasion. Medicines for Malaria Venture (MMV) released a 400-compound library in 2011, termed the Malaria Box, which contains a diverse set of compounds that display antimalarial properties (Spangenberg *et al.* 2013). Subramanian *et al.* (2018) recently screened the Malaria Box for activity against *P. falciparum* blood stage egress and merozoite invasion inhibitors (Subramanian *et al.* 2018) and identified 11 out of 26 hits that inhibited the schizont to ring stage transition at an IC_{50} of <500 nM. Upon microscopic examination of blood smears, MMV665878 and MMV006429 treated schizonts ruptured normally, but free merozoites were found attached to RBCs and few successful invasion events were evident, a phenotype typical of invasion inhibitors (Weiss *et al.* 2015). Further testing

revealed that MMV665878 and MMV006429 were potent invasion inhibitors with up to 50% of invasion events inhibited at concentrations down to 300 nM in assays using purified merozoites (Subramanian *et al.* 2018). Of the 26 compounds identified in this screen, 10 of them have been characterised as inhibitors of PfATP4, a sodium efflux pump on the parasite plasma membrane, indicating either PfATP4 is involved in egress and invasion, or that the compounds have targets additional to PfATP4 (Lehane *et al.* 2014; Subramanian *et al.* 2018).

Screens of the related Apicomplexan parasite *T. gondii* have opened up new starting points for invasion-inhibitory drug development against apicomplexan parasites. *T. gondii in vitro* motility and invasion assays were used as secondary screens against a library of 527 putative kinase inhibitors (Kamau *et al.* 2012). Of the 14 lead compounds with growth inhibitory or enhancing affects, compounds C5 (IC_{50} 1.82 μM) and C1 (IC_{50} 1.36 μM) were found to irreversibly inhibit motility or motility and invasion, respectively. A second study using a fluorescence microscopy based assay screened 1222 covalent inhibitors directly for inhibition of *T. gondii* tachyzoite attachment and invasion, identifying 5 invasion-inhibitory compounds. The leading compound, WRR-086, demonstrated low micromolar (IC_{50} of 5.7 μM) invasion-inhibitory activity. Biochemical and genetic analysis identified a homologue of human DJ-1 (TgDJ-1) as the target of WRR-086, with inhibition of TgDJ-1 linked to loss of microneme secretion and failure to invade (Hall *et al.* 2011). Screening compounds for their invasion-inhibitory activity is providing starting points for the development of drugs with novel mechanisms of action and uncovering new insights into invasion biology of apicomplexan parasites.

The clinically used antibiotic azithromycin as an inhibitor of RBC invasion

The majority of compounds identified that have invasion-inhibitory activity against malaria parasites have no record of clinical use. Recent identification of the invasion-inhibitory activity of the antibiotic azithromycin (Wilson *et al.* 2015) marks one of the few clinically used compounds that have been shown to inhibit *Plasmodium* spp. invasion of RBCs. Macrolide antibiotics are known to target the malaria parasite's remnant plastid (the apicoplast) 70S bacteria-like ribosomal complex (Sidhu *et al.* 2007; Goodman *et al.* 2013). Inhibition of the apicoplast ribosome prevents replication of this essential organelle, resulting in the loss of isoprenoid pyrophosphate (IPP) precursor synthesis and parasite death a full two cycles of growth post treatment (termed delayed death) (Dahl and Rosenthal 2007; Goodman, Su and McFadden 2007). Despite the limitations of a slow killing antimalarial for treatment of disease, azithromycin's safe clinical profile and long half-life (>50 hours) has led to the antibiotic being trialled as a partner drug in artemisinin combination therapies (Cook *et al.* 2006; Sykes *et al.* 2009).

Recently it was found that azithromycin could rapidly inhibit RBC invasion *in vitro* (IC_{50}, 10 μM, in ethanol), which is independent of apicoplast-targeted delayed death (IC_{50}, 0.04 μM, in ethanol) activity (Wilson *et al.* 2015). Although the speed of azithromycin's invasion-inhibitory activity for an otherwise slow acting drug provides a new avenue to develop the drug as an antimalarial, the requirement for a 250-fold higher concentration of azithromycin needed to inhibit invasion currently prevents clinical use of this drug as an invasion inhibitor. However, of note is the identification of several analogues that show >5-fold improvement in invasion-inhibitory activity (Wilson *et al.* 2015, Burns *et al.* Unpublished Data), indicating that improved invasion-inhibitory potency is achievable. Importantly, the most invasion-inhibitory azithromycin analogues also exhibit improved activity against short-term blood stage parasite development and retain activity against the apicoplast, suggesting that azithromycin can be developed to have both fast acting (RBC invasion inhibition and short-term parasite growth inhibition) and apicoplast-targeting delayed death properties (Wilson *et al.* 2015, Burns *et al.* Unpublished Data). Given azithromycin's history of safety, proven activity, long half-life (>50 hours), availability of modified analogues and ease of modification, the identification of azithromycin's invasion-inhibitory activity opens up an attractive starting point to develop an invasion-inhibitory antimalarial with dual-mechanisms of action.

INVASION INHIBITORS IN COMBINATION THERAPIES

A focus of antimalarial development for treatment of clinical disease is on single-dose drug combinations that act broadly across blood-stage development to quickly kill parasites (Burrows *et al.* 2017). As a standalone drug, it is unrealistic to expect an antimalarial which only targets invasion will eliminate all parasites within a matter of hours. However, such a drug could be of benefit in a combination therapy and, as demonstrated in this review, many invasion inhibitors have activity against other lifecycle stages. Combination therapies that have two (or more) safe and efficacious drugs with different mechanisms of action have significant potential advantages, including reducing the risk of developing drug resistance (Hastings 2011). Importantly, the reported mechanisms of action for invasion inhibitors developed to date are not involved in the mechanisms of action of existing antimalarials, limiting the likelihood of cross-resistance.

Evidence from rodent models of malaria suggest that antimalarial monotherapy using drugs that target intracellular parasite growth may not prevent all parasites from progressing through to the next cycle (Khoury *et al.* 2017). Failure to rapidly inhibit blood stage replication may increase the risk of selecting for drug resistance and lead to higher numbers of mosquito transmissible gametocytes posttreatment, thereby contributing to transmission. Targeting invasion directly, the first step in blood stage parasite growth, in a combination therapy would immediately stop progression of parasites into the next cycle of growth, thus limiting opportunities for drug resistance to develop and reducing the number of new gametocytes.

Combining a drug that targets intracellular parasite development (timing of action of current antimalarials) with one that inhibits RBC invasion (extracellular) has intrinsic appeal as targeting these two developmental stages could facilitate rapid clearance of disease causing blood stage parasites and increase drug efficacy. Evidence from monotherapy drug efficacy studies of severe malaria patients suggests that rapid parasite clearance after treatment results in reduced mortality (Dondorp *et al.* 2005). Complicating the speed of parasite clearance, studies have highlighted that a number of clinically used antimalarials have reduced efficacy as malaria parasites transition from mature schizonts, through invasion and into newly established ring stage infections (Painter, Morrisey and Vaidya 2010; Wilson *et al.* 2013; Dogovski *et al.* 2015; Khoury *et al.* 2017). Since each surviving *P. falciparum* schizont is capable of releasing 16–32 new RBC

invading merozoites, providing additional cover through a drug combination that includes a potent invasion inhibitor has the potential to fast-track parasite clearance.

Since *P. falciparum* invasion occurs roughly every 48 hours, this raises the question as to whether clinical treatment with a drug that has a short half-life risks being ineffective across one growth cycle if administered temporally distant from the next period of rupture and invasion. Studies evaluating circulating and sequestered populations of parasites in infected subjects have generally found a wide developmental age range for parasite populations at the time of sampling; predominantly young parasites in peripheral blood and mostly mature stages for parasites sequestered in capillaries (but younger parasites can also be at high levels) in cerebral malaria, non-cerebral malaria and placental malaria cases (MacPherson *et al.* 1985; Oo *et al.* 1987; Silamut *et al.* 1999; Beeson *et al.* 2002; Pongponratn *et al.* 2003). These studies indicate that there is limited parasite synchronicity *in vivo* and it is likely that invasion inhibitors will encounter invading merozoites soon after administration and regularly across the next 48 hours.

In terms of ideal drug properties, invasion inhibitors should have: (i) a half-life that allows the drug to be maintained at effective concentrations with a dosing regimen no more frequent than daily, and (ii) an effective concentration well below that of each dose, allowing inhibitory concentrations of drug to be available over a time period equivalent to many cycles of parasite invasion and growth. As demonstrated by the clinical use of the HIV entry inhibitor maraviroc (half-life ∼16 hours; Perry 2010), maintaining drug concentrations to inhibit pathogen host cell entry over several replication cycles is clinically achievable. In terms of clinically used compounds with invasion-inhibitory antimalarial activity, the half-life is known for azithromycin (>50 hours) and several HLMs (Heparin < 1 hour, sevuparin ∼1 hour, curdlan sulfate ∼2–3 hours and fondaparinux 17–21 hours), and the half-life will need to be a consideration for any invasion inhibitors with higher potency.

Drug resistance models suggest that the increased selective window (when drug levels fall below the minimal inhibitory concentration) of long-lasting drugs can potentially increase drug resistance selection pressure posttreatment (Stepniewska and White 2008; Kay and Hastings 2015). In contrast, drugs with a short half-life, such as the artemisinins, have a much shorter selective window and are considered less likely to select for resistance due to minimal parasite exposure to sub-inhibitory concentrations (Stepniewska and White 2008); but more frequent dosing is required to maintain treatment efficacy. Therefore, there are several important considerations in selecting ideal drug combinations with the potential impacts on clinical efficacy, reducing the risk of drug resistance and reducing transmission all needing to be assessed for combination therapies that include an invasion inhibitor. Despite the potential benefits of having an invasion-inhibitory drug in antimalarial combination therapies, the therapeutic efficacy of a drug combination featuring both an invasion inhibitor and an intracellular blood stage growth inhibitor *in vitro* or *in vivo* has yet to be assessed directly. Thus, future studies will need to assess the potential synergies of using an invasion inhibitor in a combination therapy as well as model the therapeutic and resistance-proofing benefits of doing so.

CONCLUSION

Targeting RBC invasion is a promising antimalarial drug development strategy because: (i) extracellular parasites are exposed directly to drugs in the bloodstream, (ii) most parasite proteins required for invasion lack human equivalents, offering possibilities for selective inhibition and (iii) blocking invasion immediately stops multiplication of disease causing blood stage parasites. Inhibition of host cell entry is a validated strategy for HIV combination therapies (Kilby *et al.* 1998; Dorr *et al.* 2005) and the predicted viral generation time of HIV (≥48 hours; Dixit *et al.* 2004; Murray, Kelleher and Cooper 2011; Puller, Neher and Albert 2017) is similar to the blood stage lifecycle of *P. falciparum*. Therefore, the clinical use of HIV entry inhibitors provides a proof-of-concept that inhibitors of RBC invasion can have a role in antimalarial combination therapies.

The targets of invasion-inhibitory antimalarials under development are mostly essential, conserved and non-redundant (i.e. Yeoh *et al.* 2007; Boyle *et al.* 2010; Kortagere 2010; Wilson *et al.* 2015; Pino *et al.* 2017). The conservation evident in key, drug targetable, invasion machinery between malaria isolates, different *Plasmodium* spp. and different lifecycle stages (i.e. sporozoite invasion) is leading to the development of pan-invasion inhibitors. Therapeutic inhibition of invasion is likely to have profound effects on parasite viability since the merozoite has a short half-life and failure to invade immediately ends parasite growth and multiplication. This would mitigate the risk that parasites develop drug tolerance and persist as is the case for artemisinin resistance. Merozoite invasion may be more sensitive to treatment than other intracellular RBC stages, as demonstrated by a lower IC_{50} for invasion inhibition (∼25 μM) than achieved for rupture inhibition (∼180 μM) for the PfSUB1 inhibitor MRT121113 (Yeoh *et al.* 2007). Therefore, improving a drug's activity against the process of merozoite invasion could have a significant impact on parasite clearance and clinical effectiveness. To date, a number of diverse chemotypes with different targets have been identified to inhibit RBC invasion (Supplementary Table S1), but there is tremendous scope to develop new inhibitors of this essential step in parasite growth for use in combination therapies. The search for new invasion-inhibitory targets is helped by the availability of published mature schizont stage proteomic and phosphoproteomic resources (www.plasmodb.org) that highlight potential merozoite specific therapeutic targets for assessment (Solyakov *et al.* 2011; Lasonder *et al.* 2015). Encouragingly, the development of specific assays to quantify the invasion-inhibitory activity of compounds (Wilson *et al.* 2013; Wilson *et al.* 2015; Weiss, Crabb and Gilson 2016) has led to the identification of new chemical scaffolds that inhibit invasion.

Although several compounds with invasion-inhibitory activity have achieved promising levels of potency *in vitro* and *in vivo* (Gemma *et al.* 2012; Bouillon *et al.* 2013; Giovani *et al.* 2014; Kher *et al.* 2014; Meyers *et al.* 2014; Nasamu *et al.* 2017; Pino *et al.* 2017), an important way forward for invasion inhibitor development is to optimise additional compounds with activity in the low nanomolar range to fast-track further development options. Another future research priority is the evaluation of invasion-inhibitory compounds in combination with currently used and new emerging therapeutics that target intraerythrocytic parasite development, including artemisinin. Such studies would better define the properties and timing of action of drugs to be used in optimal combinations. While proof-of-concept for invasion inhibitors has been demonstrated in animal models, further *in vivo* studies are needed to better define the therapeutic potential of the different inhibitor classes alone and in combination. Incorporation of mathematical modelling, as is increasingly being used in drug evaluation and clinical trials, to assess

the ideal properties of invasion inhibitors in combination therapies *in vivo* would be particularly valuable for informing development priorities.

Combining an invasion inhibitor with artemisinin or a similar drug that acts broadly across malaria's blood stages would provide complete drug coverage across this disease causing stage of the lifecycle. Despite the potential to identify potent, specific and broad acting antimalarials targeting invasion, the discovery and development of drugs that act against this essential and exposed step in blood stage replication has been limited. The recent identification of numerous promising drug leads and targets, combined with improved merozoite purification methods and screening strategies, has revealed promising new avenues for the development of next-generation therapeutics for malaria.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSRE* online

Authorship contributions

DWW, MJB, TdKW, PRG and JGB conceived the idea of this review; ALB, MGD MJB, JMB and DWW conducted the literature review and wrote the manuscript. JMB created the figures. All authors contributed to the design, content and editing of this manuscript.

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