REVIEW ARTICLE

Plasmodium falciparum gametocytes: with a view to a kill

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SUMMARY

Drugs that kill or inhibit the sexual stages of *Plasmodium* in order to prevent transmission are important components of malaria control programmes. Reducing gametocyte carriage is central to the control of *Plasmodium falciparum* transmission as infection can result in extended periods of gametocytaemia. Unfortunately the number of drugs with activity against gametocytes is limited. Primaquine is currently the only licensed drug with activity against the sexual stages of malaria parasites and its use is hampered by safety concerns. This shortcoming is likely the result of the technical challenges associated with gametocyte studies together with the focus of previous drug discovery campaigns on asexual parasite stages. However recent emphasis on malaria eradication has resulted in an upsurge of interest in identifying compounds with activity against gametocytes. This review examines the gametocytocidal properties of currently available drugs as well as those in the development pipeline and examines the prospects for discovery of new anti-gametocyte compounds.

Key words: malaria, *Plasmodium falciparum*, gametocytes, antimalarials, transmission blocking, drugs.

INTRODUCTION

Gametocytes are the sexual stage of the malaria parasite which develop in red blood cells and are essential for transmission to the mosquito vector. It has long been recognized that patients treated for malaria should be cleared of gametocytes in order to prevent them transmitting the infection to others (Darling, 1910). Gametocytes are also well recognized as a vulnerable stage in parasite development and therefore have become a key target in the parasite life cycle against which control strategies can be developed, for the ambitious goal of malaria elimination. There are currently no transmission-blocking vaccines or licensed antimalarial drugs with the potential to kill or inhibit gametocytes which are safe for community-wide use.

Historically antimalarial discovery and development programmes focused on asexual intra-erythrocytic stage parasites as these stages are responsible for the clinical symptoms of malaria. Compounds with activity against gametocytes were not seen as a priority and methods used to identify compounds that inhibit asexual stage parasite growth could not be used to evaluate the effect of compounds on gametocytes which are terminally differentiated. In contrast to asexual stage parasites gametocyte survival cannot be monitored using cell multiplication markers. In addition working with gametocyte stage parasites is technically challenging and until very recently methods for the production of large numbers of gametocytes suitable for use in *in vitro* screening programmes were not available. Fortunately, the renewed emphasis on the eradication of malaria that has occurred in recent years has highlighted the need for anti-gametocyte drugs and tools with which to identify them.

GAMETOCYTOGENESIS

Gametocytes are the only stage of the malaria parasite life cycle able to mediate transmission from the human host to the mosquito vector and in the case of *Plasmodium falciparum*, generally appear 10–14 days after the first appearance of asexual parasites in the host bloodstream. The process undertaken by the asexual erythrocytic stage parasites which leads to the development of these sexual stages within the host bloodstream is called gametocytogenesis. The onset of gametocytogenesis represents a transition period during which the parasite differentiates morphologically and biochemically from a life of asexual reproduction within the human host to one of

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Table 1. Morphology of gametocytogenesis based on light (Hawking *et al*. 1971) and electron microscopy (Sinden, 1982)

Stage	Time of appearance (days)	Shape/ultrastructure
I	0–2	Rounded and do not fill erythrocyte. Indistinguishable from young asexual trophozoite stage
II	1–4	Development of a new subpellicular cytoskeleton which is subtended by microtubules. As the microtubules increase in length the gametocytes develop an elongated D shape
III	2–8	Elongated with pointed or slightly rounded ends, erythrocyte slightly distorted. Subpellicular membrane and microtubule complex develop rapidly and the cell becomes greatly elongated. At this stage parasites usually have one straight side and one curved side. Males and females can be distinguished
IV	6–10	Thin and elongated with rounded ends, erythrocyte is distorted. Subpellicular membrane and microtubule complex completely surrounds the parasite. Sexual dimorphism is pronounced. Females show an increase in ribosomes, ER and mitochondria with dense pigment while in males the nucleus becomes enlarged and pigment is more scattered
V	9–23	The pointed spindle shape develops into a crescent with rounded ends due to loss of the microtubule complex. Males have scattered pigment while in females it remains fairly dense

sexual development within the mosquito vector. Asexual blood stage parasites and immature gametocytes (stage I–III) are thought to have similar metabolic profiles. As an example, both stages of development rely on haemoglobin digestion as a source of amino acids (Smalley, 1977; Hanssen *et al*. 2011) and are hence both likely to be vulnerable to drugs that affect haemoglobin metabolism. Mature (stage IV–V) gametocytes are less active metabolically than asexual parasites and are relatively insensitive to all currently used antimalarial drugs, with the exception of primaquine.

Only a small proportion of the asexual parasite population enters the sexual pathway and this number varies greatly between isolates and during the course of a natural infection (Ponnudurai *et al*. 1982; Graves *et al*. 1984; Day *et al*. 1993; Dunyo *et al*. 2006; McKenzie *et al*. 2007) (reviewed in Alano and Carter, 1990). We still do not fully understand what triggers the switch from the asexual pathway to the production of the sexual stages but we do know that this is very flexible and sensitive to environmental stimuli (Carter and Miller, 1979; Dyer and Day, 2000; Peatey *et al*. 2009*b*).

While most *Plasmodium* sexual stage parasites reach maturity within 27–30 h, *P. falciparum* gametocytes require 10–12 days to reach maturity and once mature can reportedly be carried by infected individuals for up to 55 days post clearance of asexual stages depending on treatment (Bousema *et al*. 2010). This maturation process can be divided into five distinct stages (I–V) based on light and electron microscopy (Table 1) (Hawking *et al*. 1971; Sinden, 1982). Stage I and early stage II gametocytes are indistinguishable from asexual stage parasites and only become morphologically distinguishable at late stage II–III (72–96 h post invasion) with mature stage V parasites exhibiting the characteristic crescent shape. Immature stage I–IV gametocytes sequester in host tissue including bone marrow and possibly the spleen (Smalley *et al*. 1981; Buffet *et al*. 2011; Farfour *et al*. 2012). Mature stage V gametocytes are released into the peripheral circulation and can undergo further development only when taken up by the mosquito vector.

It is known that numerous genetic and metabolic changes occur during gametocyte maturation and while we do not fully understand the biology of many of these changes it is clear that they impact on drug efficacy. While some drugs are active against immature gametocytes they are inactive against mature gametocytes. Targeting mature stage gametocytes is problematic as they are refractory to most antimalarial drugs. Transmission-blocking drugs can exert effects by both directly killing gametocytes within humans or by effecting viability or development within the vector.

An unintended effect

Field studies show that even if a drug is effective against immature gametocytes this does not always result in a reduction of mature infective gametocyte stages, demonstrating that many factors affect a patient's gametocytaemia. These include the time between acquisition of infection and treatment, the choice of drug, the dose administered and asexual parasitemia. These factors all influence the number of gametocytes circulating in a patient's bloodstream and therefore their propensity to transmit malaria. Importantly there is evidence that some drugs, such as amodiaquine and sulfadoxine–pyrimethamine, can stimulate the production of gametocytes in the field and this impacts the emergence of antimalarial resistance (Barnes *et al*. 2008; Sowunmi *et al*. 2008). Furthermore, sub-optimal concentrations of many

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antimalarials can increase the production of gametocytes *in vitro* (Buckling *et al*. 1999; Peatey *et al*. 2009*b*; Buchholz *et al*. 2011). A renewed focus on reducing gametocytaemia as well as asexual stage parasites during treatment is therefore imperative to the efforts to reduce malaria transmission.

CURRENTLY USED DRUGS WITH ACTIVITY AGAINST MATURE GAMETOCYTES

Primaquine

Primaquine is currently the only licensed antimalarial that is effective against mature stage V *P. falciparum* gametocytes *in vivo*. It is an 8-aminoquinoline that is active against gametocytes of all species. Primaquine is also effective against the exo-erythrocytic stages of *Plasmodium vivax* and *Plasmodium ovale* (Alving *et al*. 1952; Miller *et al*. 1965). However it has only weak activity against asexual stage parasites and so must be used in combination with other schizonticides (Rieckmann *et al*. 1968).

Primaquine treatment reduces gametocytaemia and results in reduced gametocyte carriage times when given singularly or with a partner drug (Burgess and Bray, 1961; Rieckmann *et al*. 1968; Kamtekar *et al*. 2004; Pukrittayakamee *et al*. 2004; Lederman *et al*. 2006; Shekalaghe *et al*. 2007; Bousema *et al*. 2010; Smithuis *et al*. 2010; Arango *et al*. 2012; Kolaczinski *et al*. 2012). The drug has been shown to block transmission in animal models (Coleman *et al*. 1992; Portela *et al*. 1999; Vale *et al*. 2009; Lelievre *et al*. 2012) and human trials (Chen *et al*. 1994; reviewed in: Jeffery *et al*. 1956; Rieckmann *et al*. 1968; Graves *et al*. 2012).

The mode of action of primaquine against *P. falciparum* gametocytes remains poorly defined but is thought to be mediated primarily by metabolites as its activity against mature stage gametocytes *in vivo* does not directly translate to the *in vitro* situation (Peatey *et al*. 2009*a*; Adjalley *et al*. 2011) . In addition, only a small percentage of primaquine is excreted unchanged (Greaves *et al*. 1979; Strother *et al*. 1981; Mihaly *et al*. 1984; Baird and Hoffman, 2004). Little is known about the metabolism of primaquine or the identity of the derived metabolite(s) responsible for its activity against gametocytes. Primaquine contains many biologically reactive groups suggesting that its metabolism is likely to be complex (Vasquez-Vivar and Augusto, 1992). Available data indicate that primaquine is metabolized into highly reactive unstable products (Strother *et al*. 1984) and only two metabolites, carboxyprimaquine and 6-methoxy-8-aminoquinoline have been isolated in humans *in vivo* (Baty *et al*. 1975; Mihaly *et al*. 1984), but other as yet unidentified metabolites are also known to exist (Mihaly *et al*. 1984). As the isolation of the active primaquine metabolites in humans has been unsuccessful, alternative systems have been used and a range of primaquine metabolites have been isolated from a variety of organisms including bacteria, yeast, rat livers, mice, dogs and monkeys (Strother *et al*. 1981; Hufford *et al*. 1983, 1986; Clark *et al*. 1984*a*, *b*; Strother *et al*. 1984; Baker *et al*. 1990; Ni *et al*. 1992; Portela *et al*. 1999). Recently Avula *et al*. (2013) developed a method of chromatography enabling the metabolites of primaquine produced after incubation with human hepatocytes to be analysed in a more sensitive way. Fourteen primaquine metabolites were detected and most interestingly carboxyprimaquine, previously thought to be inactive and benign, was shown to be further metabolized into a quinone-imine metabolite that is likely to be antimalarial and toxic (Avula *et al*. 2013).

Only a proportion of identified primaquine metabolites have been assessed for activity against parasites (Bates *et al*. 1990). When the effects of primaquine metabolites on *Plasmodium berghei* exo-erythrocytic schizonts were assessed *in vitro* it was clear that many metabolites are more active than primaquine, particularly those which are hydroxylated (Bates *et al*. 1990). However extrapolating these results into an *in vivo* setting against *P. falciparum* gametocytes is difficult; therefore direct evidence linking a specific metabolite to anti-gametocyte activity is still incomplete.

The parasite mitochondria have been implicated as the site of primaquine action with evidence coming from *in vitro* morphological studies with *P. falciparum* gametocytes and the exo-erythrocytic forms of the avian malaria parasite *Plasmodium fallax*, as well as an *in vivo* study examining murine exo-erythrocytic schizonts after treatment (Beaudoin and Aikawa, 1968; Aikawa and Beaudoin, 1970; Boulard *et al*. 1983; Lanners, 1991). These studies demonstrate that primaquine treatment causes morphological and physiological changes to the mitochondria resulting in thickened mitochondrial membranes that are often swollen and contain multiple layers (Beaudoin and Aikawa, 1968; Aikawa and Beaudoin, 1970; Boulard *et al*. 1983; Lanners, 1991). An action within mitochondria may explain why primaquine is more potent against mid to mature stage gametocytes than against asexual stage parasites. Initially it was thought that as gametocytes develop the number of mitochondria increase with mature stage parasites possessing 4–6 mitochondria compared with only 1–2 in asexual stage parasites (Krungkrai *et al*. 2000). However, recent evidence suggests that one mitochondrion is present which undergoes significant segmentation forming a highly lobed structure (Okamoto *et al*. 2009). In any case, the gametocyte mitochondria undergo significant changes during maturation (Sinden, 1982; Kato *et al*. 1990) including changes in the transcriptome of these organelles (Young *et al*. 2005). It has been hypothesized that

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primaquine effects mitochondrial function by altering electron transport (Vaidya *et al*. 1993). Hydroxylated primaquine metabolites may also inflict extensive oxidative damage to the parasite as they are known to increase the oxidative stress on human red blood cells (Baird *et al*. 1986; Fletcher *et al*. 1988; Ganesan *et al*. 2009), a factor that contributes to the toxicity of primaquine.

The fact that primaquine impacts on gametocytes and transmission and has a role to play in malaria elimination programmes is well recognized. Indeed, the World Health Organization currently recommends that a single dose of primaquine be administered after clearance of *P. falciparum* asexual stages in order to reduce subsequent malaria transmission (World Health Organization, 2011). However the effectiveness of this regimen in reducing transmission has recently been questioned (Graves *et al*. 2012).

Unfortunately there are known side-effects to treatment with primaquine that decreases its usefulness. It is known to cause haemolytic anemia and methaemoglobin formation (Anders *et al*. 1988; Bolchoz *et al*. 2001) particularly in patients with glucose-6-phosphate dehydrogenase deficiency (G6PD) (Alving *et al*. 1956) for whom primaquine is contraindicated. G6PD deficiency is highly prevalent in malaria endemic areas and severely limits the use of primaquine (Howes *et al*. 2012). So while primaquine is an effective gametocytocidal drug, its potential negative effects on health mean that continued research to find a less toxic alternative with similar efficacy against gametocytes and exoerythrocytic parasites is imperative.

Bulaquine

Bulaquine is an 8-aminoquinoline analogue of primaquine; it was formerly known as CDRI80/53 and is currently only licensed for use in India where it is used for radical cure of *P. vivax*. Data show that bulaquine has gametocytocidal activity *in vivo* against both *P. falciparum* and *Plasmodium cynomolgi* (Puri and Dutta, 2005; Gogtay *et al*. 2006), it also appears to be less toxic than primaquine in G6PD-deficient individuals and as such is deserving of further evaluation.

Artemisinin derivatives

A crude extract of the plant *Artemisia annua* has been for used for centuries in traditional Chinese medicine to treat many illnesses including malaria but the full extent of its antimalarial properties were only determined in the late 1970s (Jiang *et al*. 1982). Artemisinin, the active constituent, is relatively cheap to manufacture but it has physical properties such as poor bioavailability that limit its effectiveness. To circumvent this problem a number of derivatives including water-soluble artesunate or oil, soluble artemether and arteether (reviewed in Cui and Su, 2009) have been developed. Artemisinin and other endoperoxide derivatives have been shown to reduce gametocyte carriage in a number of field studies (Hatz *et al*. 1998; von Seidlein *et al*. 1998; Priotto *et al*. 2003; Ndayiragije *et al*. 2004; Bousema *et al*. 2010). The introduction of artemisinin for the treatment of malaria in Thailand resulted in a decline in gametocyte carriage and cases of clinical malaria (Price *et al*. 1996). Reduced transmissibility of *P. falciparum* after treatment with an artemisinin derivative has also been confirmed in a group of children treated with artesunate (Targett *et al*. 2001). In this study a low level of gametocyte carriage correlated directly with a reduction in the number of infected mosquitoes (Targett *et al*. 2001). Treatment of stage V *P. falciparum* gametocytes with artesunate *in vitro* prior to membrane feeding also reduces subsequent oocyst development in *Anopheles dirus* (Chotivanich *et al*. 2006).

The effects of artemisinin derivatives on *P. falciparum* gametocytaemia during infection could stem from the rapid clearance of asexual stage parasites, thereby reducing the potential for gametocyte formation, activity against immature sequestered gametocytes and possibly inhibition of mature gametocytes. Artemisinin has effects on stage I–III gametocytes *in vitro* with significant inhibition seen when treated with 0·1 and 1 μM drug preparations (Kumar and Zheng, 1990).

The activity of artemisinin derivatives against *P. falciparum* gametocytes has also been demonstrated *in vitro*. Many artemisinin derivatives are rapidly converted to dihydroartemisinin (DHA) *in vivo*. DHA has been shown to inhibit stage I–III gametocytes by $50 \geqslant 75\%$ at concentrations of 12–120 nM *in vitro* (Adjalley *et al*. 2011; Buchholz *et al*. 2011). It has also been shown to be active against mature gametocytes (25–50% inhibition at concentrations of 24 and 120 nM). However, other reports of the activity of DHA against mature gametocytes were reported to be lower (40% inhibition at 10 μM) (Peatey *et al*. 2012). This activity would also fit well with reports suggesting that 2–10 μM artesunate is required to inhibit mature gametocytes by 50% (Lelievre *et al*. 2012; Peatey *et al*. 2012). Artemisinin appears to be inactive against stage IV to V gametocytes at concentrations as high as 1 μM *in vitro* (Kumar and Zheng, 1990). However, the clinical relevance of these *in vitro* data are hard to predict given the short half-life of these drugs and the metabolism of artemisisin and artesunate into DHA. Interestingly, while concentrations of the artemisinin drugs may wane quickly after administration, plasma concentrations of DHA approximating 9 μM have been achieved (reviewed in Morris *et al*. 2011). These concentrations are within reason to have some effect on gametocytes given the *in vitro* data, however

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gametocytes still form and mature in patients after treatment with artesunate who were negative for gametocytaemia at the start of treatment (Targett *et al*. 2001).

The mode of action of artemisinin and its derivatives is still not well understood but is dependent on their endoperoxide dioxygen bridge (Cumming *et al*. 1997). It is believed that the endoperoxides accumulate in parasite compartments such as the cytosol and digestive vacuole and presence of haeme generates hydroperoxide, a powerful oxidizing agent which releases carbon-centred free radicals (Haynes and Vonwiller, 1994; Posner *et al*. 1994) and other metabolites (Butler *et al*. 1998). These metabolites bind with membrane-associated proteins (reviewed in Meshnick *et al*. 1996), causing damage to parasite organelles such as the mitochondria, food vacuole and the nuclear envelope (reviewed in Meshnick, 2002) although how this occurs is unclear. Most likely the free radicals attack particular proteins that effect structural function (reviewed in Price and Douglas, 2009). Gametocytes continue to digest haemoglobin and produce haeme until they reach stage IV (Hanssen *et al*. 2011), at which stage digestion ceases. This could reflect the more potent effects of artemisinin and derivatives on immature *vs* mature stage gametocytes.

DRUGS THAT ARE ACTIVE AGAINST IMMATURE GAMETOCYTES

Unlike mature gametocytes which are relatively inactive metabolically, immature gametocytes are similar to asexual stage parasites in that they are still metabolically active and are actively digesting haemoglobin. It is therefore not surprising that many drugs which are effective against asexual stage parasites also kill immature gametocytes.

Chloroquine

Chloroquine inhibits immature stage I–III gametocytes (IC_{50} 42 nM), likely affecting their ability to digest haemoglobin (Smalley, 1977; Buchholz *et al*. 2011). However, it has no effect on mature stage (IV and V) gametocytes *in vitro* (Peatey *et al*. 2012) or *in vivo* (Sowunmi and Fateye, 2003*a*; Sowunmi *et al*. 2003*b*). In addition, chloroquine can increase gametocytogenesis when given at sub-curative concentrations *in vitro* (Bucking *et al*. 1999; Peatey *et al*. 2009*b*) and in animal models (Buckling *et al*. 1997). Chloroquine treatment has also been associated with an increase in infectivity of patients to mosquitoes (Wilkinson *et al*. 1976).

Mefloquine

Mefloquine is a synthetic analogue of quinine that is active against stage I–II immature gametocytes *in vitro* at an IC_{50} of 95 nM, similar to the asexual stage IC_{50} *in vitro* at between 36–80 nM (Buchholz *et al*. 2011). Mefloquine has activity against mature stages of gametocyte development *in vitro*, however at far higher concentrations than the asexual IC_{50} *in vitro*. Studies published recently report a 50–60% inhibition of stage V gametocytes at 5–10 μM (Lelievre *et al*. 2012; Peatey *et al*. 2012) and approximately 20% inhibition at 10 μM against mixed stages III–V (Tanaka and Williamson, 2011). There is little evidence that mefloquine is active against gametocytes *in vivo* (Price *et al*. 1996; Suputtamongkol *et al*. 2003; Sowunmi *et al*. 2009).

Amodiaquine and piperaquine

Amodiaquine in the form of its active metabolite desethylamodiaquine is active against immature stage I–II gametocytes *in vitro* at clinically relevant concentrations (30 nM) (Adjalley *et al*. 2011), however *in vivo* efficacy against mature gametocytes has not been demonstrated, with some studies finding the drug actually causes an increase in gametocytaemia (Sowunmi *et al*. 2007, 2008).

Piperaquine also shows moderate efficacy *in vitro* against immature gametocyte stages at concentrations as low as 17 nM (Adjalley *et al*. 2011). Data on the effect of piperaquine against mature gametocytes in the field is limited; however studies where piperaquine was used in combination with DHA did not indicate a significant effect on the clearance of gametocytaemia in patients (Grande *et al*. 2007; Mens *et al*. 2008). The mechanism of action of amodiaquine and piperaquine is poorly defined. However as a result of structural similarities to chloroquine and additional factors such as evidence of cross resistance (Childs *et al*. 1989; Basco and Le Bras, 1993; Ochong *et al*. 2003) and similar effects on parasite morphology (Sachanonta *et al*. 2011), both drugs are thought to effect haemoglobin digestion within the parasite (Ginsburg *et al*. 1998). Such a mechanism of action would also fit well with their activity against immature gametocytes that still digest haemoglobin.

Pyronaridine

Pyronaridine has been used as an antimalarial in China for more than 30 years (Croft *et al*. 2012) and is currently being assessed for its efficacy as a partner drug for artemisinin, with a phase III clinical trial completed in 2009 (Tshefu *et al*. 2010). The drug is effective against chloroquine-sensitive and -resistant field isolates *in vitro* (Pradines *et al*. 1999; Kurth *et al*. 2009; Okombo *et al*. 2011) and clears parasites in patients with uncomplicated *P. falciparum* infections at a rate equivalent to artermether-lumerfantrine (Tshefu *et al*. 2010).

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Pyronaridine has been assessed both *in vitro* and *in vivo* for activity against *P. falciparum* gametocytes. The drug is moderately effective against stage I–II gametocytes *in vitro* demonstrating between 25–50% inhibition at the asexual IC_{50} concentration of 17 nM (Adjalley *et al*. 2011) and has an IC_{50} of between 6–20 nM against stage II–III gametocytes (Chavalitshewinkoon-Petmitr *et al*. 2000). However, reports on the effect of pyronaridine against stage V gametocytes *in vitro* are conflicting. Some studies report a significant effect against stage V gametocytes *in vitro* (IC_{50} 280 nM) (Peatey *et al*. 2012), while others do not demonstrate any effect at clinically relevant concentrations (IC_{50} 3·2 μM, approximately 700 times the asexual IC_{50}) (Adjalley *et al*. 2011; Lelievre *et al*. 2012). These studies used a variety of assay methods including microscopy (Chavalitshewinkoon-Petmitr *et al*. 2000), a gametocyte-specific GFP-luciferase reporter (Adjalley *et al*. 2011) and an ATP-based bioluminescent assay (Lelievre *et al*. 2012; Peatey *et al*. 2012) which may account for this variability.

A limited number of field studies have assessed the effect of pyronaridine on stage V gametocytes but no effect on gametocyte carriage was seen when patients were treated with this drug in Cameroon (Ringwald *et al*. 1999). Furthermore, no difference in gametocyte clearance times were seen when patients were treated with either artermether-lumerfantrine or pyronaridine-artesunate (Tshefu *et al*. 2010). Current evidence suggests pyronaridine is likely to have limited effect on gametocytes *in vivo* and therefore its use with a gametocytocidal drug such as primaquine may be warranted.

Atovaquone

Atovaquone is effective *in vitro* against stage (I–II) gametocytes, although the extent of this effect varies with different methods of assessment (Fleck *et al*. 1996; Adjalley *et al*. 2011; Buchholz *et al*. 2011). The drug has not shown efficacy against mature stage gametocytes *in vitro* (Fleck *et al*. 1996; Adjalley *et al*. 2011). However, atovaquone-proguanil (Malarone) can clear gametocytes at a faster rate than chloroquine (Enosse *et al*. 2000). Interestingly, treatment of *P. falciparum* mature gametocytes with serum taken from patients taking atovaquone does affect transmission to mosquitoes, although this may reflect inhibition of stages within the mosquito (Butcher and Sinden, 2003).

Atovaquone is a hydroxynathquinone that is thought to inhibit the mitochondrial respiratory chain and indirectly inhibit *de novo* pyrimidine synthesis, a process that is essential for the replication of DNA within the parasite (Fry and Pudney, 1992; Ittarat *et al*. 1994). As gametocytes are terminally differentiated, DNA replication is thought not to occur or is significantly reduced until exflagellation of the male gametocyte (Canning and Sinden, 1975; Sinden *et al*. 1978; Raabe *et al*. 2009). RNA replication is thought to continue until day 6 of maturation (reviewed in Baker, 2010) representing a possible target for atovaquone within the immature stages of gametocyte development.

DRUGS IN CLINICAL DEVELOPMENT WITH ACTIVITY AGAINST GAMETOCYTES

Tafenoquine

Tafenoquine is an 8-aminoquinoline analogue of primaquine that is currently undergoing Phase III clinical trials. It appears to be active against multiple stages of parasite development including liver stages of *P. vivax*. Tafenoquine, initially named WR 238605, was developed as part of a study aimed at uncovering a less toxic and longer-acting alternative to primaquine. It has many advantages over primaquine including a higher therapeutic index (Edstein *et al*. 2003), increased activity against *P. falciparum* asexual stages (Pradines *et al*. 2006) and a longer plasma half-life (12 to 16 days; 50 times that of primaquine) (Mihaly *et al*. 1985; Brueckner *et al*. 1998*b*; Edstein *et al*. 2001). Preclinical *in vivo* studies demonstrate that tafenoquine is an effective prophylactic for *P. falciparum* (Brueckner *et al*. 1998*a*; Lell *et al*. 2000; Hale *et al*. 2003; Walsh *et al*. 2004*a*) and *P. vivax* (Walsh *et al*. 2004*a*; Elmes *et al*. 2008; Nasveld *et al*. 2010). Tafenoquine is also effective in clearing exo-erythrocytic stages of *P. vivax* (Walsh *et al*. 1999, 2004*b*). However, given limited activity against *P. falciparum* asexual parasites (IC_{50} 4 μM *in vitro*) (Adjalley *et al*. 2011) tafenoquine should be used in combination with a fast-acting blood schizonticide (Fisk *et al*. 1989; Obaldia *et al*. 1997; Puri and Dutta, 2003).

Although tafenoquine is believed to be active against gametocytes there is little evidence to support this assumption, particularly for mature stage V gametocytes. *In vitro* studies demonstrate tafenoquine has no effect against stage IV and V gametocytes and has only limited effect on stages I–III (Adjalley *et al*. 2011). However there is the possibility that tafenoquine, as with primaquine, maybe active through a metabolite. *In vitro* studies demonstrate that tafenoquine is metabolized (Idowu *et al*. 1995), however, the mechanisms are poorly defined and metabolites have not been detected in humans (Charles *et al*. 2007). In the *in vitro* rat liver microsome model tafenoquine is metabolized to form aminophenolic metabolites that may be capable of redox cycling (Idowu *et al*. 1995), therefore the mode of action of tafenoquine against *Plasmodium* may be similar to that of primaquine, i.e. through oxidative stress and the disruption of mitochondrial function (Baird *et al*. 1986; Fletcher *et al*. 1988;

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Vaidya *et al*. 1993; Ganesan *et al*. 2009). This mechanism has also been indicated as the possible mode of action of tafenoquine against other protozoa. Tafenoquine inhibits cytochrome c reductase of *Leishamania* species, depolarizing mitochondrial membrane potential and increasing reactive oxygen species (Carvalho *et al*. 2010).

In vivo studies in mice do not demonstrate tafenoquine to be effective against mature gametocytes (Coleman, 1990; Coleman *et al*. 1992; Peters *et al*. 1993), however when administered at the start of *P. berghei* infection tafenoquine appears to prevent gametocytes forming (Coleman, 1990; Coleman *et al*. 1992). Administration of tafenoquine on day 2 and 4 post infection had no effect on mature stage gametocyte viability or exflagellation. In these studies tafenoquine effected *P. berghei* oocyst and sporozoite development, however only when the drug was ingested by the mosquito.

Further studies to determine the effects of tafenoquine against *P. falciparum* gametocytes *in vitro* and *in vivo* are required to conclusively establish the transmission-blocking potential of this drug.

Methylene blue

Methylene blue was the first synthetic compound to be used against malaria, but was not considered suitable for large-scale use as it causes discolouration of the eyes and skin (Wainwright and Amaral, 2005). The discovery that methylene blue is highly active against all gametocyte stages both *in vitro* at clinically relevant concentrations (Adjalley *et al*. 2011), and *in vivo* (Coulibaly *et al*. 2009) make it a promising transmission-blocking agent and has renewed interest in its potential as an antimalarial drug. It is for this reason that methylene blue has recently undergone clinical evaluation as both a monotherapy and as part of a combination (with chloroquine, artesunate or amodiaquine) (Meissner *et al*. 2006; Zoungrana *et al*. 2008; Bountogo *et al*. 2009). Results from these trials suggest methylene blue has significant potential as a component of an effective antimalarial combination therapy. Furthermore, methylene blue has been shown to significantly reduce transmission to mosquitoes in *in vitro* membrane feeding experiments at clinically relevant concentrations (Adjalley *et al*. 2011).

Methylene blue interacts with the antioxidant enzyme glutathione reductase (GR) but its mode of action or interaction(s) with GR or other potential targets is unclear. One theory is that it interferes with GR metabolism either directly or by causing NADPH (which is required for GR production) to be sequestered, altering GR levels in the parasite and also causing the production of reactive oxygen species (Kelner and Alexander, 1985; Farber *et al*. 1998; Sarma *et al*. 2003; Arora and Srivastava, 2005; Buchholz *et al*. 2008; Haynes *et al*. 2011). However, transgenic *P. berghei* blood-stage parasites lacking GR remain sensitive to methylene blue suggesting that GR may not be the primary target of this drug in these parasites (Pastrana-Mena *et al*. 2010). The oocysts of these transgenic parasites were however highly susceptible to methylene blue, suggesting it may have different targets in different stages of parasite development (Pastrana-Mena *et al*. 2010).

It has also been suggested that methylene blue may inhibit haemozoin formation in a similar method to chloroquine (Atamna *et al*. 1996; Deharo *et al*. 2002; Blank *et al*. 2012). Interestingly, the level of resistance induced experimentally in a *P. berghei* mouse model is moderate (Thurston, 1953), providing additional evidence that methylene blue may have multiple targets in parasites (Thurston, 1953; Schirmer *et al*. 2003; Blank *et al*. 2012).

The potent activity of methylene blue across multiple stages of parasite development and its reported low production cost (Schirmer *et al*. 2003) makes it an attractive potential addition to treatment formulations for malaria, particularly as a transmission-blocking agent. However, a note of caution is required as adverse effects, usually red cell haemolysis, have been reported after methylene blue is administered to G6PD-deficient patients during treatment for non-malaria ailments (Rosen *et al*. 1971; Gauthier, 2000; Foltz *et al*. 2006). Nevertheless clinical trials assessing methylene blue as an antimalarial in malaria endemic areas with high prevalence of G6PD deficiency recently demonstrated that this drug is well tolerated with only a few adverse effects seen in children (Mandi *et al*. 2005; Meissner *et al*. 2005, 2006; Muller *et al*. 2012).

Trioxaquines

Trioxaquines are dual molecule antimalarials that contain two therapeutically active moieties (Dechy-Cabaret *et al*. 2000). They combine a chemically active group of artemisinin, a trioxane motif, with a 4-aminoquinoline moiety known to enable molecules to easily enter the parasite (Dechy-Cabaret *et al*. 2000). These compounds were designed to act on the haemoglobin digestion pathway via two different mechanisms with the goal of reducing the development of resistance (Dechy-Cabaret *et al*. 2000, 2004). Trioxaquines are highly active against asexual stages of *P. falciparum in vitro*, including chloroquine-resistant strains (Dechy-Cabaret *et al*. 2000). Trioxaquines have also successfully cleared parasites in several murine models (Dechy-Cabaret *et al*. 2004; Benoit-Vical *et al*. 2007; Cosledan *et al*. 2008) and lead inhibitors are now undergoing preclinical development (Cosledan *et al*. 2008).

While widespread studies have not been performed, trioxaquine compounds have also

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demonstrated activity against all stages of gametocyte development *in vitro* (Benoit-Vical *et al*. 2007). It is not known how these agents affect mature stage gametocytes, however their activity against immature gametocytes is believed to be linked to their ability to target haemoglobin digestion. Further studies examining the potential of these drugs as anti-transmission agents are urgently required.

Endoperoxides: OZ439 and OZ277

Ozonide OZ439 is a synthetic peroxide antimalarial candidate that is in phase IIa clinical trials. Delves *et al* (2012) have shown that endoperoxides such as OZ439 are strong inhibitors of exflagellation. However activity against mature gametocytes is not proven *in vitro* or *in vivo*. OZ277 (arterolane), a synthetic endoperoxide designed on the basis of the artemisinin pharmacophore is currently in Phase III trials. OZ277 has been shown to have *in vitro* activity against stage V gametocytes (IC_{50} 6·4 μM) (Peatey *et al*. 2012) and inhibit exflagellation at 10 μM (Delves *et al*. 2012) but its effectiveness against sexual stages *in vivo* is yet to be determined.

EXPERIMENTAL DRUGS/DRUGS IN PRE-CLINICAL DEVELOPMENT WITH ACTIVITY AGAINST GAMETOCYTES

While research to discover drugs with activity against gametocytes has been ongoing for some time, it has been slow and complicated by the difficulties associated with studying these terminally differentiated parasites. The recent malaria eradication agenda has, however, spurred significant interest in this area of research. As primaquine remains the only clinically available drug with significant activity against mature stage gametocytes, it has been recognized that if eradication is to be achieved new, safer drugs with activity against mature gametocytes are required. Multiple international groups have recently begun searching for compounds with activity against mature gametocytes. Additional research examining the mode of action of active drugs is also ongoing. These studies have identified a number of agents that while still experimental may be useful as anti-gametocyte agents or tools to learn more about gametocyte biology and vulnerabilities.

Quinoline compounds: primaquine derivatives

In an effort to retain gametocyte activity while reducing the toxic side-effects of primaquine, researchers have investigated the gametocytocidal activity of primaquine analogues. While many of these derivatives retain their activity against gametocytes and have improved pharmacokinetic profiles, they often remain haemolytic in G6PD deficient individuals. The 8-aminoquinoline analogue NPC-1161C or more particularly its (−)-enantiomer, NPC-1161B, is perhaps one of the most promising primaquine derivatives currently under investigation. NPC-1161B demonstrates significantly reduced haematotoxicity and retains activity against gametocytes (*in vitro* IC_{50} 3·8 μM (Peatey *et al*. 2012)). NPC-1161C also completely inhibits *P. falciparum* exflagellation *in vitro* (10 μM) (Delves *et al*. 2012) and is more active (IC_{50} 50–500 nM) than primaquine (IC_{50} 0·5–2·5 μM) against asexual intra-erythrocytic parasites under similar conditions (Delves *et al*. 2012). These observations suggest that NPC-1161B may not require metabolic activation to be active against all stages of parasite development. Additional analogues with modifications to the terminal primary amino group of primaquine including bulaquine, imidazoquines and the trioxaquines have also shown promise as anti-gametocyte agents (Benoit-Vical *et al*. 2007; Kiszewski, 2011; Dechy-Cabaret and Benoit-Vical, 2012) and are discussed in previous sections of this review.

Decoquinate

Decoquinate, a well-known veterinary product used to control Coccidial infections in ruminants, has recently been shown to kill *P. falciparum* stage I–II gametocytes (IC_{50} 36 nM) (da Cruz *et al*. 2012). Decoquinate also inhibits the growth of asexual intra-erythrocytic and liver stage parasites. While additional studies are required to determine the activity of decoquinate against mature stage gametocytes, these initial studies are encouraging. The mode of action of decoquinate against immature gametocytes has yet to be specifically demonstrated. However, data show that it kills asexual parasites by selectively and specifically inhibiting the parasite mitochondrial bc1 complex (da Cruz *et al*. 2012). An action against bc1 might be of some concern if cross-resistance with atovaquone exists, however, data suggest that decoquinate has little cross-resistance with atovaquone. The potent and broadspectrum activity displayed by this new antimalarial lead certainly warrants further investigation.

9-Anilinoacridines

9-anilinoacridine compounds have demonstrated activity against both asexual and sexual stage malaria parasites (Figgitt *et al*. 1992; Chavalitshewinkoon-Petmitr *et al*. 2001). These agents, originally developed for use as anti-cancer agents, inhibit DNA topoisomerase II (Schneider *et al*. 1990). They have a similar core structure to the antimalarial pyronaridine which has activity against gametocytes (discussed above) and malaria parasite topoisomerase II (Chavalitshewinkoon-Petmitr *et al*. 2000).

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Pyronaridine is also known to target haematin (Auparakkitanon *et al*. 2006). While 9–anilinoacridines can inhibit gametocytes (stage II–III) at μM concentrations (IC_{50} 8–97 μM) they are more active against asexual erythrocytic parasites (IC_{50} 0·01–21 μM) (Gamage *et al*. 1994; Chavalitshewinkoon-Petmitr *et al*. 2001). There may be issues with the ability of some of these drugs to enter cells and also to be rapidly metabolized, however, this reduced activity against gametocytes is likely to be associated with their action against either haematin or DNA topoisomerase II. In comparison to asexual stages parasites, gametocytes are relatively metabolically inactive and synthesize little DNA (Sinden and Smalley, 1979). It is also important to note that some of the more active 9-anilinoacridine compounds have yet to be tested against gametocytes.

HIV-protease inhibitors

HIV protease inhibitors have been shown to inhibit malaria parasite growth at clinically relevant concentrations (Skinner-Adams *et al*. 2004; Andrews *et al*. 2006; Redmond *et al*. 2007). These drugs inhibit the growth of malaria parasites in mice (Andrews *et al*. 2006) and sera taken from HIV patients receiving HIV protease inhibitors inhibits the growth of *P. falciparum in vitro* (Redmond *et al*. 2007). Studies investigating the stage-specific activity of HIV protease inhibitors against malaria parasites have also demonstrated that they have activity against pre-erythrocytic stages (Hobbs *et al*. 2009) and can directly kill gametocytes of all stages at concentrations between 5–29 μM depending on the inhibitor (Peatey *et al*. 2009*a*, *b*; Hobbs *et al*. 2013). Furthermore, a field study conducted in Uganda indicated a significant reduction of gametocytaemia in children receiving protease inhibitor-based treatment for HIV compared with children receiving non-protease inhibitor-based drugs (Ikilezi *et al*. 2013). Such a broad spectrum of activity against a range of life-cycle stages, including gametocytes is very unusual, and while these drugs are not suitable to be used as first-line antimalarials in their own right (essentially due to cost, instability and modest activity (asexual IC_{50} range 0·4–3·8 μM) (Skinner-Adams *et al*. 2004)), these data indicate that they may represent a promising lead towards a new group of drugs that could reduce clinical disease, relapse and disease transmission.

Natural products

Natural products have traditionally been very important to the control and treatment of malaria so it is not surprising that several of the new and promising treatments with activity against gametocytes are natural products. Neem, a complex product derived from *Azadirachta india*, which has been used for many years as an insecticide and treatment for a variety of diseases is active against all gametocyte stages (Udeinya *et al*. 2006). The gametocytocidal activity of Neem has been attributed to several specific components including azadirachtin, gedunin and nimbolide. Azadiractin is believed to be the primary active component and appears to act by inhibiting the parasites' cytoskeletal system (Billker *et al*. 2002).

The proteosome inhibitor epoxomicin (Hanada *et al*. 1992) has also been identified as a potent anti-gametocyte agent (Czesny *et al*. 2009). This compound originally derived from *Actinomycetes* bacteria has activity against all stages of *Plasmodium* gametocytes (24 h IC_{50} 54 nM) and asexual intra-erythrocytic parasites (24 h IC_{50} 41 nM) (Czesny *et al*. 2009; Tanaka and Williamson, 2011). It also has limited mammalian cell toxicity and is cytotoxic to parasites, suggesting a high therapeutic index (Czesny *et al*. 2009). While epoxomicin appears to affect the morphology of gametocytes it does not inhibit exflagellation *in vitro* (Czesny *et al*. 2009). Additional proteosome inhibitors include non-specific inhibitors such as thiostepton and compounds that are already in clinical use and are now also under investigation as potential anti-gametocyte agents (Aminake *et al*. 2011).

Harmonine, a defence compound from the harlequin ladybird *Harmonia axyridis* which has activity against a broad spectrum of microbes, also has moderate activity against *P. falciparum* (Rohrich *et al*. 2012). In a recent study harmonine was shown to be active against asexual intra-erythrocytic parasites (IC_{50} 4·8–7·6 μM) and stage II gametocytes (18% reduction in gametocyte numbers when treated with 4·8 μM) (Rohrich *et al*. 2012). It also inhibited the exflagellation of microgametocytes (IC_{50} 5·8 μM) (Rohrich *et al*. 2012). While harmonine may be useful as a lead towards novel agents active against gametocytes, its moderate activity and low therapeutic index (cytotoxicity 20–60 μM) (Rohrich *et al*. 2012) suggest it will not be useful in its own right.

Riboflavin or vitamin B2 has been found to be active against *P. falciparum* gametocytes *in vitro* (Akompong *et al*. 2000*a*). It is effective against both immature and mature gametocytes. However, inhibition is highest when gametocytes are young. Riboflavin is also active against asexual erythrocytic stage parasites (Akompong *et al*. 2000*b*) with studies suggesting that activity may be mediated by an effect on haemoglobin digestion (Akompong *et al*. 2000*b*). Unfortunately, riboflavin has a short half-life (2–6 h; Jusko and Levy, 1967), and since concentrations as high as 10–100 μM are required to inhibit all parasite stages it is likely to have limited use as an antimalarial agent. Interestingly, however, riboflavin has been shown to potentiate the activity of mefloquine, pyrimethamine and quinine against asexual erythrocytic stages, suggesting that it may be valuable as a

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component of a combination therapy (Akompong *et al*. 2000*a*).

IDENTIFYING NEW DRUGS AND DRUG TARGETS

Thanks to the sustained efforts of researchers over the last decades there are currently a number of promising anti-gametocyte compounds at various stages along the development pipeline, but given the rigorous selection criteria they must pass along the way, it is certain that only a small proportion will make it through to clinical use as a licensed drug. For this reason the search for good lead compounds must continue and below we examine the prospects for discovery of new anti-gametocyte compounds.

Traditionally there are two approaches to identifying new drugs for a given disease, target-based approaches which are directed at identifying novel targets such as essential enzymes within the parasite to which new drugs can be designed (for example: McGowan *et al*. 2009; Phillips and Rathod, 2010; Skinner-Adams *et al*. 2010) and cell-based approaches based on more general phenotypic screens to identify compounds which can kill the infectious organism but whose mode of action remains undefined (Cervantes *et al*. 2012; Duffy and Avery, 2012; Guiguemde *et al*. 2012). Each has its advantages and disadvantages. While studies on the asexual stages of *P. falciparum* were greatly facilitated by the development of an *in vitro* culture system (Trager and Jensen, 1976), our limited understanding of *P. falciparum* gametocytes has made studies with these stages much more difficult. Difficulties have been predominately associated with being able to differentiate immature stage gametocytes from asexual forms and obtaining adequate numbers of pure gametocytes. As a consequence of these issues we have little information with respect to the differences in protein expression and metabolism between mature stage gametocytes and asexually replicating parasites and hence cell-based approaches are the most feasible option to identify new drugs that target gametocytes. To identify new agents that can kill gametocytes methods for cultivation of large numbers of pure gametocytes are required. A number of methodologies for small-scale gametocyte culture have been published (Campbell *et al*. 1980; Ifediba and Vanderberg, 1981; Ponnudurai *et al*. 1982), but these all have the disadvantage that they do not yield a pure gametocyte culture, but a mix of both sexual and asexual forms. Recently new methodologies have been developed to overcome some of these shortcomings (Fivelman *et al*. 2007). However, most gametocyte preparations are inherently limited by the fact that even in parasite lines with relatively high conversion rates gametocytes usually represent less than 1% of the parasitized cells with a given culture and are essentially terminally differentiated unless ingested by an *Anopheles* mosquito. While it has been reported that gametocytogenesis can be increased by the addition of drugs (Peatey *et al*. 2009*b*), or conditioned media (Fivelman *et al*. 2007) the increase in gametocyte production is generally only 2–3-fold (Dyer and Day, 2000). Dixon *et al*. reported the development of an assay utilizing a green fluorescent protein chimera of the early sexual blood stage protein Pfs16 as a marker for commitment to gametocytogenesis (Dixon *et al*. 2009). This reporter system allows accurate identification of gametocytes well before they are morphologically distinguishable from asexual parasites and made it possible for the first time to isolate relatively large numbers of pure gametocytes suitable for use in drug screening and high throughput screening assays (HTS) (Peatey *et al*. 2012). Nonetheless this method has the disadvantage of using a transgenic parasite line which alters the drug resistance profile of the parasite due to the introduction of the transgene (Peatey *et al*. 2009*b*). Gametocyte culture methods are continuously improving however, with a recent publication by Lucantoni and Avery (2012) reporting that their culture method can achieve gametocytaemia levels of 1–4%. Although limited information on this method was reported, achievement of such a high level of gametocyte production is a significant step towards the development of a HTS.

The most commonly used methods for gametocyte production start with either synchronous or asynchronous asexual stage parasite cultures which are then 'induced' to produce higher levels of gametocytes either by the use of conditioned media, reduction of culture haematocrit, or a combination of the two (Williams, 1999; Fivelman *et al*. 2007; Buchholz *et al*. 2011; Tanaka and Williamson, 2011; Peatey *et al*. 2012). Asexual parasites are removed by the use of either sorbitol (Saul *et al*. 1990) or N-acetyl glucosamine (NAG) (Fivelman *et al*. 2007). Thus it is possible to obtain a uniform population of gametocytes of one specific age. These gametocytes can then be purified from the uninfected red cells by the use of magnetic separation (MACS) (Fivelman *et al*. 2007; Ribaut *et al*. 2008). Preparations with 90% gametocyte purity can readily be achieved using this method (Fivelman *et al*. 2007). Gametocytes can then be used in drug assays immediately as in the majority of the published literature (Peatey *et al*. 2009*b* 2012; Chevalley *et al*. 2010; Buchholz *et al*. 2011; Tanaka and Williamson, 2011; Lelievre *et al*. 2012) or frozen in liquid nitrogen for later use. While freezing results in a loss of viability of the gametocytes of up to 30% (Peatey *et al*. 2011), due to variability in gametocytes numbers collected in a single culture, stockpiling frozen gametocytes may be a convenient way to perform a true high throughput screen. Large-scale production of gametocytes requires the selection of an appropriate parasite isolate as isolates differ in their propensity for producing gametocytes (Graves *et al*. 1984).

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A number of manuscripts have been published detailing different methods for undertaking drug assays on gametocytes, however assays are generally in a 96-well format so relatively few compounds are screened ($N < 50$). Methods for assessing inhibition include: ATP bioluminescence (Lelievre *et al*. 2012; Peatey *et al*. 2012), expression of a gametocyte-specific fluorescent reporter measured by flow cytometry (Peatey *et al*. 2009*a*; Buchholz *et al*. 2011), measurement of transgenic luciferase activity (Adjalley *et al*. 2011) and measurement of metabolic activity using a metabolic indicator such as alamar blue (Tanaka and Williamson, 2011) or hydroethidine (Chevalley *et al*. 2010). To date two publications have reported gametocyte assays in a 384 format (Lelievre *et al*. 2012; Lucantoni and Avery, 2012), although the report by Lucantoni and Avery (2012) mentions a 384-well formatted assay, but no experimental results are given beyond a description of the signal to noise ratio and the Z′ score of the assay. A 1534-well assay has recently been reported using alamar blue as a viability indicator. This assay requires 20 000 gametocytes per well and more importantly does not require the removal of RBCs (Tanaka *et al*. 2013). Miniaturization of the assay did not result in loss of sensitivity therefore, along with reductions in labour requirements and reagents, this assay represents a most promising step toward a HTS of a significant number of compounds (> 300 000) being performed.

While all of the described assays are useful in determining the gametocytocidal activity of known antimalarial compounds or a relatively small number of unknown compounds, none reported have yet been miniaturized enough to undertake the screening of large compound libraries where the number of compounds can exceed 300 K individual chemical entities. Most methods use relatively large numbers of gametocytes, in the order of 10^4 to 10^6 per well, thus making them difficult to use, at least in their current format, for a HTS. A method that uses gametocytes in the range of 10^2–10^3 per well is likely to be required for a HTS to be undertaken. Nonetheless, even given all currently reported methods have their limitations, these assays allow us for the first time to investigate the transmission blocking effects of current and experimental gametocytocidal agents, particularly as a number of HTS against asexual stage parasites have been undertaken (Kurosawa *et al*. 2000; Baldwin *et al*. 2005; Plouffe *et al*. 2008; Gamo *et al*. 2010; Guiguemde *et al*. 2010; Rottmann *et al*. 2010; Duffy and Avery, 2012) and large numbers of compounds with activity against asexual stage parasites identified. The most potent and diverse have been assembled into focused groups which include the GlaxoSmithKline TCAMS library (13 500 compounds), Novartis-GNF malaria box (5600 compounds) and the Medicines for Malaria Venture malaria box (400 compounds). The relatively small size of these focused libraries makes them a good starting point for screening in order to identify compounds with gametocytocidal activity and within the capacity of current assay methods. The ability to screen large (> 300 000 compounds) chemical and natural product libraries for gametocytocidal agents is now tantalizingly close, however it is important to remember that this is only the first step in a long development process albeit a step of great significance.

CONCLUSION

While very few currently available agents have activity against gametocytes, the renewed interest in studying the activity of drugs against these stages and the improved techniques developed to perform these studies are paving the way towards the identification and optimization of new gametocytocidal compounds. These drugs are urgently needed if the current eradication agenda is to be successful.

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