Functional Analysis of *Plasmodium vivax* Dihydrofolate Reductase-Thymidylate Synthase Genes through Stable Transformation of *Plasmodium falciparum*

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**Abstract**

Mechanisms of drug resistance in *Plasmodium vivax* have been difficult to study partially because of the difficulties in culturing the parasite *in vitro*. This hampers monitoring drug resistance and research to develop or evaluate new drugs. There is an urgent need for a novel method to study mechanisms of *P. vivax* drug resistance. In this paper we report the development and application of the first *Plasmodium falciparum* expression system to stably express *P. vivax* dhfr-ts alleles. We used the piggyBac transposition system for the rapid integration of wild-type, single mutant (117N) and quadruple mutant (57L/58R/61M/117T) *pvdhfr*-ts alleles into the *P. falciparum* genome. The majority (81%) of the integrations occurred in non-coding regions of the genome; however, the levels of *pvdhfr* transcript driven by the *P. falciparum* dhfr promoter were not different between integrants of non-coding and coding regions. The integrated quadruple *pvdhfr* mutant allele was much less susceptible to antifolates than the wild-type and single mutant *pvdhfr* alleles. The resistance phenotype was stable without drug pressure. All the integrated clones were susceptible to the novel antifolate JPC-2067. Therefore, the piggyBac expression system provides a novel and important tool to investigate drug resistance mechanisms and gene functions in *P. vivax*.

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**Introduction**

*Plasmodium vivax* is the most widely distributed of the five known human malaria causing parasites and accounts for 80–91 million cases of malaria annually [1,2,3]. *P. vivax* infections are responsible for considerable morbidity and economic loss in endemic countries and have previously been thought to be rarely lethal. However, recent reports revealed that severe complications and death caused by *P. vivax* are not uncommon [2,4,5,6]. Appropriate and timely treatment is the key to prevent morbidity and severe complications.

*P. vivax* parasites are susceptible to most antimalarials, particularly to chloroquine (CQ), which is used as the first line treatment for *P. vivax* infections in most areas of the world today. However, over the last twenty years there have been many reports that highlight the significant increase of resistance of *P. vivax* to CQ [7,8,9,10,11,12,13,14,15,16,17,18,19,20] and to the sulfa/antifolate combination sulfadoxine/pyrimethamine (SP) [7,21,22,23,24,25]. The spread of drug resistance in *P. vivax* makes the control and elimination of this species much more difficult.

A better understanding of mechanisms of drug resistance in *P. vivax* will facilitate the development of molecular methods for monitoring the spread and extent of resistance, which will guide national treatment policy and help the development of new drugs. However, investigations into genetic mechanisms of resistance in *P. vivax* have been severely restricted, due largely to difficulties to culture this species of parasites *in vitro*. Innovative methods are urgently needed to study drug resistance mechanism in *P. vivax*.

The genetic basis of *P. vivax* resistance to antifolates has been determined to be polymorphisms within the parasite’s dihydrofolate reductase (DHFR), a key enzyme in the folate biosynthetic pathway that is targeted by antifolates [26,27,28,29,30]. A particular set of mutations (encoding F57L + S58R+ T61M + S117T) within the *P. vivax* dhfr gene (*pvdhfr*) was shown to correlate with SP treatment failures [30,31]. The role of mutant *pvdhfr* alleles to antifolate resistance was initially demonstrated in various heterologous expression systems, such as *E. coli* [32] and yeast [33,34] because of the difficulties in culturing *P. vivax*. 
More recently, we used a homologous *P. falciparum* episomal (transient) expression system [35,36] to directly assess the effect of wild-type and various mutant *pvdhfr* alleles to parasites’ drug susceptibility. The study demonstrated that this episomal expression system has the potential to provide a rapid screening system for drug development and for studying the mechanism of resistance. However, transient *P. falciparum* expression systems tend to have copy number variability between parasite generations and rely on a constant drug selection pressure to ensure that the epiposomes are not lost over time. This constant drug pressure may also lead to other unforeseen effects on the parasite.

In recent years a *piggyBac* mediated genome integration method has been developed enabling stable transgene expression in *P. falciparum* [37,38,39]. The *piggyBac* transposition system for *P. falciparum* involves a transiently expressed helper plasmid (transposase) that is used to activate the insertion of the transposon plasmid. This transposon contains the positive selectable marker and the gene of interest expression cassette flanked by the Inverted Terminal Repeat (ITR) sequences. Upon expression in the parasite, the *piggyBac* transposase identifies a TTAA target in the *P. falciparum* genome, cuts into this position within the genome and then inserts the expression cassette from within the transposon into the *P. falciparum* genome. Due to the orientation of the ITRs in the transposon only the expression cassette is integrated and the remainder of the plasmid is left outside [40,41]. The *piggyBac* transposition system has been shown previously [41,42] to rapidly and efficiently create stable genomic integrations within a few weeks as opposed to 6–12 months compared to homologous recombination.

To date, there are limited reports of the stable transfection of *P. vivax* genes into the *P. falciparum* genome [43,44,45]. In this article we report the development of the *piggyBac* transposition system for the integration of *pvdhfr* into the *P. falciparum* genome, the transcription of the *pvdhfr* gene, and the response of the *pvdhfr* alleles to conventional antifolate drugs as a proof of concept for the utility of the system. We have also compared the phenotype stability between *piggyBac* *pvdhfr* integrants and the transiently expressed *pvdhfr* [35]. To demonstrate the usefulness of the expression system for screening and evaluating new drugs against *P. vivax* we also used this expression system to test the inhibitory effect of a new generation antifolate, JPC-2067, which is an active dihydrotriazine metabolite of JPC-2056. JPC-2056 is a third generation phenoxypyropoxybiguanide prodrug that was synthesized [46] to overcome the poor gastrointestinal tolerability associated with WR99210, as well as the safety and regulatory restrictions associated with the WR99210 precursor PS-15 [47,48]. The JPC-2067 metabolite has been found to be as potent as WR99210 in *vivo* against *P. falciparum* pyrimethamine sensitive and resistant strains and has also been selected as the lead candidate for pre-clinical development based on the equivalent efficacy to PS-15 and comparable oral tolerability in mice and monkeys to proguanil [46,49]. The findings will help the further development of this compound.

**Results**

**Transfection and Insertion Site Analysis**

Three alleles of the *pvdhfr* gene, wild-type, single mutant (117N) and quadruple mutant (57L, 58R, 61M and 117T) alleles were cloned into the *piggyBac* transposition vector and transfected into the *P. falciparum* line NF54. The transfected parasites were selected under blasticidin selection pressure. Blasticidin resistant populations were selected rapidly within 2–3 weeks and the total number of *piggyBac* insertions obtained per transfected population varied between 1 and 3. Through 6 independent transfections, blasticidin selection pressure and limiting dilution procedures, 16 unique clones of *P. falciparum* with *piggyBac* insertions in their genomes were generated. Southern blot hybridization analysis performed on these clones, revealed that a single *piggyBac* insertion occurred in each clone (Table 1) and that none of the clones retained the *piggyBac* plasmid as epiposomes indicating highly efficient transposition events (data not shown). Importantly, none of the transfected *P. falciparum* clones displayed any growth defects as judged by parasite morphology and development.

All of the *piggyBac* insertions could be mapped unambiguously on the *P. falciparum* genome by performing BLAST searches using the NCBI http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/ BlastGen.cgi?taxid = 5833 database. The sites of integration in the transformed populations were identified by either Thermal asymmetric interlaced (TAIL) PCR or vectorette PCR. The vectorette PCR was more superior to TAIL PCR in determining the integration sites in terms of sensitivity and specificity (data not shown). All *piggyBac* insertions identified were at the expected TTAA target sequence sites and their insertion sites distributed throughout the *P. falciparum* genome with no bias for any particular chromosome. Of the 16 *piggyBac* insertions 13 (81.25%) were identified in intergenic regions while the 3 remaining *piggyBac* insertions occurred within coding regions. Table 1 summarizes the *pvdhfr* allele inserted and insertion sites of these clones.

**Transcription and Expression of the pvdhfr Wild-type and Mutant Alleles in P. falciparum Erythrocyte Stage Parasites**

To demonstrate the transcription and expression levels of the integrated *pvdhfr* gene, we selected a minimum of two clones from each *pvdhfr* allele that have different insertion sites to firstly determine the transcription of the endogenous *pfdfhr*, and secondly determine the transcription and expression of the *pvdhfr* alleles in these clones by conducting both real-time RT-PCR and Western blots.

1) **Transcription of Endogenous pfdfhr Over the Erythrocytic Life Cycle**

The transcription levels of the *pfdfhr* gene relative to that of the seryl-tRNA synthetase (seryl-tRNA) gene (PR07-0073) was determined every 12 hrs, starting with rings (time 0 hrs), through trophozoites (12–24 hrs), schizonts (24–36 hrs) and ending at rings (48 hrs) (Figure 1A). The proportions of ring, trophozoite and schizont at each time point are shown in Figure 1B, C and D. In the parental NF54 line, the transcription level of *pfdfhr* increased as the parasite developed from ring stage to trophozoite stage and peaked at late trophozoite stage (at 24 hrs). All integrated clones showed a similar *pfdfhr* transcription profiles as the parental NF54 strain except clone 1 (expressing a wild-type *pvdhfr*) and clone E1 (expressing a single mutant), which had a transcription peak at 36 hrs. The overall transcription levels of *pfdfhr* in all integrated clones were lower than that of the seryl-tRNA (Figure 1A). In contrast, the overall transcription levels of *pfdfhr* in D6 and episomally transfected D6 lines (containing *pvdhfr* as epiposomes) were generally higher than or comparable to that of seryl-tRNA (Figure 1A).

2) **Transcription of Transfected pvdhfr Over the Erythrocytic Life Cycle**

To determine if the transcription and expression of *pvdhfr* driven by a *P. falciparum* *dfhr* promoter coincides with the expression of the endogenous *pfdfhr*, the *pvdhfr* transcription profile was compared to that of *pfdfhr* within one erythrocytic cycle. The *pvdhfr* transcription profiles of the integrated clones were less homogenous compared...
to the transcription of the endogenous *pfdhfr* peaking between 24 and 48 hrs, with the overall level of transcription lower than that of seryl-tRNA (Figure 1E). Episomally expressed *pfdhfr* alleles also peaked at trophozoite and schizont stages (Figure 1E). A one way ANOVA analysis showed that there was no statistically significant difference (P = 0.123) in *pfdhfr* transcription level between the differently transfected parasites.

3) Transcription of *pfvdhfr* in Different Clones

To determine if the location of the transgene integration affects expression of the parasites *pfvdhfr*, we compared clones that had integrated within the coding region to those integrants that had integrated into non-coding regions. No statistical significance (P = 0.432) of the *pfvdhfr* transcription was found.

4) Protein Expression of Transfected *pfvdhfr* Gene

The protein expression was investigated using both anti-cmyc and anti-biot antibodies. Neither antibody produced a positive signal on Western blot, while a Rex-1 antibody (kindly supplied by Dr Don Gardiner, QIMR) recognized parasite proteins on the same blot (data not shown).

Susceptibility to Conventional Antifolates

Three different clones of each *pfvdhfr* allele transfectants were assayed using the *in vitro* susceptibility assay to determine firstly, if the susceptibility profile was the same between the different clones with the same *pfvdhfr* allele and secondly, to assess the direct effect the different *pfvdhfr* alleles had on the parasites susceptibility to pyrimethamine, cycloguanil, clociguanil and WR99210 (Table 2 and Figure 2). The three clones transfected with wild-type *pfvdhfr* exhibited similar susceptibility to all drugs (P>0.05). The three clones expressing single mutant *pfvdhfr* allele showed comparable susceptibility to cycloguanil, clociguanil and WR99210 with a maximum 2.4, 2.2 and 1.9 fold difference in IC50 values between the clones (P>0.05) for cycloguanil, clociguanil and WR99210, respectively. However, one single mutant clone (E) exhibited a 10 fold higher IC50 value to pyrimethamine compared to two other clones expressing the same allele. The *pfvdhfr* quadruple mutant clones, B3 and D1 had a similar susceptibility profiles for all the antifolates (P>0.05), however, clone C3 was significantly different compared to the other two quadruple mutant clones (P<0.05).

The clones of NF54 transfected with the wild-type *pfvdhfr*-ts (WT) had a similar susceptibility profile to that of the parental NF54 strain. This demonstrated that the WT *pfvdhfr* allele was equally susceptible to antifolates compared to the wild-type *pfvdhfr*-is (P = 0.477). In contrast, the NF54 transfected with the mutant *pfvdhfr* alleles were less susceptible to antifolates compared to the WT. NF54 clones transfected with the single mutant *pfvdhfr* 117N were 5 to 50 fold less susceptible to pyrimethamine (P = <0.001) and 11 to 19 fold less susceptible to WR99210 (P = 0.013) compared to the clones with wild-type *pfvdhfr* allele. However, their susceptibilities to cycloguanil and clociguanil were not different to the clones with the wild-type *pfvdhfr* allele (P = 0.469 and P = 0.440).

Table 1. *pfvdhfr* alleles inserted and insertion sites in the integrated clones.

| Plasmid Name       | *Pvdhfr* Amino Acid Mutations | Clone       | Insertion Site     | Plasmid Name       | *Pvdhfr* Amino Acid Mutations | Clone       | Insertion Site     | Chromosome |
|--------------------|-------------------------------|-------------|--------------------|--------------------|-------------------------------|-------------|--------------------|------------|
| Truncated *Pv bd*  |                               | B8          | Intergenic GTP binding protein - tRNA binding protein | 4                   |                               |             |                     | 14         |
| *pfvdhfr* wild-type allele |                               | G3          | Intergenic MALBP1.156 - MALBP1.154 | 8                   |                               |             |                     | 8          |
| *pfvdhfr* single mutant allele |                               | K           | Intergenic PFLO190w | 9                   |                               |             |                     | 9          |
| *pfvdhfr* quadruple mutant allele |                               | D4          | Intergenic PF10_0114 - PF10_0015 | 10                  |                               |             |                     | 10         |
| *pfvdhfr* quadruple mutant allele |                               | H7          | Intergenic PF10_0182 - PF10_0183 | 10                  |                               |             |                     | 10         |
| *pfvdhfr* quadruple mutant allele |                               | A4          | Intergenic PFF0825c - PFF0830w | 6                   |                               |             |                     | 6          |
| *pfvdhfr* quadruple mutant allele |                               | C5          | Intergenic PF14_0729 - etramp14.2 | 14                  |                               |             |                     | 14         |
| *pfvdhfr* quadruple mutant allele |                               | B7          | Intergenic PF13_0061 - PF13_0062 | 13                  |                               |             |                     | 13         |
| *pfvdhfr* quadruple mutant allele |                               | D12         | Intergenic PF11_0151 - PF11_0153 | 11                  |                               |             |                     | 11         |
| *pfvdhfr* quadruple mutant allele |                               | E1          | Intergenic PF14_0071 - PF14_0072 | 14                  |                               |             |                     | 14         |
| *pfvdhfr* quadruple mutant allele |                               | B3          | Intergenic MAL13P1.114 - MAL13P1.115 | 13                  |                               |             |                     | 13         |
| *pfvdhfr* quadruple mutant allele |                               | D1          | Intergenic pHISTa-like - plasmodium exported protein | 13                  |                               |             |                     | 13         |
| *pfvdhfr* quadruple mutant allele |                               | H8          | Intergenic phosphomannomutase putative - a conserved plasmodium protein | 10                  |                               |             |                     | 10         |

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mutant, TM91c235 (P = <0.001 and P = 0.003, respectively) (Table 2). Each clone was assayed three times on three different occasions and the results were comparable in terms of the rank order and differences between alleles (data not shown).

To confirm that the above described changes in susceptibility to antifolate drugs were not due to mutations or amplification that had occurred in the endogenous \( pfdhfr \) in response to the selection pressure with blasticidin, we sequenced the \( pfdhfr \) and measured the copy number of \( pfdhfr \) for all the transfected clones. No mutations within the open reading frame of \( pfdhfr \) and no copy number changes were identified in the integrated parasites (data not shown). Furthermore, to determine if any plasmid components other than \( pvdhfr \) had any effect on the susceptibility to antifolate drugs, we compared the NF54 transfected with a truncated \( bsd \) \( pvdhfr \) to NF54 transfected with the \( P. \) \textit{vivax} wild-type \( dhfr-ts \) (WT) and found no difference (data not shown).

**Susceptibility to a Novel Antifolate, JPC-2067**

The purpose of being able to express \( P. \) \textit{vivax} drug resistance genes in \( P. \) \textit{falciparum} is so that we can better understand the effect that the various mutant alleles will have against new compounds. Table 2 and Figure 3 show the effect of JPC-2067 against the wild-type and various mutant \( pvdhfr \) integrated alleles. The \( P. \) \textit{falciparum} lines of NF54 (carrying wild-type \( pvdhfr \)) and TM91c235 (carrying

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**Figure 1. Transcription profile and parasite stages of \( dhfr \) mRNA expression over the parasite life cycle.** A) Transcription levels of the \( pf dhfr \) relative to that of seryl-tRNA gene. B) % rings over the parasite life cycle. C) % trophozoites over the parasite life cycle. D) % schizonts over the parasite life cycle. E) Transcription levels of \( pvdhfr \) relative to that of seryl-tRNA. Note: NF54 and D6 in panel E represent level of \( pf dhfr \) transcription as a comparison.

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quadruple mutant $pfdhfr$ were equally susceptible to JPC-2067 ($P > 0.05$). All of the integrated clones with different $pvdhfr$ alleles had a similar susceptibility profile as NF54 and TM91c235, with a maximum difference of 4 fold in IC$_{50}$ values between them ($P > 0.05$).

**Stability of Copy Number (CN) and the Susceptibility to Conventional Antifolates**

To determine if the removal of drug selection pressure on the transfected parasites resulted in a change in CN and/or the susceptibility of transfected parasites to antifolates, six integrated clones (two wild-type, two single and two quadruple mutant clones) were monitored for 32–53 erythrocytic cycles (64–106 days) after selection pressure was withdrawn. Sample were taken at 4–5 time points and used to assess CN and susceptibility to antifolates. D6 parasites expressing various $pvdhfr$ alleles as episomes [35,36] were also monitored in parallel for CN and susceptibility with and without drug pressure.

For the six integrated NF54 clones, the CN of $pvdhfr$ remained at approximately one throughout the 32 cycles with no significant changes in parasite susceptibility to pyrimethamine. For the episomal transfected lines, CN did not reduce with time when cultured under drug pressure. However, CN reduced over time in single and quadruple mutant transfectants when cultured without drug pressure. It was observed that approximately one episode was lost every 9 cycles for the single mutant and every 4 erythrocytic cycles for quadruple mutant transfected parasites on average (Table 3). As the CN of the quadruple mutant $pvdhfr$ epimems decreased, the susceptibility of the parasite to pyrimethamine increased significantly (Spearman test, $P = 0.0167$). The same trend was seen in the single mutant although Spearman test was just above significance ($P = 0.0833$). No significant relationship was observed between the CN of the episomes and the susceptibility to pyrimethamine for the wild-type ($P = 0.7707$). This trend was also observed for cycloguanil, clociguanil and WR99210 (data not shown). It was also observed that there was a significant difference between the IC$_{50}$ values of the integrated quadruple $pvdhfr$ allele and the episomally expressed quadruple $pvdhfr$ allele ($P = 0.001$).
Table 2. Susceptibility of parasites and transfected parasite lines to pyrimethamine, cycloguanil, clociguanil, WR99210 and JPC-2067.

| Parasite/Transfected Clone | Pyrimethamine | Cycloguanil | Clociguanil | WR99210 | JPC-2067 |
|----------------------------|---------------|-------------|-------------|---------|----------|
|                            | IC₅₀ (± SD)   | RR          | IC₅₀ (± SD) | RR      | IC₅₀ (± SD) | RR | IC₅₀ (± SD) | RR |
| Native Parasite Strain     |               |             |             |         |           |     |           |     |
| NF54                       | 0.61 (± 0.00) | 1           | 0.61 (± 0.09) | 1 | 0.05 (± 0.02) | 1 | 0.03 (± 0.00) | 1 | 0.05 (± 0.00) | 1 |
| TM91c235                   | 1276.60 (± 2.32) | 2103 | 85.58 (± 15.20) | 74 | 11.19 (± 2.10) | 211 | 5.70 (± 0.26) | 190 | 0.06 (± 0.26) | 1 |
| Clones of NF54 integrated with different pvdhfr alleles | | | | | |
| Wild-type (I)              | 0.56 (± 0.18) | 2 | 1.16 (± 0.29) | 2 | 0.06 (± 0.02) | 2 | 0.10 (± 0.01) | 4 | 0.14 (± 0.01) | 3 |
| Wild-type (K)              | 0.94 (± 0.01) | 2 | 1.10 (± 0.12) | 2 | 0.08 (± 0.06) | 2 | 0.16 (± 0.00) | 5 | 0.12 (± 0.00) | 3 |
| Wild-type (D4)             | 0.61 (± 0.01) | 1 | 0.57 (± 0.12) | 1 | 0.05 (± 0.02) | 1 | 0.01 (± 0.02) | 0 | 0.09 (± 0.02) | 2 |
| Single mutant (E)          | 30.20 (± 7.32) | 50 | 1.92 (± 0.37) | 3 | 0.11 (± 0.04) | 2 | 0.53 (± 0.09) | 18 | 0.12 (± 0.09) | 3 |
| Single mutant (E1)         | 3.31 (± 1.51) | 5 | 1.03 (± 0.11) | 2 | 0.08 (± 0.01) | 1 | 0.32 (± 0.25) | 11 | 0.07 (± 0.25) | 2 |
| Single mutant (D12)        | 5.85 (± 0.09) | 10 | 0.81 (± 0.02) | 1 | 0.05 (± 0.02) | 1 | 0.57 (± 0.27) | 19 | 0.11 (± 0.27) | 2 |
| Quadruple mutant (B3)      | 75.06 (± 37.00) | 134 | 87.51 (± 24.67) | 144 | 4.04 (± 1.92) | 76 | 7.23 (± 0.09) | 241 | 0.03 (± 0.02) | 1 |
| Quadruple mutant (D10)     | 93.02 (± 30.05) | 166 | 73.21 (± 20.23) | 121 | 5.14 (± 1.86) | 97 | 6.68 (± 0.38) | 223 | 0.06 (± 0.04) | 1 |
| Quadruple mutant (C3)      | 57.11 (± 16.97) | 94 | 119.00 (± 3.90) | 196 | 1.02 (± 0.10) | 19 | 1.57 (± 0.01) | 52 | 0.05 (± 0.01) | 1 |
| Clones of D6 episomally expressing different pvdhfr alleles | | | | | |
| Wild-type                  | 0.17 (± 0.01) | 4 | | | | | | | |
| Single mutant              | 0.19 (± 0.02) | 4 | | | | | | | |
| Quadruple mutant           | 1.03 (± 0.30) | 22 | | | | | | | |

IC₅₀s are means of triplicate data points and are expressed as mean nM ± SD. The relative resistance index (RR) was determined in comparison to NF54.
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Figure 3. In vitro susceptibility profiles of *P. falciparum* NF54, NF54 stably transfected parasites, D6, D6 episomally transfected parasites and TM91c235 to JPC-2067. The symbols represent the means of triplicate data points.
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Table 3. Copy number of *pvdhfr* (CN) and IC50 to pyrimethamine of transfected parasites at five time points over 53 parasite life cycles.

| Parasite strain/transfected clones | Number of days since the start of experiment | 0 | 24 | 46 | 64 | 106 |
|------------------------------------|---------------------------------------------|----|----|----|----|-----|
|                                    | CN ± SD                                      | CN ± SD | CN ± SD | CN ± SD | CN ± SD | CN ± SD |
| Native parasite strains            |                                             |        |        |        |        |       |
| D6                                 |                                             | 1.41 (±0.42) | 0.95 (±0.32) | 1.19 (±0.17) | 1.19 (±0.52) | 0.81 (±ND) |
| NF54                               |                                             | 0.78 (±0.05) | 0.94 (±0.03) | 0.74 (±0.15) | 0.64 (±0.16) | 0 (ND)  |
| TM91c235                           |                                             | 2067.00 (±162.00) | 2526.00 (±420.00) | 2760.00 (±72.00) | 1126.00 (±27.30) | 2252.00 (ND) |
| D6 parasites episomally expressing various *pvdhfr* alleles without and with drug selection | | | | | | |
| Wild-type                          | 1.65 (±0.07) | 2.03 | 0.54 (±0.26) | 2.59 | 0.59 (±0.11) | 2.61 | 0.59 (±0.16) | 2.39 | 0.56 (±0.04) |
| Wild-type + blasticidin pressure | 1.65 (±0.07) | 2.17 | 0.69 (±0.13) | 1.98 | 0.73 (±0.12) | 1.92 | 0.72 (±0.17) | 2.16 | 0.35 (±0.01) |
| Single mutant                      | 11.94 | 1.57 (±0.50) | 8.10 | 0.20 (±0.06) | 8.90 | 0.87 (±0.07) | 7.00 | 0.32 (±0.10) | 5.70 | 0.13 (±0.02) |
| Single mutant + blasticidin pressure | 11.94 | 1.57 (±0.50) | 13.83 | 0.45 (±0.09) | 16.95 | 1.08 (±0.08) | 15.75 | 0.99 (±0.13) | 19.00 | 0.41 (±0.12) |
| Quad mutant                        | 15.99 | 1698.00 (±547.00) | 6.94 | 725.00 (±128.00) | 4.71 | 636 (±81.00) | 4.52 | 507.00 (±63.00) | 1.24 | 94.71 (±4.40) |
| Quad mutant + pyr pressure          | 15.99 | 1698.00 (±547.00) | 11.09 | 1179.00 (±74.00) | 10.42 | 1393.00 (±224.00) | 11.35 | 1156.00 (±186.00) | 33.00 | 1011.18 (±110.00) |
| NF54 parasite clones integrated with different *pvdhfr* alleles without drug selection | | | | | | |
| Wild-type (I)                      | 0.99 | 0.93 (±0.39) | 1.18 | 0.54 (±0.47) | 0.74 | 2.82 (±0.93) | 1.20 | 0.46 (±0.93) | ND | ND |
| Wild-type (D4)                     | 0.63 | 0.71 (±0.34) | 1.19 | 0.56 (±0.24) | 1.19 | 1.50 (±0.34) | 1.19 | 0.53 (±0.07) | ND | ND |
| Single mutant (E)                  | 0.50 | 79.40 (±8.66) | 0.80 | 43.2 (±7.32) | 0.72 | 65.58 (±3.84) | ND | 42.3 (±6.35) | ND | ND |
| Single mutant (E1)                 | 0.60 | 8.31 (±1.51) | ND | 7.89 (±0.83) | 0.54 | 4.10 (±0.16) | ND | 1.41 (±0.30) | ND | ND |
| Quad mutant (B3)                   | 1.15 | 68.42 (±6.78) | 0.70 | 137.08 (±0.93) | 0.92 | 84.93 (±6.78) | 0.83 | 201 (±51.99) | ND | ND |
| Quad mutant (C3)                   | 0.84 | 69.38 (±2.62) | 0.86 | 53.64 (±2.62) | 1.20 | 94.6 (±59.86) | 0.82 | 183.00 (±22.13) | ND | ND |

Discussion

This study demonstrates the integration and stable expression of *pvdhfr* alleles in a heterologous system of *P. falciparum*. It provides evidence that the transposon-mediated genomic integration method, *piggyBac*, is a useful method to create stable transformed clones for studying *P. vivax* drug resistance mechanisms and gene functions in *P. falciparum*. This new system will enable studies into the biology of *P. vivax* parasites which had been significantly lacking due, partially to, our inability to culture this pathogenic parasite species.

The *piggyBac* transfections inserted a single copy of *pvdhfr* into one of the *P. falciparum* chromosomes. The insertion sites appear to be random with the majority of sites identified in non-coding regions of different chromosomes as previously described [41,42]. The insertion of *pvdhfr* did not affect the timing and level of endogenous *pfdhfr* as the transcription timing and levels of endogenous *pfdhfr* in all clones were comparable to those of the parental untransfected parasites. The location of the insertions, which were identified in this study, also did not appear to affect the transcription level of transfected *pvdhfr* because the transcription levels were not significantly different between clones that were inserted in different locations on different chromosomes. Interestingly, the peak transcription time of *pvdhfr* (~36 hrs in the experiment) appears to lag behind that of the endogenous *pfdhfr* (~24 hrs in the experiment). Since the promoter used to transcribe *pvdhfr* is the *pfdhfr* promoter, it is not clear why this shift of timing has occurred. Differences in developmental stages of parasites should not have resulted in this shift as the transcription of *pfdhfr* and *pvdhfr* were measured in the same sample. One possibility may be due to the epigenetic control at the *pfdhfr* locus.

The protein expression was also investigated using both anti-cmyc and anti-bsd antibodies. Unfortunately, neither antibody produced a positive signal on Western blot. There are several possible explanations for negative Western blot. Firstly, the protein expressed was too small for these antibodies to bind and generate a positive signal. Earlier reports [50,51,52] also described failure to obtain positive signals on Western blot and demonstrated that this was due to DHFR-TS being a low abundance protein. Secondly, the expressed PvDHFR maybe conformationally folded such that the cmyc and bsd were not accessible to the antibodies. Nevertheless, the transfected parasites had a susceptibility profile corresponding to the transfected *pvdhfr* allele, indicating the successful expression of functional PvDHFR in the transfected *P. falciparum* parasites.

As was seen previously in the episcopal expression of the *pvdhfr* alleles [33,36], the same in vitro susceptibility phenotypic profile was observed for the integrated wild-type and mutant *pvdhfr* alleles. That is, the wild-type *pvdhfr* transfected clones were as susceptible to antifolates as the susceptible parent strain while the mutant *pvdhfr* alleles conferred significantly reduced susceptibility to the antifolate drugs; with the quadruple mutant *pvdhfr* allele conferring a higher resistance than the single mutant *pvdhfr* allele tested. This increase in resistance to the antifolates was not due to mutations in
the endogenous \textit{pfdhfr}, nor was it due to amplification of the endogenous \textit{pfdhfr} gene or changes in its expression as all clones had a comparable timing and level of \textit{pfdhfr} transcription.

Three different clones of the same \textit{pvdhfr} allele (wild-type, single and quadruple mutant) were used to determine the susceptibility phenotype of the transfected parasites to rule out the possible effect of other unintended mutations or unintended positional effects caused by the integration of the transfected allele into the parasite genome [53]. Indeed, while consistent susceptibility phenotype profiles were observed between the three wild-type clones (I, K and D4), between two single mutant clones (D12 and E1) and between two quadruple mutant clones (B3 and D10), significantly different phenotypes were observed in one of the three single mutant clones (E) and one of the three quadruple mutant clones (C3). This highlights the importance of using multiple clones with different insertion sites to determine the phenotype profile of transfected parasites.

Interestingly, the level of resistance transferred by two systems was not identical, particularly in susceptibilities to antifolates between episomal and integrated quadruple mutant \textit{pvdhfr} allele (57L/58R/61M/117T) transfected parasites. The integrated parasite clones showed a lower level of resistance to all antifolate drugs compared to episomal transfected parasites. The \textit{IC}_{50} values to pyrimethamine was 75.06±37.00 nM, 57.11±16.97 nM and 95.02±30.05 nM for integrated clones and 1698±547 nM for the episomal transfected parasite line. The quadruple mutant \textit{pvdhfr} integrated clones also showed a lower level of resistance to the new antifolate compound JPC-2067 (resistance index 0.72–1.26) than the episomal transfected parasites (resistance index 22) [19]. The difference in susceptibility to these drugs is likely resulted from difference in \textit{pvdhfr} copy number between the episomal and integrated quadruple mutant parasites. When the copy number of \textit{pvdhfr} episomes reduced to 1.24 after culturing 106 days without drug selection, the \textit{IC}_{50} value of the episomal quadruple mutant parasite decreased to 94.71±4.54 nM, comparable to that of integrated quadruple mutant parasite.

The relationship between copy number and susceptibility to antifolate drugs has been studied in the past without a clear conclusion. Results of our previous study [35], suggested that the susceptibility of the antifolates was not affected by the copy number of the episomes carrying the wild-type and mutant \textit{pvdhfr} alleles. In the current study, we conducted a test over 106 days on the same parasite line and identified a good correlation over time between episome copy number and \textit{IC}_{50} values of pyrimethamine for both the single and quadruple mutant \textit{pvdhfr} transfected parasites, while no such a correlation was identified for the wild-type \textit{pvdhfr} transfected parasites. The results demonstrate that variation in copy number within the same transfected parasite line will cause changes in parasite susceptibility to antifolates.

The development of a homologous system to stably express \textit{P. vivax} genes removes the variability and uncertainty in parasite susceptibility caused by episomal copy number and allows us to determine more accurately the susceptibility of antifolates against parasites with mutant \textit{pvdhfr} alleles. This system has a better potential to provide a rapid screening system for drug development and for studying drug resistance mechanisms in \textit{P. vivax} than the episomal expression system. To demonstrate this potential we tested a novel antifolate compound JPC-2067 against parasite clones transfected with various \textit{pvdhfr} alleles. This compound when given to monkeys as a pro-drug (JPC-2056) has been shown to be highly potent against \textit{P. falciparum} parasite carrying wild-type and mutant \textit{pfdhfr} alleles [49]. However, its effect against \textit{P. vivax} carrying different \textit{pvdhfr} was unknown causing concerns on the further development of this compound. Using the stably transfected parasites we demonstrated that the compound was highly effective in killing parasites integrated with \textit{pvdhfr} quadruple mutant allele with the same potency as against parasites integrated with wild-type \textit{pvdhfr}. These results provide evidence and support for the future development of JPC-2056 for the treatment of both \textit{P. falciparum} and \textit{P. vivax} infections. In addition to assisting drug development, this stable \textit{P. falciparum} expression system has the potential to identify and confirm other \textit{P. vivax} drug targets, as well as elucidate the biological function of other \textit{P. vivax} genes.

Materials and Methods

Parasites

\textit{P. falciparum} lines NF54 and TM91c235 were used in this study as antifolate susceptible and resistant controls, respectively. Parasite lines were cultured continuously in a 4% hematocrit blood-LPLF RPMI 1640 as previously reported [54]. Parasite cultures were synchronized at the ring stage by repeated D-Sorbitol treatment [55] and were allowed to grow for one cycle after the last treatment and then used for transfection or \textit{in vitro} susceptibility testing. \textit{P. falciparum} D6 and D6 episomally expressing various alleles of \textit{pfdhfr} [35] were used in copy number, transcription and susceptibility tests. For CN and transcription analyses, aliquots of parasite cultures were taken every 12 hrs, starting from early ring stage, over a period of 48 hrs.

Plasmid Construction

The open reading frames (ORFs) of wild-type, single mutant (117N) and quadruple (57L/58R/61M/117T) mutant alleles of \textit{pvdhfr-ts} were amplified from genomic DNA of \textit{P. vivax} isolates [56] by PCR using primers and conditions described previously [30,56]. The plasmid construction was performed as previously described [35,56] with minor modifications. The \textit{pfdhfr} PCR product was inserted between the \textit{P. falciparum} dhfr-ts promoter and \textit{P. vivax} thymidylate synthase \textit{pvdhfr} allele; the \textit{pvdhfr} cassette had the 0.4-kb \textit{Saccharomyces cerevisiae} boi ORF inserted in frame between the promoter and \textit{dhfr-ts} ORF as described previously [36]. The \textit{pvdhfr} cassette was then cloned into the pXL-BacII vector [42] at the HindIII sites between ITTR2 and 3’/his residue-rich protein-2 (Figure 4).

The pHTH and pDCTH transposases were constructed as previously described in [41,42], respectively. The plasmids for the parasites with transient expression of the \textit{pvdhfr} were developed and transfected as described previously [35].

Transfections

Transfection of \textit{P. falciparum} NF54 was achieved by parasite invasion of plasmid DNA-loaded RBCs as described earlier [41,42] with minor modifications. Briefly, mature blood-stage parasites were purified on a MACS magnetic column (Miltenyi Biotec) and 1 million purified parasites were added to erythrocytes loaded with either 300 µg of the transposon plasmid containing either wild-type or various mutant \textit{pvdhfr} and 150 µg of the transposase plasmid pHTH [42] or 300 µg of the transposon plasmid and 300 µg of the transposase plasmid pDCTH [41] to start a 5 ml parasite culture. Individual clones were obtained by limiting dilution of parasites post-drug selection and parasite clones were detected as previously described [57].

DNA and RNA Extractions

Genomic DNA was extracted from parasites using QIAamp DNA Blood Mini Kits (QIAGEN, USA) and used for: Southern blot hybridisation to confirm the \textit{pigg}Bac integration in the \textit{P. falciparum} genome [42]; inverse PCR [42,56] or vectorette PCR.
reactions to identify the piggyBac insertion sites; quantitative real-time PCR to determine copy number of the pvdhfr gene [35].

Total RNA was extracted from parasites using Nucleospin RNA II kits (Macherey-Nagel, Germany) with an additional 1 u/ml DNase I (New England Biolabs, USA) treatment at 37°C for 3 hrs, followed by a second round of RNA purification using the same kit. Total RNA was used in quantitative real-time reverse transcription PCR (RT-PCR) to determine the transcription of both pfdhfr and pvdhfr genes [59].

Determining CN of pfdhfr and pvdhfr

The CN of the pfdhfr and pvdhfr was determined by a quantitative real-time PCR in an Mx4000 multiplex quantitative PCR system (Stratagene) using a SYBR green-based assay and pfdhfr and pvdhfr primers. The single copy gene eba175 gene was used as a reference (normaliser) gene for estimating the CN for pfdhfr and pvdhfr, respectively (the target). Primers used to amplify fragments of pvdhfr and the eba175 genes were published previously [35].

The sizes of the boxes are not proportional to the lengths of the genes. The pfdhfr promoter drives the expression of bsd-cmyc-pvdhfr-ts fused proteins.

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Sequencing of pfdhfr

To exclude the possibility that altered drug phenotypes could be due to the mutations or an increase in the copy number of the endogenous pf dhfr gene, the pf dhfr gene was amplified and sequenced using the primers and PCR conditions described previously [61].

Analysis of pvdhfr and pfdhfr Transcription

To determine the transcription levels of endogenous pf dhfr and transfected pvdhfr, quantitative real-time reverse transcription PCR (RT-PCR) assays were performed as described previously [59], using primers specific for pvdhfr as described previously [35] and for pfdhfr (PfDHFR Fwd 5'ACCTGGCGAGTTCCATACAGG3' and PfDHFR Rev 5'TCTTGGAAATGAGTGGGATTCTCTGT3'). The statistical significance of the mRNA expression between the transfected parasite lines and their parental strain was determined by either the paired t-test or one way analysis of variance (ANOVA) provided by Sigma Plot for Windows, version 11 (Systat Software, Inc., San Jose, CA).

In vitro Susceptibility, Bioassay and Time Course Studies

WR99210 and JPC-2067 were kindly supplied by Jacobus Pharmaceutical Company (Princeton, N.J.), Cycloguanil and clociguanil, was purchased from ICI Pharmaceuticals (Macclesfield, United Kingdom) and pyrimethamine, was purchased from Hoffmann-La Roche (Switzerland). All compounds were initially
dissolved in dimethyl-sulfoxide (DMSO) and diluted in plasma free LPLF 1640 RPMI. The susceptibility assays were performed as described previously [35,62]. The bioassays were performed as described previously [49] with minor modifications. Briefly, JPC-2067 working drug solutions (50 μl) were serially diluted by 2-fold on 96 well microtitre plates using complete LPLF 1640 RPMI followed by the addition of 50 μl of infected red blood cells suspended in culture medium. The final cell suspension (100 μl) had a haematocrit of 2%, of which 0.5% were infected erythrocytes (>95% rings). A solution of 10 μl of 1:100 dilution of 14.0 Ci/mmol [3H] hypoxanthine (Perkin-Elmer): PRPMI (final concentration of 1 μCi/well) was added after 48 hrs when parasites had already reinvaded the red blood cells and were at ring stages, and the parasites were harvested after 96 hrs. Incorporation of [3H] by intra-erythrocytic malaria parasites (measured in cpm) was recorded for each well, and the concentrations of drugs that inhibited 50% of parasite growth (IC50) when compared with drug-free serum samples (controls) were determined [49]. That is, reduction = 100 x [(mean counts per minute no drug-control samples – mean counts per minute test samples)/mean counts per minute no-drug-control samples]. The percentage of growth inhibition was plotted for each drug concentration. The IC50 was determined using nonlinear regression analysis provided by Sigma Plot for Windows, version 11. All assays were performed in duplicate on at least three different occasions. The time point susceptibility assays were conducted as described previously [35]. However, the assays were only performed once in triplicate at each time point.

References
1. Mendis K, Sina BJ, Marchesini P, Carter R (2003) The neglected burden of Plasmodium vivax malaria. Am J Trop Med Hyg 64: 97–106.
2. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, et al. (2007) Vivax malaria: neglected and not benign. Am J Trop Med Hyg 77: 79–87.
3. Hay S, Guerra CA, Tatem AJ, Noor AM, Snow RW (2008) The global distribution and population at risk of malaria: past, present, and future. Lancet Infect Dis 4: 327–336.
4. Poespoprodjo JR, Fobia W, Kenangalem E, Lampah DA, Hasanuddin A, et al. (2009) Vivax malaria: a major cause of morbidity in early infancy. Clin Infect Dis 48: 1784–1792.
5. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, et al. (2009) Key gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite. The Lancet Infectious Diseases 9: 555–566.
6. Tjitra E, Anstey NM, Sugarto P, Wariker N, Kenangalem E, et al. (2008) Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med 5: e128.
7. Tachiw A, Sinwantorho H, Kenangalem E, Wuwung M, Broekman A, et al. (2007) Therapeutic response of multidrug-resistant Plasmodium falciparum and P. vivax to chloroquine and sulfadoxine-pyrimethamine in southern Papua, Indonesia. Trans R Soc Trop Med Hyg 101: 351–359.
8. Cowie GR (1963) Pitfalls in a discovery: the chronicle of chloroquine. Am J Trop Med Hyg 12: 121–128.
9. Peters W (1998) Drug resistance in malaria parasites of animals and man. Adv Parasitol 41: 1–61.
10. Pukrittayakamee S, Chantra A, Simpson JA, Vanjanonta S, Clemens R, et al. (2008) Therapeutic responses to different antimalarial drugs in vivax malaria. Antimicrob Agents Chemother 54: 1600–1603.
11. Wellems TE, Plowe CV (2001) Chloroquine-resistant malaria. J Infect Dis 184: 770–776.
12. Rieckmann K, Davis DR, C HD (1989) Plasmodium vivax resistance to chloroquine? Lancet ii: 1183–1884.
13. Feyssa DJ, Tuni S, Maredi A, Masbar S, Patipolphi R, et al. (1998) Chloroquine-resistant Plasmodium vivax in transmigration settlements of West Kalimanatan, Indonesia. Am J Trop Med Hyg 59: 513–518.
14. Dua VK, Kar PK, Sharma VP (1996) Chloroquine resistant Plasmodium vivax malaria in India. Trop Med Int Health 1: 816–819.
15. Marlar T, Myat PK, Aye YS, Khang KG, Ma S, et al. (1995) Development of resistance to chloroquine by Plasmodium vivax in Myanmar. Trans R Soc Trop Med Hyg 89: 307–308.
16. Soto J, Toledo J, Gutierrez P, Lazz M, Llanos N, et al. (2001) Plasmodium vivax clinically resistant to chloroquine in Colombia. Am J Trop Med Hyg 65: 90–93.
17. Phillips EJ, Keystone JS, Kain KC (1996) Failure of combined chloroquine and high-dose primaquine therapy for Plasmodium vivax malaria acquired in Guyana. South America. Clin Infect Dis 23: 1171–1173.
18. Alercio MG, Alercio W, Macedo V (1999) Plasmodium vivax resistance to chloroquine (R2) and mefloquine (R3) in Brazilian Amazon region. Rev Soc Bras Med Trop 32: 67–68.
19. Kurcer MA, Simek Z, Zeryck FY, Atay S, Celik H, et al. (2004) Efficacy of chloroquine in the treatment of Plasmodium vivax malaria in Turkey. Ann Trop Med Parasitol 98: 447–451.
20. Phan GT, de Vries PJ, Tran BQ, Le HQ, Nguyen NV, et al. (2002) Artemisinin or chloroquine for blood stage Plasmodium vivax malaria in Vietnam. Trop Med Int Health 7: 858–864.
21. Darlow B, Vibhuta H, Gibney S, Jolley D, Stace J, et al. (1982) Sulfadoxine-pyrimethamine for the treatment of acute malaria in children in Papua New Guinea. II. Plasmodium vivax. Am J Trop Med and Hyg 31: 10–13.
22. Alam MT, Bora H, Bharti PK, Sahu MA, Das MK, et al. (2007) Similar trends of pyrimethamine resistance-associated mutations in Plasmodium vivax and P. falciparum. Antimicrob Agents Chemother 51: 857–863.
23. Dobrobyen EB, Terekartiamjorn C, Andre RG, Phantuyohin P, Noepyapamoonth S (1979) Treatment of vivax malaria with sulfadoxine-pyrimethamine and with pyrimethamine alone. Trans R Soc Trop Med Hyg 73: 15–17.
24. Baird JK (2009) Resistance to therapies for infection by Plasmodium vivax. Clin Microbiol Rev 22: 508–534.
25. Young MD, Burgess RW (1959) Pyrimethamine resistance in Plasmodium vivax malaria. Bull World Health Organ 20: 27–36.
26. de Pecoulas PE, Basco LK, Tahar R, Ouatas T, Mazabraud A (1998) Analysis of the Plasmodium vivax dhfr dfr51 gene and with pyrimethamine resistance. Mol Biochem Parasitol 92: 267–273.
27. de Pecoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco LK (1998) Sequence variations in the Plasmodium vivax dhfr dfr51 gene and with pyrimethamine resistance. Mol Biochem Parasitol 92: 267–273.
28. Imwong M, Pukrittayakamee S, Louarreseauv S, Pasvol G, Poiriez J, et al. (2001) Association of genetic mutations in Plasmodium vivax dhfr with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother 45: 3122–3127.
29. Imwong M, Pukrittayakamee S, Renia L, Leutonneur F, Charlier JP, et al. (2003) Novel point mutations in the dhfr-thsd gene of Plasmodium vivax revealed by sequencing-selected mutations, drug pressure. Antimicrob Agents Chemother 47: 1514–1520.
30. Tjitra E, Baker J, Suprianto S, Cheng Q, Anstey NM (2002) Therapeutic efficacies of artesunate-sulfadoxine-pyrimethamine and chloroquine-sulfadoxine-
alcohol.
pyrimethamine in vivax malaria pilot studies: relationship to Plasmodium vivax dhfr mutations. Antimicrob Agents Chemother 26: 3947–3953.

31. Hastings MD, Porter KM, Maguire JD, Susanti I, Kania W, et al. (2004) Dihydrofolate reductase mutations in Plasmodium vivax from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. J Infect Dis 189: 744–750.

32. Leartsakolpachich U, Inwong M, Pukritthayakam S, White NJ, Supomou G, et al. (2002) Molecular characterization of dihydrofolate reductase in relation to antifolate resistance in Plasmodium vivax. Mol Biochem Parasitol 119: 63–73.

33. Hastings MD, Sibley CH (2002) Pyrimethamine and WR99210 exert opposing selection on dihydrofolate reductase from Plasmodium vivax. Proc Natl Acad Sci USA 99: 13157–13161.

34. Hastings MD, Maguire JD, Bangs MJ, Zimmerman PA, Zimmerman PA, et al. (2005) Molecular characterization of dihydrofolate reductase in relation to antifolate resistance in Plasmodium vivax. Mol Biochem Parasitol 149: 53–61.

35. Auliff AM, Adams JH, O’Neil MT, Cheng Q (2010) Defining the role of mutants P. vivax dihydrofolate reductase-thymidylate synthase gene using an episomal Plasmodium falciparum transfection system. Antimicrob Agents Chemother 54: 3927–3932.

36. O’Neil MT, Korinczky ML, Gresty KJ, Auliff A, Cheng Q (2007) A novel Plasmodium falciparum expression system for assessing antifolate resistance caused by mutants P. vivax dihydrofolate reductase-thymidylate synthase. J Infect Dis 196: 467–474.

37. Bhattacharjee S, van Ooij C, Balu B, Adams JH, Haldar K (2008) Maurer’s clefts of Plasmodium falciparum are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. Blood 111: 2418–2426.

38. van Ooij C, Tames PA, Bhattacharjee S, Miller NL, Harrison T, et al. (2008) The malaria secretome: from algorithms to essential function in blood stage infection. PLoS Pathog 4: e1000084.

39. Tames PA, Bhattacharjee S, van Ooij C, Miller NL, Lijanas M, et al. (2008) An erythrocyte vesicle protein exported by the malaria parasite promotes tubovesicular lipid import from the host cell surface. PLoS Pathog 4: e1000118.

40. Balu B, Adams JH (2007) Advancements in transfection technologies for Plasmodium. International Journal for Parasitology 37: 1–10.

41. Balu B, Chauhan C, Mahler SP, Shoue DA, Kissinger JC, et al. (2009) piggyBac is an effective tool for functional analysis of the Plasmodium falciparum genome. BMC Microbiol 9: 83.

42. Balu B, Shoue DA, Fraser MJI, Adams JH (2005) High-efficiency transformation of Plasmodium falciparum by the lepidopteran transposable element piggyBac. Proc Natl Acad Sci U S A 102: 16391–16396.

43. Pfahler JM, Galinski MR, Barmwell JW, Lanzer M (2000) Transient transfection of Plasmodium falciparum blood stage parasites. Molecular and Biochemical Parasitology 149: 99–101.

44. Sa JM, Yamamoto MM, Fernandez-Becerra C, de Azevedo MF, Papakrivos J, et al. (2006) Expression and function of pfmdr1, a Plasmodium vivax ortholog of pfmdr1, in Plasmodium falciparum and Desmodium discolor. Mol Biochem Parasitol 150: 219–228.

45. Somvong Y, Uthaiwipul C, Prommana P, Sriratanakool S, Yuthavong Y, et al. (2011) Transgenic Plasmodium parasites stably expressing Plasmodium vivax dihydrofolate reductase-thymidylate synthase as in vitro and in vivo models for antifolate screening. Malar J 10: 291.

46. Shereet TW, Kozar MP, O’Neil MT, Smith PL, Schielser GA, et al. (2005) In vitro metabolism of phenoxypyropylbiguanide analogues in human liver microsomes to potent antimalarial dihydrofolates. J Med Chem 48: 2015–2013.

47. Canfield CJ (1986) Geneva: World Health Organization Monograph Series; Bruce-Chwatt LJ, editor. 99–100 p.

48. Canfield CJ, Milhous WK, Ager AL, Rossan RN, Sweeney TR, et al. (1993) PS-15: a potent, orally active antimalarial from a new class of folic acid antagonists. Am J Trop Med Hyg 49: 121–126.

49. Edstein MD, Kotecka BM, Ager AL, Smith KS, DiTusa CA, et al. (2007) Antimalarial pharmacodynamics and pharmacokinetics of a third-generation antifolate PJP2056 in cynomolgus monkeys using an in vivo in vitro model. J Antimicrob Chemother 60: 718–818.

50. Zhang K, Rathod PK (2002) Divergent regulation of dihydrofolate reductase between malaria parasite and human host. Science 296: 545–547.

51. Nirmalan N, Wang P, Sims PF, Hyde JE (2002) Transcriptional analysis of genes encoding enzymes of the folate pathway in the human malaria parasite Plasmodium falciparum. Mol Microbiol 46: 179–190.

52. Nirmalan N, Sims PF, Hyde JE (2004) Translational up-regulation of antifolate drug targets in the human malaria parasite Plasmodium falciparum upon challenge with inhibitors. Mol Biochem Parasitol 136: 63–70.

53. Goldberg DE, Jane C, Cowman AF, Waters AP (2010) Has the time come for us to complement our malaria parasites? Trends Parasitol.

54. Teager W, Jensen JB (1976) Human malaria parasites in continuous culture. J Parasitol 62: 484–486.

55. Lambros C, Vanderberg JP (1979) Synchronization of Plasmodium falciparum erythrocyte stages in culture. J Parasitol 65: 418–420.

56. Auliff A, Wilson DW, Russell B, Gao Q, Chen N, et al. (2006) Amino acid mutations in Plasmodium falciparum DHFR and DHPS from several geographical regions and susceptibility to antifolate drugs. Am J Trop Med Hyg 75: 617–621.

57. Mahler SP, Balu B, Shoue DA, Weissenbach ME, Adams JH (2008) A highly sensitive PCR-based method for the detection of Plasmodium falciparum clones in microtiter plates. Malar J 7: 222.

58. Liu YG, and Huang N (1998) Efficient Amplification of Insert End Sequences from Bacterial Artificial Chromosome Clones by Thermal Asymmetric Interlaced PCR. Plant Molecular Biology Reporter 16: 175–181.

59. Chavchich M, Greven L, Peters J, Chen X, Cheng Q, et al. (2010) Role of fhsn1 amplification and expression in induction of resistance to artemisinin derivatives in Plasmodium falciparum. Antimicrob Agents Chemother 54: 2455–2464.

60. Arnold C, Hodgson JJ (1991) Vectorial PCR: a novel approach to genomic walking. PCR Methods Appl 1: 39–42.

61. Duraisingh MT, Curtis J, Warhurst DC (1998) Plasmodium falciparum: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. Exp Parasitol 85: 1–8.

62. Edstein MD, Bahr S, Kotecka B, Shank D, Rieckmann KH (1997) In vitro activities of the biguanide PS-15 and its metabolite, WR99210, against cycloguanil-resistant Plasmodium falciparum isolates from Thailand. Antimicrob Agents Chemother 41: 2300–2301.