Role of Known Molecular Markers of Resistance in the Antimalarial Potency of Piperaquine and Dihydroartemisinin In Vitro

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Using a range of laboratory-adapted and genetically modified Plasmodium falciparum parasite isolates, we investigated the interaction between dihydroartemisinin and piperaquine (PIP), the individual components of an artemisinin combination therapy currently under development, in addition to the role of known drug resistance genes in parasite susceptibility in vitro. All but one parasite line investigated displayed an interaction of dihydroartemisinin and PIP that was antagonistic, although the degree of antagonism was isolate dependent. In terms of resistance markers, the pfcrt haplotypes CVIET and SVMNT were positively associated with reduced sensitivity to PIP, with parasites carrying the South American CQR (SVMNT) allele being generally less sensitive than CVIET parasites. Parasites carrying the CQS (CVMNK) allele displayed a further increase in PIP sensitivity compared with CVIET and SVMNT parasites. Our data indicate that PIP sensitivity was not affected by pfmdr1 sequence status, despite positive correlations between the structurally related compound amodiaquine and pfmdr1 mutations in other studies. In contrast, neither the pfcr nor pfmdr1 sequence status had any significant impact on susceptibility to dihydroartemisinin.

Malaria remains a major disease causing significant health problems in many parts of the world, especially Africa. Disappointingly, it is argued that more people are infected with malaria now than was the case 20 years ago, with approximately 200 million infected persons and 2 million deaths each year (31). This remains the case despite recent reports of a falling incidence of Plasmodium falciparum infection following deployment of artemisinin combination therapies (ACTs) and insecticide-treated bed nets in specific geographical settings. There are a number of factors that contribute to these figures, but the most important is parasite resistance to existing and affordable drugs and an absence of alternatives.

Historically, communities have adopted monotherapy strategies for the treatment of malaria. History has proven that this is a poor strategy, and if we look at other infectious diseases, such as tuberculosis and human immunodeficiency virus infection, combination chemotherapy is routine as a means of slowing resistance development. The rationale for combination chemotherapy in malaria is simple: if you have two or more drugs with independent mechanisms of action, then the probability of a parasite emerging that is resistant to both mechanisms at the same time is reduced significantly, provided that parasites resistant to either component drug are rare in the population (35).

The WHO recently championed the use of combination chemotherapy for the treatment of malaria. Furthermore, it is argued that these combinations should include an artemisinin-based drug, such as artesunate, artemether, dihydroartemisinin (DHA), or artemisinin itself (36). This recommendation is based on the fact that these drugs appear to kill parasites more efficiently than any other class of antimalarial drug, thereby rapidly reducing parasite biomass and fever in patients, together with the ability to kill multidrug-resistant parasites.

There are a number of ACT therapies currently available for use. The first commercially registered fixed-dose combination was Coartem. This is a combination of the quinoline-like drug lumefantrine with artemether. Clinical trials with this drug demonstrated good efficacy in many settings where malaria is endemic (9a, 9b, 23a). Two ACT combinations currently under development are pyronaridine plus artesunate (Pyramax) and dihydroartemisinin plus piperaquine (DHA-PIP; marketed as Artekin and Eurartesim), both of which recently completed phase III trials in humans. DHA-PIP is currently awaiting FDA approval, but Artekin is currently used in many malaria-infected countries and forms part of the Vietnamese National Malaria Control Programme.

DHA is highly effective against Plasmodium falciparum both in vivo and in vitro. However, when used alone this drug is associated with a very high rate of parasite recrudescence, and in order to increase the chance of clinical cure, the drug needs to be taken for at least 7 days to achieve a maximum cure rate (20a, 20b). The low clinical cure rate is a consequence of the very short half-life of DHA coupled with poor compliance with the 7-day dosage requirement. PIP is a bis-quinoline compound that belongs to the 4-aminouquinoline class of antimalarials. In contrast to DHA, this drug has a very long half-life of approximately 30 days (12; S. Muangnoicharoen et al., unpublished observations). The drug was widely used in China, beginning from 1980, as monotherapy to replace chloroquine (CQ) for the treatment and prophylaxis of malaria and was shown to be...
TABLE 1. Functional PfCRT and PfMDR1 haplotypes and in vitro drug susceptibilities of P. falciparum lines to PIP, DHA, and CQ

<table>
<thead>
<tr>
<th>Line</th>
<th>Parental line</th>
<th>Functional PfCRT haplotype (amino acid at position)</th>
<th>PfMDR1 haplotype (amino acid at position)</th>
<th>In vitro susceptibility (IC50 [nM])</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>COR</td>
<td>C I E T S E S I I Y Y S N D</td>
<td></td>
<td>293.3 ± 28.7; 13.4 ± 2.4; 1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>TM6</td>
<td>COR</td>
<td>C I E T S E S I I Y - - - -</td>
<td></td>
<td>139.8 ± 10.7; 15.8 ± 4; 1.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>7G8</td>
<td>COR</td>
<td>S M N T S Q D L R N F C D Y</td>
<td></td>
<td>160.3 ± 12.7; 11.2 ± 1.7; 1.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>3D7</td>
<td>COR</td>
<td>C M N K A Q N I R N F S D D</td>
<td></td>
<td>16.7 ± 1.5; 3.4 ± 1.3; 0.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>C2G6 CQ03</td>
<td>GCO3</td>
<td>C M N K A Q N I R N F S D D</td>
<td></td>
<td>22.9 ± 2; 3.9 ± 0.4; 0.8 ± 0.1</td>
<td>29</td>
</tr>
<tr>
<td>C3G14</td>
<td>GCO3</td>
<td>C I E T S E S T I N F S D D</td>
<td></td>
<td>143.8 ± 11.4; 11.5 ± 1.8; 1.1 ± 0.4</td>
<td>29</td>
</tr>
<tr>
<td>C6G7</td>
<td>GCO3</td>
<td>C M N T S Q D L R N F S D D</td>
<td></td>
<td>126.9 ± 17.2; 6.6 ± 1.58; 0.3 ± 0.1</td>
<td>29</td>
</tr>
<tr>
<td>D1G10 D10</td>
<td>D10</td>
<td>C M N K A Q N I R N F S N D</td>
<td></td>
<td>45.2 ± 3.7; 8.1 ± 1.3; 0.7 ± 0.3</td>
<td>26</td>
</tr>
<tr>
<td>D1G7G8</td>
<td>D10</td>
<td>C M N K A Q N I R N Y C D Y</td>
<td></td>
<td>52.7 ± 4.8; 10.4 ± 1.1; 0.7 ± 0.3</td>
<td>26</td>
</tr>
<tr>
<td>7G8G7D10</td>
<td>7G8</td>
<td>C M N T S Q D L R N F S C D Y</td>
<td></td>
<td>389.5 ± 12.4; 9.1 ± 1.3; 0.6 ± 0.2</td>
<td>26</td>
</tr>
<tr>
<td>7G8D10</td>
<td>7G8</td>
<td>C M N T S Q D L R N F S N D</td>
<td></td>
<td>204.1 ± 1.2; 12 ± 3; 1.3 ± 0.2</td>
<td>26</td>
</tr>
</tbody>
</table>

* Amino acids in bold represent those that differ from the canonical CQS allele. Assays were performed at 1% hemotocrit and 1% parasitemia. IC50s are presented as means ± standard errors of the means (n = 3).

very safe and effective against *P. falciparum* malaria both in vitro and in vivo (4). Unfortunately, resistant parasite strains emerged relatively quickly, although the mechanism of resistance remains largely unresolved (6).

Although individually both drugs have their pros and cons, in combination this ACT showed excellent cure rates in many clinical trials conducted in Southeast Asia and Africa (1, 14, 18, 19, 22, 30, 37, 38). Importantly, these trials suggest that the combination is very safe, with minimal adverse events. However, there remain a number of issues that need to be addressed with respect to the combination of PIP and DHA. The drug has entered clinical trials with limited recognized preclinical evaluation, a decision based on the facts that there was considerable human experience with the artemisinin derivative DHA and that PIP was used successfully in China as a monotherapy for many years until unacceptable resistance emerged.

As a consequence, there is insufficient published preclinical pharmacology on this combination therapy, with few data on the mechanisms of action and interactions between the two components in terms of antimalarial activity or the influence of known resistance mechanisms on parasite susceptibility. Some of these deficiencies are addressed in this study.

MATERIALS AND METHODS

**P. falciparum strains and culture.** The CQ-resistant (CQR) K1, TM6, and 7G8 strains and the CQ-sensitive (CQS) 3D7 parental strain of *P. falciparum* were used in this study. The *pfcr t* and *pfmdr1* recombinant parasite lines were kindly provided by David Fidock (Columbia University, New York, NY) and Alan Cowman (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), respectively (26, 29). The *pfcrt* and *pfmdr1* haplotypes for the *P. falciparum* lines used in these studies are shown in Table 1. Cultures contained a 2% suspension of O+ erythrocytes in RPMI 1640 (R8758) medium supplemented with 10% pooled human AB serum, 25 mM HEPES (pH 7.4), and 20 mM gentamicin sulfate (33).

In vitro drug susceptibility assays and isologoblast analysis of PIP and DHA interactions. The sensitivity of *P. falciparum*-infected erythrocytes to various drugs was determined using the [3H]hypoxanthine incorporation method (7) with an inoculum size of 1% parasitemia (ring stage) and a 1% hemotocrit. Fifty percent inhibitory concentrations (IC50s) were calculated by using the four-parameter logistic method (Grafit program; Erithacus Software, United Kingdom). To determine whether the antimalarial activity of two drugs was additive, antagonistic, or synergistic, parasite growth was tested by titration of the two drugs at fixed ratios proportional to their IC50s. The fractional inhibitory concentrations (FICs) of the resulting IC50s were plotted as isologograms, where an FIC of 0.8 to 1.2 was classified as additive, one of 1.2 to 2 was considered mildly antagonistic, one of >2 was considered antagonistic, and one of <0.8 was classified as synergistic (2). Statistical significance was determined by an unpaired two-tailed t test.

DHA and PIP were both obtained from Guangzhou Holleykin Pharmaceutical Company Limited, The Republic of China (batch 20040201), and checked for purity by Paul O’Neill (Department of Chemistry, University of Liverpool). Drug stocks of DHA were prepared by dissolving a known amount of solid material in 100% dimethyl sulfoxide, and drug stocks of PIP were prepared by dissolving a known amount of solid material in 90% methanol and 10% 1 M hydrochloric acid to make a final concentration of 10 mM. All drug stocks were serially diluted with culture medium to the desired concentration.

RESULTS

In vitro drug susceptibility of parental lines of *P. falciparum*. In vitro drug susceptibilities to PIP and DHA were determined for a range of isolates, including parental and genetically modified lines of *P. falciparum*. The CQS strain 3D7 exhibited a PIP IC50 of 3.4 ± 1.3 nM, with the CQR lines all exhibiting significant decreases in drug susceptibility (Table 1) (K1 IC50 13.4 ± 2.4 nM [P = 0.02]; TM6 IC50 15.8 ± 4.0 nM [P = 0.01]; and 7G8 IC50 11.2 ± 1.7 nM [P = 0.05]). There was no significant difference in the in vitro drug susceptibility for DHA for all parental lines tested, although the CQR lines showed a trend toward a decreased susceptibility to DHA (Table 1).

Effects of *pfcr t* and *pfmdr1* alleles on PIP and DHA susceptibility. The cross-resistance of PIP and CQ in standard laboratory lines of *P. falciparum* was suggestive of an influence of *pfcr t* alleles on parasite PIP drug susceptibility. Utilizing *pfcr t* recombinant parasite lines, it was possible to test this hypothesis. In vitro drug susceptibility for PIP was significantly decreased in the recombinant C3Dd2 and C6G7G8 lines, carrying the Dd2 and 7G8 pfcr t alleles, respectively, versus the control C2G6CQS line, which carries the canonical CQS pfcr t allele; the IC50s were 6.6 ± 1.58 nM, 11.5 ± 1.8 nM, and 3.9 ± 0.4 nM, respectively, with significance determined by comparison to the C2G6CQS line (P values of 0.05 and 0.08, respectively) (Table 1). As with the parental lines, there were no significant differences in the different parasite line responses to DHA (Table 1).

Genetic modification of the *pfmdr1* gene (parasite lines D10D10, D107G8, 7G8G7, and 7G8D10) had little effect on both PIP and DHA drug susceptibilities, with all lines tested show-
TABLE 2. FICs of DHA and PIP in combination against a range of P. falciparum lines

<table>
<thead>
<tr>
<th>Line</th>
<th>(\Sigma) FIC*</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>1.27</td>
<td>Mildly antagonistic</td>
</tr>
<tr>
<td>TM6</td>
<td>1.25</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>7G8</td>
<td>2.86</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>3D7</td>
<td>2.40</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>C2G3C3</td>
<td>1.59</td>
<td>Mildly antagonistic</td>
</tr>
<tr>
<td>C2D2C2</td>
<td>1.70</td>
<td>Mildly antagonistic</td>
</tr>
<tr>
<td>C6G8</td>
<td>2.51</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>D10D10</td>
<td>1.48</td>
<td>Mildly antagonistic</td>
</tr>
<tr>
<td>D10G10</td>
<td>1.34</td>
<td>Mildly antagonistic</td>
</tr>
<tr>
<td>7G8G10</td>
<td>0.80</td>
<td>Mildly synergistic</td>
</tr>
<tr>
<td>7G8D10</td>
<td>1.34</td>
<td>Mildly antagonistic</td>
</tr>
</tbody>
</table>

* Sum of the FICs for each drug tested in combination.

ing similar IC\(_{50}\)s and no clear trend toward a particular allele (Table 1).

In vitro interactions of PIP and DHA. The interaction between PIP and DHA was determined by isobologram analysis with a range of parental and genetically modified lines of P. falciparum. From this study, it was observed that the interaction between the two drugs was mainly antagonistic, with some lines (7G8, 3D7, C2G3C3, and C6G8) showing a more pronounced antagonism (Table 2). In the case of the C3D2D, D10D10, D10G10, and 7G8G10 lines, the antagonism, although still apparent, was less marked (Table 2). Line 7G8G10 displayed an isobologram that was indicative of a mildly synergistic interaction.

DISCUSSION

The continued spread of parasite drug resistance to monotherapies has forced a shift toward the use of ACTs. However, resistance to at least one component of many of the ACTs currently in clinical use has been documented, and it is feared that in widespread use ACTs will gradually lose their clinical efficacy. Artekin is one such ACT, comprised of the artemisinin derivative DHA and the bis-quinoline PIP. The use of the PIP and DHA combination as a treatment for uncomplicated malaria is on the rise, but unfortunately clinical resistance to PIP has been reported in all areas of China where it has been used extensively as a monotherapy (4, 6). This study set out to investigate the molecular determinants of parasite susceptibility to DHA and PIP and the pharmacodynamic interaction between the two components of this ACT.

Utilizing pfcr (29) and pfmrd1 (26) genetically modified parasites, this study provides the first evidence that resistance to PIP is conferred by mutations in pfcr that are most commonly associated with CQR. The pfcr modified lines were generated by replacing the entire pfcr allele in a CQS parasite with that from a CQR parasite (29). The resulting parasite exhibited all of the characteristics of a CQR parasite and confirmed a major role for pfcr in CQR. Phenotyping of this parasite line (C3D2D) showed an approximately threefold decrease in susceptibility to PIP compared to that of the control line, C2G3C3 (11.5 nM versus 3.9 nM, respectively), with a similar result being obtained using standard laboratory CQR and CQS lines (Table 1). Interestingly, there was a trend, albeit a slight one, toward a reduced PIP susceptibility in parasites harboring the CVIET PfCRT haplotype. Although no definitive causal relationship was determined, earlier studies have shown a cross-resistance of PIP and CQ (10, 34). In our limited panel of parasite lines, genetic modification of the pfmrd1 locus had little effect on parasite susceptibility to PIP (Table 1), despite observations from a number of studies showing a positive correlation between the YYY PfMDR1 haplotype and amodiaquine susceptibility (amodiaquine is structurally related to PIP) (8, 11). However, the contributions of pfmrd1 mutations to PIP susceptibility cannot be ruled out and warrant further investigation with a larger set of P. falciparum lines, including the pfmrd1 genetically modified lines of Sidhu et al. (27, 28).

The use of pfcr modified parasites has provided a clear role for this gene in determining parasite susceptibility to PIP. This observation is novel yet not surprising, since it has been well documented that pfcr can influence parasite susceptibility to a range of structurally unrelated antimalarials, including CQ, amodiaquine, halofantrine, and mefloquine (15, 20, 29).

At the clinical level, PIP cross-resistance with CQ is a concern. When PIP was first deployed as a monotherapy for the treatment of malaria in China, reports of high-level clinical failure due to parasite resistance appeared within less than a decade. This was against a backdrop of existing CQR, although there was no link made between the two phenotypes at that time. Despite this knowledge, it has been argued that since the drug has never been used outside of China as a monotherapy and would now be deployed only as a combination, resistance is less likely to evolve. The data presented in this study suggest that this is incorrect, as it is based on the assumption that the resistance mechanism operating in China does not exist elsewhere in the world. However, since we have provided clear evidence for reduced PIP susceptibility being conferred by mutations in pfcr and based on the simple fact that PFCR-dependent CQR is present at high levels in almost all of the world where malaria is endemic, it can be assumed that this will provide a platform for the rapid development of resistance to PIP when the drug is eventually in widespread clinical use (see reference 9 and references contained within). Despite the overwhelming evidence supporting the potential for the rapid dissemination of PIP resistance, the relevance of this to clinical treatment outcome is not known based on the modest changes in PIP susceptibility observed in our panel of parasite lines.

DHA is a very potent antimalarial, with IC\(_{50}\) usually in the low nanomolar range (0.13 to 23.3 nM) (16, 21). Many studies have assessed the influence of mutations in pfmrd1 and pfcr and the parasite response to the natural product artemisinin, and although we cannot guarantee that DHA kills parasites in exactly the same way as artemisinin, the close structural similarity of the compounds suggests that this is the case. However, in the present study, DHA was equipotent against all lines tested irrespective of pfcr or pfmrd1 sequence status.

At the molecular level, it has been shown in a number of in vitro studies that both mutations in and the expression level of pfmrd1 have a significant impact on artemisinin susceptibility to artemisinin (27–29). However, the only clear association that was found in these studies was for a general trend toward decreased artemisinin susceptibility in parasite lines that harbor the pfmrd1 SND haplotype (28). In fact, the greatest impact on artemisinin susceptibility was produced by
genetically modifying a parasite line to express significantly lower levels of PfMDR1, with the resultant parasite being twice as susceptible to artesiminin (27). Our in vitro data suggest that neither pfmdr1 nor pfcrト mutation influences parasite susceptibility to DHA. This suggests that the clinical efficacy of the DHA component of any ACT should be maintained despite the growing prevalence of pfmdr1 and pfcrト mutations in regions where malaria is endemic, although the clinical impact on the ACT may still cause problems for successful therapy.

The interaction between DHA and PIP in vitro was antagonistic in a range of parasite isolates, in agreement with earlier reports. Davis et al. (5) have shown antagonism between DHA and PIP against the 3D7 and K1 laboratory isolates, and similarly, Synder et al. (32) demonstrated antagonism between PIP and the synthetic peroxide OZ277 or the semisynthetic compound artemether (both related to DHA) against the K1 and NF54 laboratory isolates. The antagonistic interaction between quinoline-based drugs such as PIP and peroxide-based drugs such as DHA is well accepted but is not considered to be a problem for their clinical use as combination therapy. This is clear from the very high efficacies of ACTs such as lumefantrine-artemether (Coartem), mefloquine-artesunate, and amodiaquine-artesunate, all of which are in clinical use. The fact that this antagonism is not an issue clinically is important and might reflect the fact that the artemisinin component is very potent yet is eliminated quickly from the body (3, 13, 24, 25).

The mechanism behind this interaction is not clear, but there is strong evidence that drugs such as PIP, like CQ, interact with heme in the food vacuole of the parasite, and similarly, DHA and the artemisinins are able to become activated by ferrous iron and heme (17, 23). This common requirement for heme may be the basis for this antagonism.

In this study, the excellent antimalarial activities of the ACT components PIP and DHA were confirmed in a range of parasite isolates, and the antagonistic interaction between the two drugs was confirmed. Importantly, the potential cross-resistance between PIP and CQ was demonstrated definitively and suggests, at the molecular level, a role for PfCRT in determining parasite susceptibility to PIP. This association with mutations in pfcrト, which are already prevalent worldwide, does raise concerns about the development of resistance to this drug when it becomes deployed extensively for clinical case management. Population exposure to PIP and DHA will increase substantially once the drug combination is formally licensed. It is anticipated that this ACT will prove to be a safe and highly effective drug against malaria. It will be important to ensure that mechanisms are in place to continually monitor the emergence of resistance to this drug, and the link with CQR mutations in pfcrト and clinical failures will be a useful molecular marker to incorporate into these monitoring initiatives.

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