Clinical and \textit{in vitro} resistance of \textit{Plasmodium falciparum} to artesunate-amodiaquine in Cambodia

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Summary

Artesunate-amodiaquine has inadequate efficacy in Cambodian patients with uncomplicated falciparum malaria. Amodiaquine resistance is present in Cambodia and not associated with molecular markers reported in Africa and South America.
Abstract

Background

Artesunate-amodiaquine is a potential therapy for uncomplicated malaria in Cambodia.

Methods

Between September 2016 and January 2017, artesunate-amodiaquine efficacy and safety were evaluated in a prospective, open-label, single-arm observational study at health centers in Mondulkiri, Pursat and Siem Reap Provinces, Cambodia. Adults and children with microscopically-confirmed Plasmodium falciparum malaria received oral artesunate-amodiaquine once daily for three days plus single-dose primaquine, with follow-up on Days 7, 14, 21 and 28. The primary outcome was Day-28 PCR-adjusted adequate clinical and parasitological response (ACPR). An amodiaquine parasite survival assay (AQSA) was developed and applied to whole genome sequencing results to evaluate potential amodiaquine resistance molecular markers.

Results

In 63 patients, Day-28 PCR-adjusted ACPR was 81.0% (95% confidence interval [CI], 68.9–88.7). Day 3 parasite positivity rate was 44.4% (28/63; 95%CI, 31.9–57.5). All 63 isolates had the K13(C580Y) marker for artemisinin resistance; 79.4% (50/63) had Pfpm2 amplification. The AQSA resistance phenotype (≥45% parasite survival) was expressed in 36.5% (23/63) of isolates and was significantly associated with treatment failure ($P = 0.0020$). Pfmdrl mutant haplotypes were N86/184F/D1246 and Pfcrt was CVIET or CVIDT at positions 72–76. Additional Pfcrt mutations were not associated with amodiaquine resistance, but the G353V mutant allele was associated with ACPR compared to Pfmdrl haplotypes harboring F1068L or S784L/R945P mutations ($P = 0.030$ and $P = 0.0004$, respectively).

Conclusions

For uncomplicated falciparum malaria in Cambodia, artesunate-amodiaquine had inadequate efficacy owing to amodiaquine-resistant P. falciparum. Amodiaquine resistance was not associated with previously identified molecular markers.

Keywords: artesunate-amodiaquine, artemisinin, Plasmodium falciparum, Cambodia, drug resistance.
Introduction

Artemisinin-based combination therapy (ACT) includes a rapid-acting artemisinin with a longer-acting partner drug. ACTs support effective malaria treatment globally, contributing to recent declines in mortality [1]. In 2006, artemisinin-resistant Plasmodium falciparum was confirmed in Cambodia’s western provinces [2], subsequently verified in multiple studies [3]. Artemisinin resistance delays parasite killing, but resistance to the partner drug is required before treatment failure rates increase [4, 5]. Unfortunately, P. falciparum resistant to artemisinins and partner drugs (piperaquine, mefloquine) circulate in Cambodia and the Greater Mekong sub-region, undermining clinical efficacy and limiting treatment options [1, 6-8].

Artesunate-amodiaquine was not deployed systematically in Cambodia and requires evaluation as a potential replacement for failing ACTs. In Africa, artesunate-amodiaquine is used extensively with 98.5% clinical efficacy [1]. P. falciparum with multi-drug resistance 1 (Pfmdr1) alleles N86Y/Y184/D1246Y (YYY haplotype) is associated with amodiaquine treatment failures in Africa [9], but has not been detected in Cambodia [10]. The most prevalent chloroquine resistance transporter gene (Pfcrt) haplotype in Cambodia is CVIET at positions 72-76 (wild-type CVMNT) [10], which is also prevalent in Africa [11, 12], and insufficient to confer amodiaquine resistance in vivo [13, 14]. In Vietnam, two artesunate-amodiaquine clinical trials showed encouraging results with 98% efficacy [15, 16]. However, there are no recent data from South East Asia on artesunate-amodiaquine efficacy.

This study investigated artesunate-amodiaquine clinical efficacy for uncomplicated falciparum malaria in Cambodia. In the event of clinical failures, molecular markers associated with amodiaquine resistance were to be investigated.

Methods

Study design

This prospective, single-arm, open-label therapeutic efficacy trial of artesunate-amodiaquine plus single-dose primaquine was conducted between September 2016 and January 2017 at three health centers in Cambodia: Koh Gnek (Koh Gnek district, Mondulkiri province), Promoy (Veal Veng district, Pursat province), and Khvav (Chi Kraeng district, Siem Reap province) (Supplementary material Figure S1).

The study confirmed to Good Clinical Practice and the Declaration of Helsinki (2000). The protocol followed the standard World Health Organization protocol for the surveillance of antimalarial treatment efficacy [17], and was approved by National Cambodian Ethical Board and the World Health Organization Regional Office, Western Pacific Region. Owing to an administrative error, this study was registered retrospectively at https://www.anzctr.org.au (identifier...
ACTRN12619001628134). All patients or their guardians provided written informed consent. Additionally, assent was obtained from children aged 12 years and above.

**Patients**

Eligible patients were aged 5–60 years with microscopically confirmed *P. falciparum* mono-infection (1000–250,000 µL⁻¹ blood), fever or history of fever during the past 24 h, who could swallow oral medication. Unmarried girls and women aged 12–18 years were excluded because a pregnancy test would be culturally unacceptable; pregnant and lactating women were also excluded. All other women of child-bearing potential had a pregnancy test. Exclusion criteria were severe falciparum malaria, severe malnutrition, febrile conditions other than malaria or other underlying chronic illness, medication that might interfere with antimalarial pharmacokinetics, or a history of hypersensitivity to artemisinin or amodiaquine.

**Treatment**

Artesunate-amodiaquine (ASAQ Winthrop®, Sanofi, Paris, France) was administered under supervision once-daily for three days. Doses were determined by bodyweight to achieve 4 mg/kg/day (range 2–10 mg/kg) artesunate and 10 mg/kg/day (range 7.5–15 mg/kg) amodiaquine. Primaquine was given as a single 15 mg dose (0.25 mg base/kg). All patients were treated as in-patients with out-patient follow-up visits on Days 7, 14, 21, and 28. Any recurrence during follow up was treated with artesunate-mefloquine.

**Procedures**

At enrollment, a clinical examination was performed and a full medical history taken. Adverse events were recorded at all study visits. Parasitemia and *Plasmodium* species identification was assessed using Giemsa stained thick and thin blood films obtained at screening, every day following the first treatment dose until samples were parasite negative, at each weekly follow-up visit, and if clinically indicated. Parasite counts were recorded as the average from two microscopists using standard methods [17]. Treatment failures were verified as recrudescence using polymerase chain reaction (PCR) genotyping by comparing *P. falciparum* genes msp1, msp2 and glurp in pre-treatment blood samples versus those obtained at recurrence [18].

**Molecular surveillance**

Using samples collected on day 0, the *Kelch13 (K13)* gene was sequenced to identify mutations associated with artemisinin resistance [19], and gene copy numbers for *P. falciparum plasmepsin 2/3 (Pfpm2)* and *Pfmdr1* were determined, as per published methods [20]. The threshold for gene amplification was defined as >1.5 copies.
**Amodiaquine susceptibility in vitro**

Pre-treatment blood samples were collected into acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) and processed within 48 h at Institut Pasteur, Cambodia. Clinical isolates were culture adapted using standard methods [4]. *P. falciparum* reference strains 3D7 (amodiaquine susceptible, from MR4) and 7G8 (amodiaquine resistant, from the European Malaria Reagent Repository) were similarly maintained and used as controls. Molecular markers obtained from day 0 samples were confirmed as identical to those obtained from the corresponding culture-adapted parasites via whole genome sequencing except for one isolate that had *Pfpm2* amplification at day 0, which reverted to a single gene copy under culture.

*In vitro* susceptibility to mono-desethyl-amodiaquine (from the WorldWide Antimalarial Resistance Network) was assessed using the \[^{3}H\]-hypoxanthine assay, according to published methods [4]. Inhibitory concentration values (IC\(_{50}\)) were determined using ICEstimator software (http://www.antimalarial-icestimator.net).

The amodiaquine survival assay (AQSA) was based on a similar assay for piperaquine [21]. Tightly synchronized ring-stage parasites (0–3 h post-invasion) were exposed to 200 nM mono-desethyl-amodiaquine for 48 h and maintained for a further 24 h in drug-free medium. Live parasites were then enumerated microscopically from Giemsa-stained thin blood films on examination of ≥10,000 erythrocytes. Parasite survival following exposure to mono-desethyl-amodiaquine was determined as a percentage relative to untreated controls.

**Amodiaquine resistance and association with molecular markers**

Investigation of potential molecular markers associated with amodiaquine resistance used an expanded data set including culture-adapted *P. falciparum* isolates from this study plus 34 culture-adapted clinical isolates collected from sentinel sites between February 2017 and February 2018 (n=10 Kampong Speu, n=13 Mondulkiri, n=3 Pursat, n=8 Ratanakiri).

*Pfcrt* and *Pfmdr1* were sequenced using whole genome sequencing with Illumina paired-reads sequencing, according to published protocols [19, 20]. After processing, data were integrated into the Whole-genome Data Manager (version 2.0) [19, 20]. Single nucleotide polymorphisms (SNPs) were investigated using Phen2gen software [20].
Outcomes

The primary efficacy outcome was Day-28 adequate clinical and parasitological response (ACPR) adjusted for reinfection using PCR-genotyping. Day-3 parasite positivity rate was the secondary efficacy outcome. Safety outcomes were the frequency of adverse events, serious and severe adverse events.

Statistical analysis

Data were analyzed with Excel StatX and Graphpad Prism (version 8.3.0). ACPR was evaluated using Kaplan–Meier survival curves and associated 95% confidence intervals (95%CI), and compared using the log-rank test (Mantel–Cox). IC\textsubscript{50} and AQSA values versus clinical outcome were compared using the Mann–Whitney test. Resistance thresholds for IC\textsubscript{50} and AQSA parasite survival were determined with receiver operating characteristic (ROC) analysis. Kruskal–Wallis tests were used to identify significant differences in Pfcrts–Pfmdr1 haplotype AQSA parasite survival results. Significant P values were <0.05.

Results

Patients

Most patients were adult males (87.3% 55/63) (Table 1). There were no withdrawals or patients lost to follow up; all 63 patients were included in the analysis. Thirty-one patients were from Mondulkiri, 29 from Pursat and 3 from Siem Reap.

Therapeutic efficacy and molecular surveillance

Day-28 ACPR was 81.0% (51/63). All recurrences were late clinical failures (days 21–28) and PCR-confirmed as recrudescence; nine in adults (18–52 years), three in children (8–15 years). The Kaplan–Meier Day-28 ACPR estimate was 81.0% (95%CI, 68.9–88.7) (Figure 1); 77.4% (95%CI, 58.4–88.5) for Mondulkiri, 86.2% (95%CI, 67.3–94.6) for Pursat, and 66.7% (95%CI, 5.4–94.5) for Siem Reap. No severe or serious adverse events were reported during the study.

Day-3 parasite positivity rate was 44.4% (28/63; 95%CI, 31.9–57.5); 22.6% (7/31; 95%CI, 9.6–41.1) for Mondulkiri, 69.0% (20/29; 95%CI, 49.2–84.7) for Pursat, and 33.3 (1/3, 95%CI, 0.8–90.6) for Siem Reap. All 63 isolates had the K13(C580Y) marker for artemisinin resistance. None had increased Pfmdr1 copy number, but 85.7% (54/63) had Pfpm2 amplification.

In vitro amodiaquine resistance

In the \[^3\text{H}\]-hypoxanthine assay, the mono-desethyl-amodiaquine median IC\textsubscript{50} for the 63 clinical isolates was 174.5 nM (interquartile range [IQR] 90.7–213.1). Isolates from patients with recrudescence (n=12) had a median IC\textsubscript{50} of 193.8 nM (IQR 156.6–240.3) versus 165.0 nM (IQR 88.3–212.0) for patients with Day-28 ACPR (n=51) (P = 0.084, Mann–Whitney) (Figure 2A). ROC analysis indicated an IC\textsubscript{50} threshold value most strongly correlated with Day-28 ACPR of <181 nM.
(Supplementary material Figure S2); sensitivity was 61% (95%CI, 47–73) at a specificity of 75% (95%CI, 47–91). Area under the curve (AUC) was 0.66 (95%CI, 0.49–0.83; P = 0.083). Thus, IC₅₀ had inadequate discriminatory value for predicting clinical outcome.

**Amodiaquine survival assay (AQSA)**

In the 63 clinical isolates, AQSA median parasite survival was 23.5% (IQR 1.6–65.5). Quality control values were 72.8% parasite survival for the amodiaquine-resistant 7G8 strain and 0% for the susceptible 3D7 strain. There was a strong positive correlation between IC₅₀ and AQSA parasite survival (Pearson r value 0.75 [95%CI, 0.62–0.84] P < 0.0001) (Figure 3A). Recrudescent isolates had a significantly higher median survival (64.0% [IQR 29.1–77.0]) versus those from patients with Day-28 ACPR (13.3% [IQR 0.9–48.1]) (P = 0.0054, Mann–Whitney) (Figure 3B). ROC analysis indicated an AQSA threshold predictive of Day-28 ACPR of <45% survival, with 75% sensitivity (95%CI, 47–91) and 73% specificity (95%CI, 59–83). AUC was 0.75 (95%CI, 0.60–0.91; P = 0.0063) (Supplementary material Figure S3). ACPR occurred with 92.5% (37/40) of isolates with <45% AQSA parasite survival but decreased significantly to 60.9% (14/23) for those with ≥45% survival (P = 0.0020, log-rank Mantel–Cox) (Figure 3C). Therefore, ≥45% parasite survival in the AQSA was a clinically relevant resistance phenotype.

**Molecular signature associated with amodiaquine resistance**

In the expanded dataset of 97 clinical isolates, median AQSA parasite survival was 8.0% (IQR 1.0–50.2), and 28.9% (28/97) had ≥45% parasite survival. Ninety-six isolates were K13(C580Y) and one was K13(Y493H). One isolate had Pfmdr1 amplification, whereas 78.4% (76/97) had Pfpm2 amplification. There was no significant difference in the median AQSA value of strains with single-copy Pfpm2 (6.3% [IQR 1.1–56.4]) versus those with multiple copies (12.3% [IQR 0.91–47.6]) (P = 0.74, Mann–Whitney).

The frequency of SNPs for the key *P. falciparum* resistance genes Pfcr and Pfmdr1 are shown in Supplementary material Figure S4. For isolates with complete Pfcr haplotypes (N = 94), 12 different haplotypes were identified, of which 97.9% (92/94) were either Dd2 or on the Dd2 background (Table 2). For Pfmdr1, six different haplotypes were found, with 51.1% (48/94) having Y148F plus at least one other mutation (Table 2).

AQSA results for isolates with complete Pfcr–Pfmdr1 sequences (N = 92) indicated significant differences in survival between four haplotypes: Dd2–Y184F/S784L/R945P versus Dd2 F145I–Y184F and versus Dd2 G353V–Y184F (P = 0.0075 and P = 0.0003, respectively; Kruskal–Wallis); and between Dd2 T93S–Y184F/F1068L versus Dd2 F145I–Y184F and versus Dd2 G353V–Y184F (P = 0.0003 and P < 0.0001, respectively; Kruskal–Wallis) (Figure 4A). When examining clinical outcomes, although the data set was smaller (N=60), there was still a significant difference between Dd2 G353V–Y184F versus Dd2–Y184F/S784L/R945P or Dd2 T93S–Y184F/F1068L (P = 0.0004 and P = 0.0304, respectively; log-rank Mantel–Cox) (Figure 4B).
**Discussion**

Antimalarial drug resistance curtails ACT efficacy for uncomplicated malaria in Cambodia, with the emergence of triple mutants (artemisinin, piperaquine and mefloquine resistant) underlining the need for new therapeutic options [22-24]. High artesunate-amodiaquine efficacy in Africa and Viet Nam, and the absence of known amodiaquine resistance markers in Cambodia, suggested that this ACT would be efficacious. Surprisingly, 19.0% of patients had recrudescence; sufficient to exclude artesunate-amodiaquine as an uncomplicated malaria treatment in Cambodia.

The K13(C580Y) artemisinin resistance marker was ubiquitous in this study and is the predominant K13 mutant in Cambodia [25]. Consistent with this, the Day-3 parasite positivity rate was 44.4%. However, artemisinin resistance increases recrudescence probability only if there is also partner drug resistance [4, 5]. Thus, the high treatment failure rate is most likely explained by amodiaquine resistance.

Previous studies in Africa indicated a mono-desethyl-amodiaquine IC$_{50}$ resistance threshold of >60 nM with amodiaquine monotherapy [26], compared with >180 nM for artesunate-amodiaquine in the current study. This could be because artemisinin resistance is partial, and a higher degree of amodiaquine resistance is necessary to support parasite recrudescence following artesunate-amodiaquine versus amodiaquine monotherapy.

In the current study, IC$_{50}$ lacked sufficient discriminatory power to differentiate between ACPR and recrudescence. This lack of correlation between IC$_{50}$ and clinical outcome was observed previously for artemisinin and piperaquine, promoting the development of parasite survival assays measuring cytocidal activity [4, 21, 27]. This study validated the AQSA, a novel amodiaquine parasite survival assay, which correlated well with clinical outcome, and was sufficiently sensitive and specific to use as a resistance phenotype to investigate potential amodiaquine resistance molecular markers. Consequently, we propose AQSA ≥45% parasite survival as a novel definition for amodiaquine resistance.

In Africa, Pfmdr1 N86 and D1246 selection following artemether-lumefantrine improves clinical outcomes with artesunate-amodiaquine in *P. falciparum* malaria [28]. This suggests that an artemether-lumefantrine-amodiaquine triple therapy could counter-select for resistance, with ongoing initiatives to develop the combination [29]. However, the rationale for artemether-lumefantrine-amodiaquine in Cambodia has not been demonstrated. Our data show that amodiaquine resistance in Cambodia exists in the absence of the African amodiaquine-resistant haplotype Pfmdr1 86Y/Y184/1246Y [10], as all mutant Pfmdr1 haplotypes were N86/184F/D1246. Also, mutations common in South American *P. falciparum* Pfmdr1 (S1034C, N1042D and D1246Y) were absent. Molecular markers for lumefantrine resistance are not validated for South East Asia,
and our data do not support counter selection for amodiaquine susceptibility. Rather, resistance to both amodiaquine and lumefantrine appears possible in Cambodian *P. falciparum*.

In the expanded parasite data set, all except two *Pfcrtn* haplotypes were on the Dd2 background (i.e. C72/V73/741/75E/76T/220S/271E/326S/356T/371I). The amodiaquine-resistant 72–76 SVMNT haplotype reported in South American parasites was absent, consistent with previous data [10].

In Asia, novel *Pfcrtn* mutations have emerged on the Dd2 chloroquine-resistant allelic background, in contrast to Africa where 3D7, GB4 and Cam783 haplotypes predominate [30]. In clinical isolates and gene edited parasites, *Pfcrtn* Dd2 decreases *in vitro* susceptibility to amodiaquine relative to *Pfcrtn* 3D7, so can be considered an amodiaquine-tolerant background [30, 31]. In the current study, 85.9% (79/92) of the Dd2-based haplotypes had additional mutations. The *Pfcrtn* mutations G353V and F145I were significantly associated with amodiaquine sensitivity in the AQSA, and G353V was associated with ACPR. These findings are consistent with data in gene-edited parasites showing that these mutations confer resistance to piperaquine, but sensitize parasites to amodiaquine, chloroquine and quinine [32, 33]. Notably, in gene edited parasites neither G353V nor F145I had any impact on lumefantrine susceptibility and counter selection between lumefantrine and amodiaquine appears unlikely [32]. In previous studies, *Pfcrtn* T93S has been associated with piperaquine resistance and its prevalence has been increasing in Cambodia [33–35]. In the current study, *Pfcrtn* T93S was the most common *Pfcrtn* haplotype, and was associated with both amodiaquine-susceptible and amodiaquine-resistant haplotypes. Thus, elevated AQSA was not associated with an identified polymorphism in *Pfcrtn*. Rather, key mutations in this gene appear to be associated with sensitization to amodiaquine.

*Pfmdr1* showed six haplotypes in this study, and 51.1% (48/94) were Y184F plus another mutation. Of these, S784L/R945P and F1068L were associated with clinical and *in vitro* resistance. *Pfmdr1* S784L has previously been noted from several locations in Cambodia at frequencies between 0.5–29.8%, but was not associated with R945P [10]. *Pfmdr1* F1068L was reported from Pailin at a low frequency (4.2%) [10], versus 20.2% (19/94) in the current study. This study is the first report associating these *Pfmdr1* haplotypes with amodiaquine resistance. However, our data set is insufficient to show causality or to perform multivariate analysis. Thus, extended genome-wide association studies and genome editing are required to validate our findings. The single amodiaquine-susceptible *Pfmdr1* S784L/R945P mutant in the AQSA was the only isolate with *Pfmdr1* amplification. Conclusions cannot be drawn from one isolate, but additional investigations may be valuable.

The origin of amodiaquine resistance in Cambodia is unclear. This drug was not used recently and saw only limited implementation in the 1990s. Piperaquine resistance selection is unlikely to be associated with amodiaquine resistance emergence, as the associated genotype i.e. *Pfpm2* amplification and *Pfcrtn* mutations have either no effect or sensitize parasites to amodiaquine [32]. We could thus hypothesize that amodiaquine resistance emerged in Cambodia in the past.
consecutively to extensive chloroquine use, and since then has been perpetuated by an unidentified mechanism.

While confirming artesunate-amodiaquine resistance in Cambodia, this was a small study conducted in a limited geographical region. Although significant relationships between SNPs in key resistance genes and amodiaquine resistance phenotypes was observed, causality cannot be determined. For example, the associations could result from the close relatedness of parasites in this study. Further investigations are required to confirm the putative amodiaquine resistance markers and assess their relevance to other malaria endemic areas. In the absence of molecular markers, the AQSA provides a novel methodology to assess clinically relevant amodiaquine resistance. However, AQSA specificity and sensitivity were determined according to the limited study size and location and the ≥45% parasite survival AQSA resistance phenotype may require revision with additional data.

This study highlights the need for careful assessment of therapeutic outcomes and molecular markers before introducing a new antimalarial treatment in Cambodia. Resistance to amodiaquine was unexpected and was not associated with any known resistance genotype from other malaria endemic areas. Our findings indicate that clinical resistance was linked to the acquisition of high-level resistance against an amodiaquine-tolerant background. Thus, any amodiaquine-based combination would place partner compounds under a high selective pressure and be inappropriate in Cambodia.
Notes

Contributions

RL, PR, FA & BW contributed to the concept and design of the study. MMK, CM, NK, SK, SKe, CK, NKl, RE, SC & BI were involved in data acquisition and MMK, RL, CM, NK, DMB, MDB, PR, FA & BW in data analysis and interpretation. All authors critically reviewed the manuscript, approved the final version and take full responsibility for the publication.

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Disclaimer

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Declaration of interests

MDB, DMB and PR are a staff member of the World Health Organization. The authors alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the World Health Organization. All other authors have no conflicts of interest to report.
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Figure legends

Figure 1. Kaplan–Meier estimates of ACPR with artesunate-amodiaquine for uncomplicated malaria.
There were no re-infections on PCR-adjustment and no patients were censored.

Figure 2. Relationship between mono-desethyl-amodiaquine IC50 determined in the [3H]-hypoxanthine uptake inhibition assay and clinical outcome at Day 28 following treatment with artesunate-amodiaquine for 63 P. falciparum clinical isolates.
Open circles represent P. falciparum isolates and black horizontal bars and I bars indicate the median and interquartile range, statistical comparison used the Mann–Whitney test.

Figure 3. Parasite survival in the amodiaquine survival assay (AQSA).
A) Correlation between AQSA parasite survival and IC50 determined in the [3H]-hypoxanthine uptake inhibition assay.
B) Relationship between AQSA parasite survival and clinical outcome at Day 28 following treatment with artesunate-amodiaquine. Open circles represent P. falciparum isolates and black horizontal bars and I bars indicate the median and interquartile range, statistical comparison used the Mann–Whitney test.
C) Kaplan–Meier estimates of ACPR for parasites with the AQSA resistance phenotype (≥45% survival) versus those with the susceptible phenotype (<45% survival), statistical comparison used the log-rank test (Mantel–Cox).

Figure 4. Effect of P. falciparum Pfcrt–Pfmdr1 haplotype on resistance phenotype.
A) Parasite survival in the amodiaquine survival assay (AQSA) for Cambodian clinical isolates (N = 92), the horizontal red dotted line indicates the AQSA resistance phenotype (≥45% parasite survival). Symbols represent P. falciparum isolates and black horizontal bars and I bars indicate the median and interquartile range, statistical comparison used the Kruskal–Wallis test.
B) Kaplan–Meier plots for Day-28 ACPR in 60 patients with P. falciparum malaria treated with artesunate-amodiaquine for amodiaquine-resistant and -susceptible Pfcrt–Pfmdr1 haplotypes.
Table 1. Baseline characteristics of the safety and efficacy population.

| Characteristic                                      | Study population (N=63) |
|-----------------------------------------------------|------------------------|
| Males/females, n                                    | 61/2                   |
| Adults aged >15 years, n                            | 56                     |
| Children aged 5–15 years, n                         | 7                      |
| Mean age (SD) [range], years                        | 28.2 (11.9) [5–56]     |
| Mean weight (SD) [range], kg                        | 53.7 (10.5) [23–78]    |
| Geometric mean parasitemia (range) µL⁻¹ blood       | 22,695 (1,756–248,000) |

SD, standard deviation
Table 2. *Pfcrt* or *Pfmdr1* haplotype and parasite survival in the amodiaquine survival assay (AQSA).

| Haplotype | n (%) | Median parasite survival, % (IQR) |
|-----------|-------|----------------------------------|
| **Pfcrt** |       |                                  |
| Dd2†      | 13 (13.8) | 48.1 (1.5–77.2)                  |
| Dd2 F145I | 7 (7.4)    | 0.0 (0–1.8)                      |
| Dd2 G353V | 13 (13.8)  | 0.4 (0–1.0)                      |
| Dd2 G367C | 1 (1.1)    | 0.4                              |
| Dd2 H97Y  | 14 (14.9)  | 15.9 (4.5–46.8)                  |
| Dd2 I210F | 13 (13.8)  | 8.0 (3.8–31.5)                   |
| Dd2 I218F | 1 (1.1)    | 0.0                              |
| Dd2 M343I | 2 (2.1)    | 1.6 (0.2–3.0)                    |
| Dd2 N88K  | 1 (1.1)    | 0.7                              |
| Dd2 T93S  | 27 (28.7)  | 57.8 (22.6–71.7)                 |
| Cam734†   | 1 (1.1)    | 11.1                             |
| GB4†      | 1 (1.1)    | 0.0                              |
| **Pfmdr1**|       |                                  |
| Wild type | 3 (3.2)    | 4.8 (1.4–11.1)                   |
| Y184F     | 43 (45.7)  | 1.3 (0–6.3)                      |
| P72S/Y184F| 3 (3.2)    | 24.7 (0.6–32.9)                  |
| Y184F/F1068L| 19 (20.2)  | 64.2 (46.0–74.7)                 |
| Y184F/G1314D| 18 (19.1)  | 13.7 (3.6–42.8)                  |
| Y184F/S784L/R945P| 8 (8.5) | 67.8 (48.1–81.6) |

†Mutations for *Pfcrt* D2d: M74I/N75N/K76T/A220S/Q271E/N326S/I356T/R371I; *Pfcrt* Cam734: M74I/N75D/K76T/A220S/Q271E/T333S; *Pfcrt* GB4: 74I/75E/76T/A220S/Q271E/R371I.

*Pfcrt*, *P. falciparum* chloroquine resistance transporter; *Pfmdr1*, *P. falciparum* multidrug resistance 1.
PCR-adjusted Day-28 ACPR
81.0% (95% CI 68.9–88.7)
Day-28 ACPR (n=12) Recrudescence (n=51)

Clinical outcome

IC$_{50}$ (nM)

$P = 0.084$
A. Pearson $r$ value 0.75 (95% CI 0.62–0.84) ($P < 0.0001$)

B. $P = 0.0054$

C. Cumulative incidence of ACPR

Day-28 ACPR (n=12) Recrudescence (n=51)

Clinical outcome

Cumulative incidence of ACPR (%)

- <45% survival
- ≥45% survival

| Study day | 0  | 21 | 23 | 24 | 25 | 28 |
|-----------|----|----|----|----|----|----|
| Number at risk <45% | 40 | 40 | 38 | 38 | 38 | 37 |
| parasite survival in AQSA |
| Number at risk ≥45% | 23 | 23 | 19 | 17 | 16 | 16 |
| parasite survival in AQSA |
Dd2–Y184F
Dd2–P72S/Y184F
Dd2–Y184F/S784L/R945P
Dd2 T93S–Y184F
Dd2 T93S–WT
Dd2 T93S–Y184F/F1068L
Dd2 T93S–Y184F/G1314D
Dd2 H97Y–Y184F
Dd2 H97Y–WT
Dd2 H97Y–P72S/Y184F
Dd2 H97Y–Y184F/G1314D
Dd2 F145I–Y184F
Dd2 I210F–Y184F
Dd2 I218F–Y184F/G1314D
Dd2 M343I–Y184F
Dd2 G353V–Y184F
Cam734–WT
GB4–Y184F

Different Pfcr–Pfmdr1 haplotypes

Significant differences:
Other haplotypes versus Dd2–Y184F/S784L/R945P: \( P = 0.0108 \)
Dd2 T93S–Y184F/F1068L versus Dd2 G353V–Y184F: \( P = 0.0304 \)
Dd2–Y184F/S784L/R945P versus Dd2 G353V–Y184F: \( P = 0.0004 \)