Assessing the Cost-Benefit Effect of a *Plasmodium falciparum* Drug Resistance Mutation on Parasite Growth *In Vitro*

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Plasmodium falciparum mutations associated with antimalarial resistance may be beneficial for parasites under drug pressure, although they may also cause a fitness cost. We herein present an *in vitro* model showing how this combined effect on parasite growth varies with the drug concentration and suggest a calculated drug-specific cost-benefit index, indicating the possible advantage for mutated parasites. We specifically studied the D-to-Y change at position 1246 encoded by the *pfmdr1* gene (*pfmdr1* D1246Y) in relation to amodiaquine resistance. Susceptibilities to amodiaquine, desethylamodiaquine, and chloroquine, as well as relative fitness, were determined for two modified isogenic *P. falciparum* clones differing only in the *pfmdr1* 1246 position. Data were used to create a new comparative graph of relative growth in relation to the drug concentration and to calculate the ratio between the benefit of resistance and the fitness cost. Results were related to an *in vivo* allele selection analysis after amodiaquine or artesunate-amodiaquine treatment. *pfmdr1* 1246Y was associated with decreased susceptibility to amodiaquine and desethylamodiaquine but at a growth fitness cost of 11%. Mutated parasites grew less in low drug concentrations due to a predominating fitness cost, but beyond a breakpoint concentration they grew more due to a predominating benefit of increased resistance. The cost-benefit indexes indicated that *pfmdr1* 1246Y was most advantageous for amodiaquine-exposed parasites. *In vivo*, a first drug selection of mutant parasites followed by a fitness selection of wild-type parasites supported the *in vitro* data. This cost-benefit model may predict the risk for selection of drug resistance mutations in different malaria transmission settings.

Artemisinin-based combination therapy (ACT) is recommended as first-line therapy against uncomplicated *Plasmodium falciparum* malaria worldwide (1). The artesunate and amodiaquine (AQ) combination (ASAQ), which has proven efficacious in Africa (2), is one of the essential ACTs. AQ and its slowly eliminated active metabolite desethylamodiaquine (DEAQ) are 4-aminoquinolines structurally related to chloroquine (CQ). AQ has been used previously in monotherapy for uncomplicated *P. falciparum* malaria in parts of Africa, where it has remained relatively efficacious despite the similarities and putative cross-resistance with CQ (3, 4).

Several genetic alterations have been associated with tolerance/resistance to different long-acting ACT partner drugs. Resistance to CQ, AQ, and DEAQ has been associated with the K76T mutation encoded in the *P. falciparum* CQ resistance transporter gene (*pfcrt*) and with the N86Y mutation encoded in the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) (5–13). Further, the D1246Y mutation encoded in *pfmdr1* (*pfmdr1* D1246Y) has been selected for after treatment with AQ monotherapy and ASAQ combination therapy in East Africa (8, 14).

Mutations may also affect parasite fitness, however. Biological fitness is essentially defined as the ability of an organism to survive and propagate its genes to the next generation (15). It has been shown for several organisms that the wild-type genotype is normally the most viable and that mutations that are advantageous for surviving environmental challenges are acquired at a fitness cost (16–19). This may also apply to drug resistance in malaria, where mutations might be disadvantageous in the absence of drug pressure. This is supported by an *in vitro* study where transfected isogenic parasites with a triple mutation in the *pfmdr1* gene produced fewer viable merozoites (20). Further, lower parasite den-

sities *in vivo* were associated with T at position 76 encoded by *pfcrt* (*pfcrt* 76T) and Y at position 86 encoded by *pfmdr1* (*pfmdr1* 86Y) in Guinea Bissau (21), and their prevalences decreased after discontinued use of CQ in Malawi (22).

The beneficial effect of resistance and a possible fitness cost counteract each other. The eventual effect on parasite growth and a possible selection varies upon different settings of malaria endemicity and local drug policies. Mathematical models suggest that an important factor determining the spread of drug resistance is the proportion of infected people being treated, i.e., the fraction of the total parasite population under drug pressure (23). In areas of low endemicity, such as South-East Asia, where a majority of the infected individuals are symptomatic and treated, the resulting high-drug-pressure environment is favorable for the selection and spread of drug-resistant mutated parasites (24, 25). Conversely, in area of high endemicity, such as sub-Saharan Africa, a large proportion of malaria-infected individuals are semi-immune and asymptomatic and thus are not treated with antimalarial drugs. In the latter areas, mutated parasites with significantly reduced fit-

ness might be continuously deselected and the spread of drug resistance hampered (23).

Our objectives were to analyze how this combined cost-benefit effect on parasite growth varies with the drug concentration and to suggest a calculated drug-specific cost-benefit index, indicating the possible advantage for mutated parasites. This may be useful to predict the potential risk for selection of drug-resistant mutations in different malaria transmission settings.

MATERIALS AND METHODS

***P. falciparum* clones for *in vitro* analyses.** Two *P. falciparum* clones differing only in the *pfmdr1* 1246 position were chosen for this study (26). They were obtained through genetic modification of the CQ-sensitive D10 clone from Papua New Guinea, i.e., the transfection control, D10 *pfmdr1* SND, representing the wild-type *pfmdr1* D1246 clone (D10^{wt}), and D10 *pfmdr1* SNY, with a single modification, representing the mutated *pfmdr1* 1246Y clone (D10^{mut}). Both clones (reference no. MRA-563 and MRA-564, respectively) were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). Their respective genotypes were confirmed with PCR-restriction fragment length polymorphism (RFLP) and genomic DNA (gDNA) sequencing.

Drug susceptibility analyses. The effect of the *pfmdr1* D1246Y mutation on parasite susceptibility to CQ, AQ, and DEAQ was determined through an HRP2 enzyme-linked immunosorbent assay (HRP2-ELISA)-based *in vitro* assay as previously described (27). *In vitro*-cultured parasite clones (D10^{wt} and D10^{mut}) were diluted to initial parasitemias of 0.05% and aliquoted into microculture 96-well plates predosed with ascending concentrations of 0 to 404 nM CQ, 0 to 67 nM AQ, or 0 to 156 nM DEAQ. After incubation at 37°C for 72 h, the samples were freeze-thawed, transferred, and processed into precoated ELISA plates (Cellabs, Sidney, Australia) for spectrophotometric analysis (Multiskan EX; Thermo Labsystems, Helsinki, Finland) of HRP2 produced during parasite growth. The 50% and 90% inhibitory concentration (IC$_{50}$ and IC$_{90}$) values were calculated using the software program HN-NonLin V1.05 Beta (H. Noedl, 2001) (http://malaria.farch.net). The IC$_{20}$ values were calculated by applying a polynomial trend line on the average HRP values and solving its equation at $y = 80\%$ (SigmaPlot 11.0; Systat Software Inc., Chicaco, IL). All cultures were run in duplicate and independently tested for their drug susceptibilities six times.

Relative growth and fitness analyses. The effect of the *pfmdr1* D1246Y mutation on relative parasite growth was determined in a competition experiment between synchronized ring-staged cultures of D10^{wt} and D10^{mut} clones *in vitro*. The cultures were initially subcultivated to 0.5% parasitemia. Equal aliquots of each culture were added into a 1:1 mixed culture and run in medium containing drug concentrations corresponding to the approximate IC$_{20}$ value (CQ, 15 nM; AQ, 10 nM; or DEAQ, 10 nM) or IC$_{50}$ (CQ, 30 nM; AQ, 20 nM; DEAQ, 20 nM) for the two clones. In the IC$_{20}$-based cultures, drug exposure was removed after 6 days, while in the IC$_{50}$-based cultures, drug exposure was removed after 4 days, when the parasite densities had decreased to approximately 0.1%. After drug removal, all cultures were washed thoroughly and then run in drug-free medium until 6 days after the time of parasite recovery, when regrowth of remaining parasites was established. The time of recovery was defined as the day when growing ring-stage trophozoites were first observed microscopically after drug removal. In parallel to the drug-exposed cultures, control cultures were run in drug-free medium for a total of 12 days, including a subcultivation to 0.5% parasitemia after 6 days. Blood medium was collected for microscopy and spotted on filter paper (3MM Whatman) for genotyping every second day. All mixed cultures were run in duplicate.

DNA was extracted from the filter papers using a QIAamp DNA mini-kit (Qiagen). Amplification was performed through nested PCR using previously published first primers (28) and specifically designed nest primers, i.e., forward (5'-TTTTCAAACCAATCTGGATCTG-3') (biotinylated) and reverse (5'-CTCTGTTTTTGTCCACCTGATAAG-3'). All

PCRs included 1× *Taq* polymerase reaction buffer, 3 mM magnesium chloride, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.4 μM (each) primer, and 0.75 U of *Taq* DNA polymerase (Promega Corporation, Madison, WI). All PCR products were visualized in an ethidium-bromide-stained 2% agarose gel under UV transillumination (GelDoc 2000; Bio-Rad, Hercules, CA). Allele quantification was performed through gDNA pyrosequencing as previously described (Pyro Gold reagents and PSQ 96MA and PyroMark MD systems; Biotage AB, Uppsala, Sweden) (29). Specifically for *pfmdr1* D1246Y, we used a reverse sequencing primer (5'-TTGAAAATAAGTTTCTAAGA-3') and the dNTP dispensary entry CTACGTCTA. Results were adjusted against a standard curve, derived from a scale of established mixes of the two alleles, and corrected for the allele exchange output, i.e., the presence of two *pfmdr1* fragments in the transfected genome (26). All samples were run in triplicate. Thus, all samples were separately run a total of six times for each time point.

Allele selection analysis *in vivo*. To explore whether a cost-benefit effect of *pfmdr1* D1246Y might be reflected in the parasite selection dynamics after AQ or ASAQ therapy *in vivo*, we analyzed the allele frequencies in baseline infections compared with those in recurrent and more specifically recrudescent infections during follow-up in three previously conducted drug efficacy trials in children <5 years old with uncomplicated *P. falciparum* malaria in East Africa. These include an AQ monotherapy study in Kenya in 2003 ($n = 53$) (7) and two ASAQ combination therapy studies in Zanzibar in 2002-2003 ($n = 198$) and 2005 ($n = 174$) (14, 30). DNA from blood samples collected on filter papers (3MM; Whatman, United Kingdom) had previously been extracted (Bloodprep; Applied Biosystems). *pfmsp2* genotyping was performed to distinguish between recrudescent and reinfecting parasites (31). PCR-RFLP was used for *pfmdr1* D1246Y genotyping (5, 28), and mixed results were excluded since we did not have information on the separate allele proportions within such infections.

Data analysis and statistics. *In vitro* susceptibilities were compared using Student's *t* test (SigmaPlot 11.0; Systat Software Inc., Chicago, IL). Statistical significance was defined as a *P* value of ≤0.05 and controlled according to the Bonferroni method.

The relative growth rate (*g*) of the mutant clone in the competition experiment was calculated from the following equation: log(mut$_t$/wt$_t$) = log(mut$_0$/wt$_0$) + [$t * $log(*g*)]. "mut" and "wt" represent the proportions of the mutant and the wild-type alleles initially (t_0) and after a defined number (*t*) of asexual generations during the course of the experiments. *g* for the wild-type clone was defined as 1.0, whereas *g* for the mutant clone under drug-free conditions corresponds to the relative fitness (*w*), and the fitness cost was calculated as $1 - w$ (20, 32).

For comparison, the relative absorbance (abs) of the mutant clone in the HRP2-ELISA susceptibility analyses was calculated by normalizing against the background absorbance in the following equation: $g = (10$^abs$ $ well A_{mut}/10^abs$ $ well $H_{mut})/(10$^abs$ $ well A_{wt}/10^abs$ $ well $H_{wt})$.

Ethical considerations. All clinical trials were performed in accordance with the Declaration of Helsinki (33) and ICH good clinical practice (34). Informed consent was obtained from parents/guardians of all enrolled participants. Ethical approvals were obtained from the Research Ethics Committee at Karolinska Institutet in Stockholm, Sweden (KI Dnr 03-545, KI Dnr 03-753, and KI Dnr 2005/57-31), the Ethical Research Committee, Nyanza Province, Kenya (GN.153, vol. 1/50), and the Zanzibar Health Research Council (ZHRC/GC/2002 and ZMRC/RA/2005).

RESULTS

***pfmdr1* 1246Y is associated with decreased susceptibilities to AQ/DEAQ.** In the drug susceptibility analyses, the profiles differed for the three drugs (see Table S1 in the supplemental material). For CQ, no difference in the IC$_{50}$ was found between the D10^{wt} and D10^{mut} clones, whereas for AQ and DEAQ, significantly higher IC$_{50}$s ($P < 0.001$) were found for D10^{mut} (28.7 nM; 95% confidence interval [CI$_{95}$], 26.8 to 30.6; and 20.5 nM; CI$_{95}$,
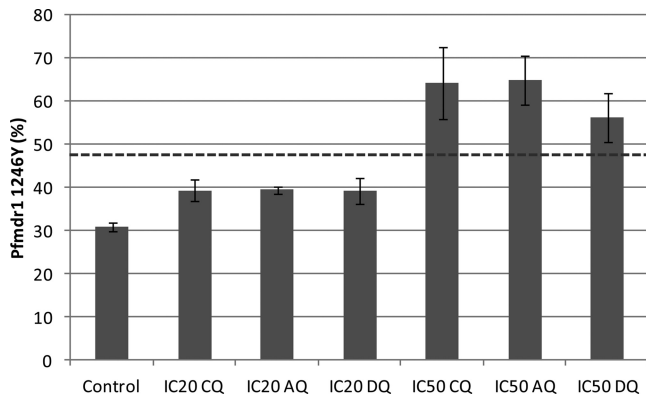
FIG 1 Proportion (%) (CI$_{95}$) of the *pfmdr1* 1246Y allele in the non-drug-exposed control culture and cultures exposed to an approximate IC$_{20}$ (6 days) or IC$_{50}$ (4 days) of CQ, AQ, or DEAQ. The dashed line is the average original proportion of the *pfmdr1* 1246Y allele.

19.4 to 21.5 [respectively]) compared with D10^{wt} (15.1 nM; CI$_{95}$, 13.1 to 17.1; and 15.3 nM; CI$_{95}$, 13.5 to 17.0 [respectively]).

The cost-benefit effect of *pfmdr1* 1246Y on parasite growth varies with the drug concentration. In the competition experiments, parasites exposed to IC$_{20}$ drug concentrations grew 10 to 15% less than non-drug-exposed parasites and recovered without delay upon drug removal. Parasites exposed to IC$_{50}$s of CQ, AQ, or DEAQ grew 70 to 80% less than non-drug-exposed parasites and did not recover until day 6, 14, or 18 after drug removal, respectively.

The fact that the cultures did not grow exactly 20% and 50% less might be a result of an unequal effect of the approximate IC$_{20}$ or IC$_{50}$ on the D10^{wt} and D10^{mut} clones included in these mixed cultures.

The relative growth of the D10^{mut} clone differed depending on the degree of drug exposure (Fig. 1; see also Table S2 in the supplemental material). In the drug-free control cultures, the proportion of D10^{mut} continuously decreased, from 48.0% to 30.9% over the full culture period of 12 days ($P < 0.001$), which corresponds to a negative relative fitness compared with findings for D10^{wt} ($w = 0.89$). In the cultures exposed to IC$_{20}$ values of CQ, AQ, or DEAQ, the proportions of D10^{mut} also decreased, but to a lesser extent, i.e., from 47.3% to 39.3%, 39.5%, or 39.2%, respectively. This also corresponds to negative relative growth rates ($g = 0.95$ 0.95, or 0.94, respectively). In contrast, in the cultures exposed to IC$_{50}$ concentrations of CQ, AQ, or DEAQ, the proportion of D10^{mut} increased, i.e., from 48.8% to 64.2%, 65.0%, or 56.3%, respectively. This instead corresponds to positive relative growth rates ($g = 1.11$, 1.12, or 1.05, respectively). The observation that D10^{mut} also increased under CQ exposure might be due to the similar IC$_{50}$s between the clones, and under such circumstances, minimal differences in synchronization may interfere.

Model to assess cost-benefit effect of *pfmdr1* 1246Y. The relative growth rate of D10^{mut} was thus negative in the drug-free and IC$_{20}$-exposed cultures and positive for the IC$_{50}$-exposed cultures. To analyze how the relative growth for D10^{mut} versus that for D10^{wt} varies with the drug concentration, we developed a new comparative cost-benefit graph (Fig. 2). Under drug-free conditions, the maximum growth rate for D10^{wt} was defined as 100% ($g = 1.0$), and for D10^{mut}, it was defined as 89%, corresponding to the relative fitness ($w = 0.89$) derived from the competition ex-
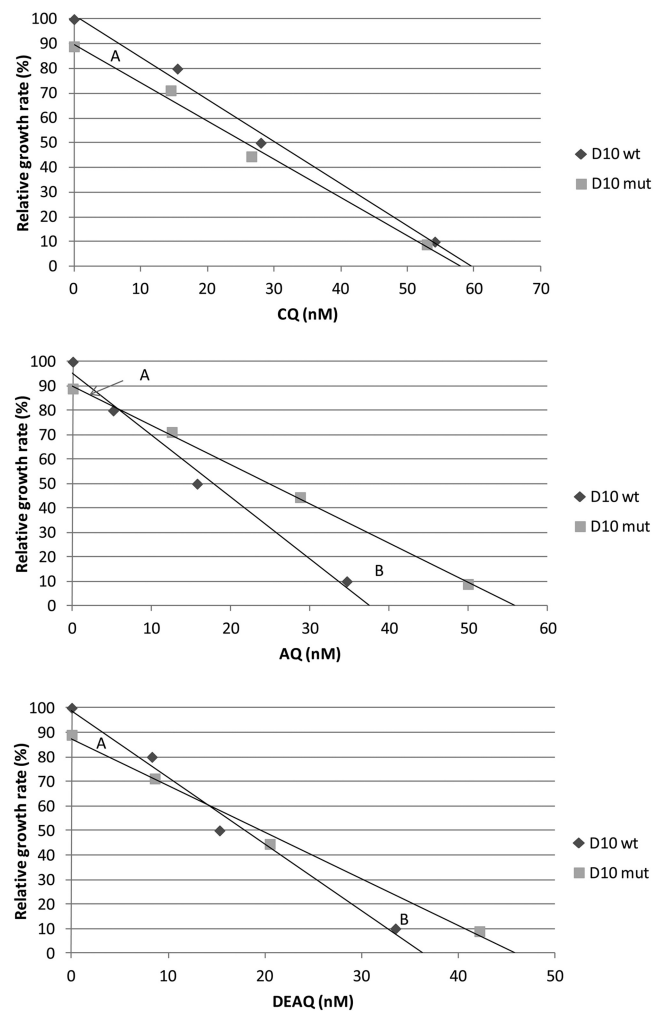

FIG 2 Relative growth rates (%) of D10^{mut} versus D10^{wt} at different concentrations of CQ, AQ, and DEAQ (nM). The maximum growth rate for D10^{mut} without drug exposure was based on the relative fitness ($w = 0.89$). The other plots are based on the IC$_{20}$, IC$_{50}$, and IC$_{90}$ values. The trend line intercept indicates the breakpoint where the drug resistance benefit equals the fitness cost of *pfmdr1* 1246Y. Area *A* represents the fitness cost, and area *B* represents the drug resistance benefit of *pfmdr1* 1246Y over the full concentration range.

periment. For each clone, the 80%, 50%, and 10% relative growth rates were then plotted against the respective IC$_{20}$, IC$_{50}$, and IC$_{90}$ values derived from the susceptibility analyses.

Applied trend lines showed that D10^{mut} grew least compared with D10^{wt} under drug-free conditions. With increasing drug concentrations, the difference in the growth rate continuously decreased until the trend lines potentially crossed at a breakpoint concentration, after which D10^{mut} grew continuously more. Hence, the relative growth rates of D10^{mut} parasites were thus negative in drug concentrations lower than the breakpoint concentration, where the benefit of resistance equals the fitness cost, while in higher concentration the relative growth rates were positive. The areas between the trend lines correspond to the fitness cost (area *A*) and the benefit of resistance (area *B*) over the full drug concentration range. The ratio between area *B* and area *A* divided by the breakpoint concentration represents a cost-benefit index. This index might correspond to the overall advantage of

pfmdr1 1246Y in relation to the breakpoint concentration and as such its selection potential.

For CQ, the two trend lines never crossed, while the trend lines crossed at breakpoint concentrations of approximately 5 nM for AQ and 14 nM for DEAQ. By normalizing the values for relative growth and drug concentration against those for the D10^{wt} clone and using the trend line equations, we could geometrically calculate the cost-benefit indexes to zero (no area *B*) for CQ, 37.9 for AQ, and 12.0 for DEAQ.

The results were confirmed experimentally by comparing the relative parasite growth rates with the relative absorbencies derived from the HRP2-ELISA susceptibility analyses, where we found a similar pattern (data not shown).

The cost-benefit effect influences the selection of the *pfmdr1* D1246Y alleles after AQ/DEAQ exposure *in vivo*. The treatment outcomes of the AQ and two ASAQ efficacy studies have been described elsewhere (see Table S3 in the supplemental material) (7, 14, 30). In summary, the crude treatment failure rates were 36%, 40%, and 29%, while the PCR-adjusted treatment failure rates were 19%, 8%, and 9%, respectively. No significant differences in crude or PCR-adjusted treatment failure rates were found between the two ASAQ studies. Aggregate analysis of the *pfmdr1* D1246Y allele frequencies over time in all recurrent parasitemias during follow-up after ASAQ therapy showed two selection peaks, i.e., *pfmdr1* 1246Y on days 8 to 14 and *pfmdr1* D1246 on days 22 to 28 (Fig. 3). This was followed by a return to the baseline frequencies between days 29 and 42. A similar pattern was also observed in recrudescent infections specifically, although the number of infections was small, and after AQ monotherapy, even though the follow-up duration was only 21 days in this study.

The prevalence of children with recurrent parasitemia during the first selection peak of *pfmdr1* 1246Y was statistically significantly lower after ASAQ combination therapy (7/294; 2%) than with AQ monotherapy (12/28; 43%) (*P* < 0,001) (Fig. 3; see also Table S3 in the supplemental material).

DISCUSSION

AQ is an essential ACT partner drug, since ASAQ is presently a first-line treatment of uncomplicated *P. falciparum* malaria in more than 20 African countries (35), although resistance against AQ/DEAQ both *in vitro* and *in vivo* has been described (6–8, 10, 11, 13, 14). The sustained efficacy of AQ as a long-acting partner drug in ACT is therefore a matter of concern. Understanding the pharmacodynamic basis of selecting resistance to this quinoline is thus fundamental. Our *in vitro* results with allele-exchanged parasites confirm that *pfmdr1* 1246Y is associated with decreased susceptibility to AQ and DEAQ. This was further supported by its selection upon drug pressure in the competition experiments. These results, together with our previous findings of its selection after AQ/ASAQ treatment *in vivo* (7, 14), therefore support *pfmdr1* 1246Y as a molecular marker associated with resistance to AQ/DEAQ.

In the competition experiments, the fitness cost for parasites carrying the *pfmdr1* 1246Y mutation was determined to 11% per asexual generation. This cost-benefit effect of the *pfmdr1* D1246Y mutation on protein function is supported by results from a recent bioinformatic analysis (36). PfMDR1 is located in the membrane of the digestive vacuole with its nucleotide binding site facing the parasite cytoplasm (37). Subcellular studies of the distribution of fluorescein derivatives (Fluo-4) (38) suggest that
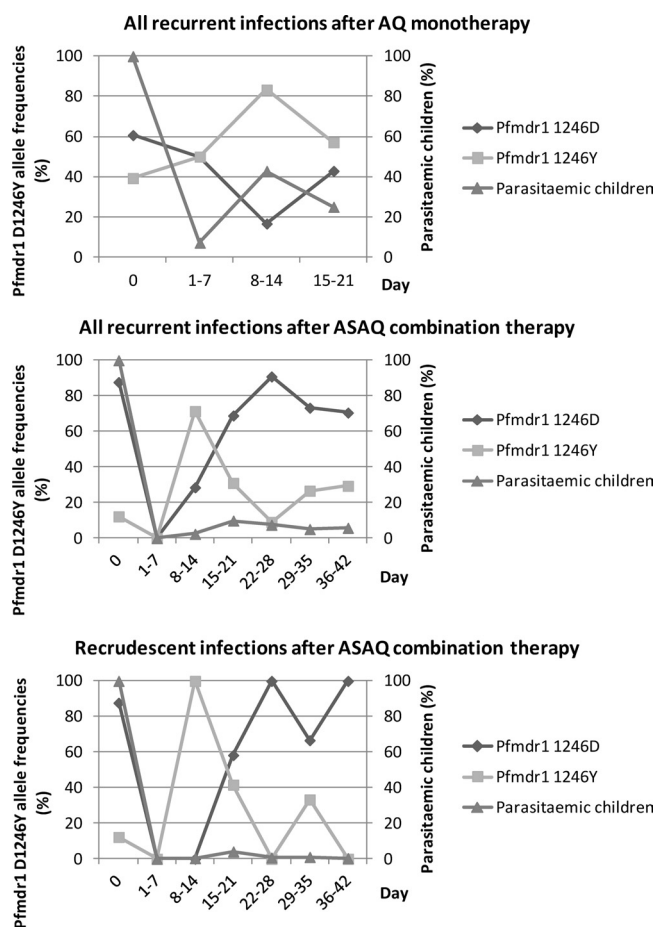

FIG 3 Frequencies (%) of the *pfmdr1* D1246Y alleles (first axis) in the proportion (%) of children (second axis) with recurrent infections during follow-up (days) after AQ monotherapy in Kenya 2003 (*n* = 53), as well as recurrent and recrudescent infections after ASAQ combination therapy in Zanzibar, 2002-2003 and 2005 (*n* = 372).

PfMDR1 is an importer of diverse substrates into this organelle. The localization of *pfmdr1* D1246Y in the structure of the transporter suggests a functional impact on the influx capacity of the mutated protein (36). This might decrease the import of aminoquinoline compounds and hence their access to their active site inside the digestive vacuole, thus reducing drug susceptibilities (38). However, the effect might vary due to differences in the chemical structure and binding sites, which might interact unequally with an altered protein structure and function. A reduced capacity may also affect the import of essential substrates. This might affect fundamental processes inside the food vacuole. Consequently, parasites carrying the *pfmdr1* D1246Y mutation may be less competitive than wild-type parasites in the absence of aminoquinoline drug pressure.

We have herein described a new cost-benefit graph, where the relative growth of parasites with a particular mutation corresponds to a concentration-dependent balance between the specific benefit of resistance and the fitness cost. Mutated parasites grow less in low drug concentrations due to a predominating fitness cost, but beyond a breakpoint concentration they potentially grow more due to a predominating benefit of increased resistance. Based on the D10^{mut}-versus-D10^{wt} data, we calculated the cost-
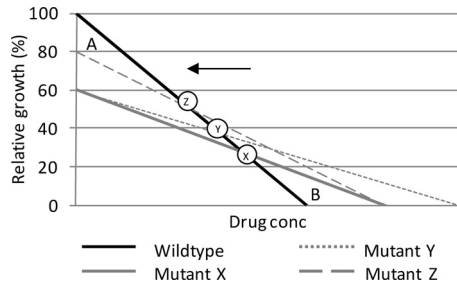
FIG 4 Hypothetical graph of relative growth of a wild-type clone compared with that of a mutant clone (mutant X). The breakpoint concentration (X to Z) moves toward lower concentrations with the addition of synergistic mutations resulting in an increased resistance benefit (mutant Y) and/or compensating mutations resulting in a decreased fitness cost (mutant Z).

FIG 5 Hypothetical graph of the relative growth rate for a wild-type clone and a mutant clone in relation to the drug concentration over days after treatment *in vivo*. The mutant clone benefits during higher concentrations due to decreased drug susceptibility (drug selection phase), while during lower concentrations beyond the cost-benefit breakpoint, it forfeits due to a fitness cost (fitness selection phase).

benefit indexes representing the drug-specific advantage of *pfmdr1* 1246Y in relation to the breakpoint concentrations, which indicate the risk for selection. *Pfmdr1* 1246Y was most advantageous for parasites exposed to AQ and second most for parasites exposed to DEAQ. For parasites exposed to CQ, no similar advantageous effect of this mutation could be observed. Such cost-benefit indexes may potentially be used as a tool to compare the risk for selection of different mutations associated with tolerance/resistance to different ACT partner drugs.

The *in vitro* findings were consistent with our findings from the clinical trials (Fig. 3). AQ is rapidly and extensively metabolized by the hepatic enzyme system CYP2C8 to DEAQ, with the peak concentration in plasma within 4 to 6 h and a half-life in plasma of 10 to 14 days (39). Due to the longer half-life, it is probable that DEAQ is mainly responsible for the antimalarial activity. AQ/DEAQ, as well as CQ, has large volumes of distribution, and concentrations in blood have been shown to vary significantly (7). A pharmacokinetic study of ASAQ *in vivo*, however, registered average maximum DEAQ concentrations of 350 to 400 ng/ml on day 3 and 40 to 50 ng/ml on day 14 after treatment (40). This suggests that the registered selection of *pfmdr1* 1246Y during the first 14 days after AQ/ASAQ treatment is associated with a higher concentration of DEAQ, defining the drug selection phase (corresponding to area *B in vitro*). The selection of *pfmdr1* D1246 during the following 14 days suggests an association with lower concentrations of the drug, defining the fitness selection phase (corresponding to area *A in vitro*). This phase extends beyond the follow-up under drug-free conditions. The breakpoint concentration *in vitro* would thus correspond to the intercept between the two selection phases *in vivo*.

Mutations associated with drug resistance might be supported by compensatory and/or synergistic mutations (14, 41). In our cost-benefit graph, this will hypothetically result in a decreased fitness cost (area *A*) and/or increased resistance benefit (area *B*), along with a leftward motion of the breakpoint concentration, i.e., toward a lower concentration, as well as an increased cost-benefit index *in vitro* (Fig. 4). An equivalent rightward motion of the selection intercept is expected to happen *in vivo*, as well as an increased drug selection phase (Fig. 5). However, if synergistic mutations are associated with a further fitness cost, the breakpoint concentration *in vitro* and selection intercept *in vivo* might be unaffected, and they may not be more selected for.

The frequency of recurrent parasitemias during the critical drug selection phase was significantly lower after ASAQ than with
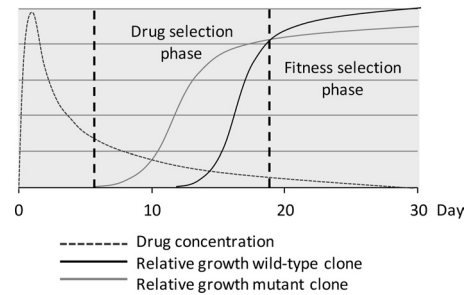
AQ monotherapy (Fig. 3; see also Table S3 in the supplemental material). This may suggest that the addition of artesunate reduces the risk of a *pfmdr1* 1246Y selection, which eventually may result in an overall selection of the wild-type allele in the population. These results may explain our findings from a recent study in Zanzibar in 2010, where the frequency of *pfmdr1* 1246Y in baseline infections actually had decreased despite 7 years' use of ASAQ as a first-line treatment (42).

In summary, the results indicate that *pfmdr1* 1246Y is associated with decreased susceptibility to AQ/DEAQ but at a significant fitness cost to the parasite. *Pfmdr1* 1246Y is clearly selected for after AQ/ASAQ therapy during the drug selection phase, while artesunate seems to decrease such selection through the effective reduction of early recurrent parasitemias. In addition, *pfmdr1* 1246Y appears to incur a too-substantial fitness cost to the parasite to be sustained in competition with wild-type parasites in the absence of treatment (drug pressure) (23). This may prevent a selection of *pfmdr1* 1246Y in the parasite population and development of resistance, possibly supporting AQ as a valuable and potentially sustainable partner drug option for ACT in East Africa.

Finally, the described model may be useful to evaluate the cost-benefit effects of other mutations associated with resistance to ACT partner drugs to predict their potential risk for selection in different malaria transmission settings. Such prediction is important for future optimized use of partner drugs and as such the conditions for sustained ACT efficacy.

ACKNOWLEDGMENTS

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