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Selection of *pfmdr1* mutations after amodiaquine monotherapy and amodiaquine plus artemisinin combination therapy in East Africa

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Abstract

Despite the pharmacodynamic advantages with artemisinin-based combination therapy (ACT) and some potentially opposite molecular mechanisms of tolerance to amodiaquine (AQ)/desethylamodiaquine (DEAQ) and artesunate (ART), there is a risk for rapid decay in efficacy if the two drugs are unable to ensure mutual prevention against a selection and spread of drug-resistant parasites.

We have studied if mutations in the *pfcrt* and *pfmdr1* genes selected in recurrent infections after AQ monotherapy are also selected after AQ plus ART combination therapy.

Samples for molecular analysis were derived from three clinical trials on children <5 years old with uncomplicated *Plasmodium falciparum* malaria; one AQ monotherapy study conducted in Kenya 2003 and two AQ plus ART combination therapy studies conducted in Zanzibar 2002–2003 and 2005, respectively.

The PCR-adjusted treatment failure rates in the three studies were 19%, 8% and 9%, respectively. After monotherapy there was a significant selection of *pfcrt* 76T in re-infections (OR not calculable; $p = 0.048$) and of *pfmdr1* 86Y in recrudescent infections (OR 8.0; $p = 0.048$). No such selection was found after combination therapy. A selection of *pfmdr1* 1246Y and the *pfmdr1* haplotype (a.a 86, 184, 1246) YYY was found in recrudescent infections both after monotherapy (OR 7.6; $p = 0.009$ and OR 3.1; $p = 0.029$) and combination therapy in 2005 (OR 3.6; $p = 0.017$ and OR 5.4; $p < 0.001$).

Hence, *pfmdr1* 1246Y with synergistic or compensatory addition of *pfmdr1* 86Y and 184Y appears to be involved in AQ/DEAQ resistance and treatment failure. Our results suggest that ART may protect against a selection of these SNPs initially, but maybe not after continuous drug pressure in a population. However, treatment failure rate and spread of *pfmdr1* SNPs may remain at a low level because of the suggested opposite selection by ART and the pharmacodynamic advantages with ACT.
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1. Introduction

The development and expansion of resistance to the mainstay antimalarials chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) is a major cause for the increased morbidity and mortality of *Plasmodium falciparum* malaria in Africa (Bjorkman and Bhattarai, 2005). In response to the increasing resistance, artemisinin-based combination therapy (ACT) is now advocated as first line therapy on the African continent (WHO, 2001). The hypothesis is that the artemisinin-derivative (ART), which causes rapid and effective reduction of parasite biomass and gametocyte carriage, and the partner drug, which has a longer duration of action, will achieve effective clinical and parasitological cure, protect each other from the development of resistance and reduce the overall transmission of malaria (White, 1998). However, the choice of partner drug is critical for ACT to endure.

AQ is a 4-aminoquinoline related to CQ that has been used as treatment for uncomplicated *P. falciparum* malaria in parts of

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Africa for decades. Despite the widespread CQ resistance in Africa AQ has remained effective in many areas (Olliaro and Mussano, 2003) and with its long half-life metabolite desethylamodiaquine (DEAQ) as well as in vitro reports on a synergistic relationship with ART (Gupta et al., 2002; Mariga et al., 2005) it is presently a main partner drug option either for first- or second-line ACT.

In vitro decreased susceptibility to DEAQ has been correlated with decreased mean hydrophobicity of the haplotype (a.a 72–76) SVMNT peptide in the *P. falciparum* CQ resistance transporter (*pfcrt*) gene (Warhurst, 2003; Menard et al., 2006). Data from one of our own in vitro studies on Colombian samples have also shown a possible association between reduced susceptibility to AQ and *pfcrt* 326S, 356T, as well as an 86Y, 184Y, 1042N and 1246Y SNP in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene, while reduced susceptibility to DEAQ was associated with *pfcrt* 326D, 334N and 356L (Echeverry et al., submitted).

In vivo *pfcrt* 76T and *pfmdr1* 86Y, 1246Y, as well as the *pfmdr1* haplotype (a.a 86, 184, 1246) YYY have been associated with treatment failure after AQ monotherapy (Ochong et al., 2003; Happi et al., 2006; Holmgren et al., 2006; Dokomajilar et al., 2006; Humphreys et al., 2007).

There are so far no reports on true resistance to ART in vivo, but reduced susceptibility to ART in vitro has been associated with *pfATP6* S769N (Krishna et al., 2006) and opposite to AQ resistance, with *pfmdr1* 86N, 1246D and its amplification (Price et al., 1999, 2004; Duraisingh et al., 2000; Reed et al., 2000; Ashley and White, 2005; Duraisingh and Cowman, 2005).

Despite the pharmacodynamic advantages of ACT and some potentially opposite molecular mechanisms of tolerance to AQ and ART respectively, there is a risk for rapid decay in efficacy if the two drugs are unable to ensure mutual prevention against a selection and spread of drug-resistant parasites.

Our aim in this study was to evaluate if SNPs in the *pfcrt* and *pfmdr1* genes selected in recurrent infections after AQ monotherapy are also selected after AQ plus ART combination therapy.

2. Materials and methods

2.1. Samples

Samples for molecular analysis were derived from three clinical trials on children <5 years old with uncomplicated *P. falciparum* malaria in East Africa:

1. AQ monotherapy study conducted between November and December 2003 in Western Kenya (Holmgren et al., 2006).
2. AQ plus ART (artesunate) combination therapy study (ASAQ I) conducted between November 2002 and February 2003 in Zanzibar, Tanzania (Martensson et al., 2005).
3. AQ plus ART (artesunate) combination therapy study (ASAQ II) conducted between January and July 2005 in Zanzibar, Tanzania.

During the studies in Kenya and Zanzibar 2002–2003 the first-line treatment for uncomplicated malaria was SP and CQ, respectively, and second-line treatment was AQ and SP, respectively. The first-line treatment was then changed to Artemether-Lumefantrine (Coartem®) in Kenya (April 2004) and to ART plus AQ in Zanzibar (September 2003). Hence, at the time for the second study in Zanzibar 2005 ART plus AQ had been the first-line treatment for about a year.

2.2. Molecular analyses

Parasite molecular genetic analyses were performed at the Malaria Research Unit, Karolinska Institute in Stockholm, Sweden. Genotyping analysis of *pfcrt* K76T and *pfmdr1* N86Y was done by a PCR-RFLP method (Djimde et al., 2001). As for the AQ monotherapy study from Kenya these results are described in a previous report (Holmgren et al., 2006) and extracted DNA from blood samples on filterpapers (3MM®, Whatman, United Kingdom) from 60 out of 72 patients from the same study were available for genotyping of *pfcrt* N326S/D, T333S, S334N, I356T/L, *pfmdr1* F184Y, S1034C, D1042N and D1246Y. For genotyping of *pfmdr1* D1246Y previously published primers were used (Cox-Singh et al., 1995) and for the remaining *pfcrt* and *pfmdr1* SNPs novel primers were designed. All PCR reactions included 1× *Taq* polymerase reaction buffer, 2.5–3 mM magnesium chloride, 0.2 mM dNTP, 0.5–1 μM of each primer and 1.25 units of *Taq* DNA polymerase (Promega Corporation, Madison, USA). For the *pfcrt* SNPs a nested PCR at a low 60 °C elongation temperature was used and the 992 and 939 bp long products were sent for sequencing analysis (Macrogen Inc., Seoul, South Korea). For the *pfmdr1* SNPs a nested PCR was used followed by RFLP with the enzymes *Apo* I, *Tsp509* I, *Dde* I, *Ase* I and *EcoR* V. The products were resolved on 2% ethidium bromide stained agarose gels and visualized under UV transillumination (Bio Rad GelDoc System, Biorad, USA).

As for the ASAQ I and ASAQ II studies from Zanzibar extracted DNA from blood samples on filterpapers (3MM®, Whatman, United Kingdom) from 206 and 174 patients, respectively, were available for genotyping of the SNPs *pfcrt* K76T, *pfmdr1* N86Y, F184Y and D1246Y. Genotyping analysis of *pfcrt* K76T and *pfmdr1* N86Y was done by a multiplex PCR-RFLP method (Veiga et al., 2006). *Pfmdr1* F184Y genotyping was done by a nested PCR with a 5′-biotinylated primer, followed by pyrosequencing (Pyro Gold Reagents PSQ™ 96MA and PyroMark™ MD System, Biotage AB, Uppsala, Sweden) and allele quantification, with a pure/mix allele limit of 90%, based on a standard curve of a series of two mixed clones (PSQ™ Assay Design software, Uppsala, Sweden). *Pfmdr1* D1246Y genotyping was done as described above.

Pfmsp2 genotyping was performed on all recurrent infections to distinguish between a recrudescent infection (relapse of the same infection as compared with the first infection) and a re-infection (relapse of a different infection as compared with the first infection) (Snounou et al., 1999). In areas with high transmission rate and parasite diversity, such as our study areas in Kenya and Zanzibar, the risk would be very small for a re-infection to have the same genotype as the first infection and misinterpreted as a recrudescent infection. On the

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