Polymorphisms in the *Plasmodium falciparum pfcrt* gene and clinical response to amodiaquine in *P. falciparum* malaria infection.

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A. Study Purpose and Rationale

Malaria is the most devastating of human parasitic diseases. It is currently estimated that there are more than 300 million clinical cases of malaria each year, resulting in over 1 million deaths, primarily in children under the age of five.[1] Chloroquine (CQ), the mainstay of antimalarial chemotherapy and prophylaxis for decades, now fails frequently against *P. falciparum*, the deadliest of the human malarias. Increasing resistance to chloroquine has created an urgent need for evaluation of alternative antimalarials and alteration of antimalarial drug policy in endemic regions to stem the recent dramatic increases in malaria morbidity and mortality caused by *P. falciparum* malaria. While the molecular mechanism of chloroquine resistance (CQR) is not entirely understood, recent identification of mutations in a transporter, PfCRT, shown to be related to the resistance mechanism has allowed for development of molecular epidemiologic surveillance tools based on critical *pfcrt* mutations to screen for CQR in endemic regions.[2] Evaluation of alternative antimalarials, including the chloroquine analog amodiaquine (AQ), are likewise ongoing in endemic regions with a focus toward combination chemotherapy and the goal of preserving of the therapeutic lifespan of remaining agents. In Uganda, high rates of CQR have recently dictated a change in national drug policy to recommend the anti-folate sulfadoxine/pyramethamine (SP) as first line therapy for uncomplicated falciparum malaria, yet rapid development of resistance to SP throughout sub-Saharan Africa indicates the need to implement combination therapy.[3] Thus among currently ongoing therapeutic efficacy studies is the evaluation by Staedke and colleagues of SP in combination with AQ in children under 5 years of age. Based on reports of possible in vivo cross resistance between CQ and the related aminoquinolone amodiaquine[4] and recent in vitro data of decreased AQ susceptibility in CQR parasites harboring the *pfcrt* mutation K76T[5], this study will assess the efficacy of amodiaquine in parasite isolates harboring the CQR mutation K76T utilizing blood samples from the ongoing clinical efficacy study of SP +AQ for the treatment of uncomplicated symptomatic P falciparum infection.

B. Baseline drug resistance

Field studies evaluating the clinical efficacy alternative antimalarial combinations are currently ongoing in several endemic regions of varying degrees of CQR. Baseline estimates of CQR in endemic regions are not well established but are estimated to range from 5-95%.[6] In Uganda, CQ has, until recently, been the recommended first-line agent for uncomplicated falciparum malaria. Conservative estimates of CQR based on a recent metanalyis of multiple clinical CQ efficacy studies conducted over the last decade in Uganda indicate a rapid increase in overall CQR, from 25% in 1988 to 54% in 1996.[6] Studies done after 1996 indicate a prevalence in urban areas. Kampala is an urban center where malaria is highly endemic, occurring perennially with peaks during the 2 rainy seasons. The most recent data from the capital city of Kampala have demonstrated 83-96% parasitologic resistance, correlating with 62-76% clinical treatment failure, highest in children under five years of age, a subset of patients lacking acquired clinical immunity.[6] It has been suggested that CQ should be abandoned when clinical failure rates reach 25%, thus the government of Uganda has recently changed its national antimalarial policy to advocate use of the anti-folate SP.[7] However, recent further evaluation likewise indicates that resistance to SP has emerged and is quickly spreading in this and other endemic regions where SP monotherapy is being utilized.[3] Experience with AQ is limited, therefore estimates of AQ resistance are only recently

emerging but indicate as much as 22% parasitologic resistance in some areas, correlating with 3-10% clinical treatment failure.[6]

C. Methods

Utilizing patient blood samples from concurrent field studies of clinical antimalarial efficacy in Kampala, Uganda, an area of known high prevalence of in vivo CQ treatment failure, prevalence of the critical *pfcrt* mutation K76T will be evaluated in pre and post treatment isolates of study subjects treated with SP or SP+AQ. Full details of the primary clinical study that will provide the samples for analysis in this study are similar to those detailed in Staedke et al.[8], and briefly described below.

Primary outcome will be a proportional increase in the prevalence of the K76T mutation detected in parasite isolates obtained before and after antimalarial drug therapy with AQ. This will be measured by nested PCR of parasite isolates obtained from blood samples collected in the primary study at day 0, 7, 14. Secondary outcomes will be correlation with clinical response to treatment as assessed in the primary study by at each of the primary endpoints.

D. Study design

This is a prospective molecular epidemiologic observational cohort study based on data and samples from a primary randomized placebo controlled double blinded trial of patients with uncomplicated falciparum malaria randomized to treatment with either SP or SP+AQ similar to that previously described in detail in Staedke et al.[8]

E. Statistical analysis

Using a baseline parasitologic AQ resistance of 20% and CQ resistance of 85%, estimates of clinical failure for each of the treatments were used to calculate the sample size of the study that would have 80% or greater power to detect a 10% difference in the proportion of the K76T *pfcrt* mutants among clinical failures between any two of the treatment groups by proportional X^2 test. The calculated sample size for each arm by this method is approximately 300 patients. Samples from all patients with known outcomes will be included in the analysis. Statistical associations between point mutations and in vivo outcomes will be assessed using the Fisher's exact test (2 tailed). An association between the molecular marker and resistance to a given drug will be considered to be significant if the p value is less than 0.05.

F. Subjects

Because age younger than 5 years had been identified as the strongest predictor of CQ treatment failure in Kampala, patients recruited for this study are all <5 years of age. As previously described by Staedke[8], patients in the primary study are drawn from those presenting to urban clinics in Kampala with symptoms suggestive of malaria and a positive screening thick blood smear. These patients are then referred to the study clinic for further assessment/enrollment in the trial based on the following inclusion criteria: 1)age >6mos and <5years, 2)weight over 5kg; 3)tympanic temperature >38.0^{o}C or febrile symptoms in the prior 48hours; 4) acute uncomplicated P falciparum monoinfection with a parasite density of >2000 parasites/ul; 5)residence in Kampala 6) willingness to participate in the study for 14 days; 7) written informed consent provided by parent/guardian. Patients were excluded if they had 1)a history of allergic reactions to any of the study drugs or sulfa drugs; 2)danger signs of severe malaria (including Hct <15%, persistent vomiting, coma, seizure, lethargy, inability to stand or drink); 3)an alternate explanation for febrile illness, 4)participation in a clinical study in the prior 3 months; 5)self medication with other antimalarial drugs.

In a double-blinded manner, enrolled patients are then randomized to receive either single dose SP (25mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine) or SP + AQ 25 mg/kg in three divided doses

given on day 0, 1 and 2. Patients in the SP group are given lactose placebo tablets in place of AQ on d0, 1 and 2. All treatments are directly observed.

Clinical follow up on days 1, 2, 3, 7 and 14 after treatment, with blood sample collection by finger stick with microscopic examination is performed on days 3, 7 and 14 and whenever further symptoms were reported or fever detected. The primary endpoint of the study, parasitologic resistance is assessed using classic WHO classic definitions of resistance and sensitivity of S or RI, II, or III treatment failures as published, defining early and late treatment failure.[9] Patients who met criteria for treatment failure on d14 were subsequently treated with quinine.

Baseline frequency of mutations will be determined from samples taken before treatment and selected randomly without knowledge of clinical outcome.

G. DNA analysis

DNA will be extracted per standard protocols from dried filter papers that had been soaked in blood obtained from patients before and after treatment.[2] Analysis of *pfcrt* K76T mutants will be carried out on extracted DNA by nested mutation specific PCR or PCR with mutation specific RFLP with resolution of amplified DNA fragments by electrophoresis on agarose gel. Wild type and mutant genotypes are distinguished based on predicted size by the appropriate primers or digestion products against controls of known in vitro CQ phenotype.

H. Study Drugs

SP is a fixed combination of 25mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine which are inhibitors of two folate pathway enzymes DHFR and DHPS respectively. SP is an approved drug for the treatment of malaria currently marketed as Fansidar (Roche). It is absorbed from the GI tract and excreted renally. The half life of sulfadoxine is 169 hours and pyrimethamine 111 hours. It is generally well tolerated with minimal side effect profile beyond that of major reactions to sulfonamides in general. It is pregnancy class C and is contraindicated in infants <2months of age.

Amodiaquine hydrochloride is a 4-aminoquinolone chloroquine analog that differs from chloroquine by the presence of an aromatic ring in its side chain. It is a prodrug for the active metabolite desethylamodiaquine. It is absorbed from the GI tract and rapidly converted by first pass metabolism. Dosage recommendations are variable but the standard for therapeutic efficacy trials is 10mg/kg for the first two days and 5mg/kg on the 3rd day. As for chloroquine, its mechanism of action is not entirely known but appears to be involved in preventing polymerization of toxic heme breakdown products within the parasitic food vacuole. Its terminal elimination half life is 1-3 weeks. It is mainly excreted in the bile. Because severe adverse effects of agranulocytosis and hepatitis were noted in some patients after prolonged prophylaxis, it is currently not FDA approved in the US but has been recently added to the WHO list of essential medications for the treatment of severe malaria and is approved and distributed in the UK under the brand name Camoquin (Parke-Davis). There is little or no evidence that it is similarly toxic when used for treatment dosages up to 25mg/kg for 3days. It is contraindicated in pregnancy.

I. Miscellaneous

The primary study will be approved by the institutional review boards of UCSF and Makerere University in Kampala. There are no additional potential risks and no direct benefits to the study subjects incurred by this adjunctive molecular analysis of samples from the primary study. Approval for the use of blood samples for additional analysis is included in the informed consent signed by patients upon enrollment in the primary study. Therefore, no further consent form is included for submission with this proposal.

Subjects will not be compensated for their participation in this study or the primary study.

J. References

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