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Antimalarial Drug Efficacy in *Plasmodium falciparum* Infections in Malawi, Seven Years After Switching From Chloroquine to Sulfadoxine/Pyrimethamine

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In Malawi chloroquine was replaced by sulfadoxine/pyrimethamine (SP) in 1993 because of increasing chloroquine treatment failures in *Plasmodium falciparum* (*P. falciparum*) patients. Seven years after this change, we studied *in vitro* and *in vivo* efficacies of different antimalarial drugs and mutations of dihydrofolate reductase (*dhfr*)/dihydropteroate synthase (*dhps*) genes in *P. falciparum* infections of asymptomatic school children in Salima. The included children were randomly allocated to either treatment group with a standard dose of 3-days chloroquine (n = 50) or a single dose of SP (40) and followed up for 28 days. The *in vivo* sensitivity rate of chloroquine and SP were 92% and 83% respectively. *P. falciparum* isolates were successfully evaluated for *in vitro* drug sensitivity to SP (n = 52), pyrimethamine (52), amodiaquine (14), quinine (36), and mefloquine (17). Although 92% of the isolates were resistant to pyrimethamine, 85% showed *in vitro* sensitivity to SP. All isolates assessed for quinine and mefloquine and 93% of the isolates for amodiaquine showed *in vitro* sensitivity. A high prevalence rate (78%) of parasites with triple Asn-108/Ile-51/Arg-59 *dhfr* and double Gly-437/Glu-540 *dhps* mutations was found in 173 *P. falciparum* infections. Our results suggest that the reduced drug pressure accompanying the policy change consequently resulted in recovery of chloroquine sensitivity in parasites. The high *in vitro* pyrimethamine resistance was consistent with the high prevalence of the *dhfr* triple mutant. However, the high efficacy of SP confirmed the important role of synergism between pyrimethamine and sulfadoxine in the treatment of highly pyrimethamine-resistant parasites.

Key words: *Plasmodium falciparum* malaria, *dhfr* / *dhps*, chloroquine, sulfadoxine / pyrimethamine, Malawi

Introduction

Malaria kills more than 3,000 children per day in Africa and an increasing prevalence of drug resistant strains exacerbates this situation. In Malawi the *in vivo* sensitivity of *Plasmodium falciparum* (*P. falciparum*) to chloroquine decreased

from 59% in 1984[1] to less than 20% in 1990[2]. The *in vitro* sensitivity to chloroquine was 53% in 1988[3]. These results prompted the official change of the first line drug for treatment of uncomplicated malaria from chloroquine to sulfadoxine/pyrimethamine (SP)[4]. *In vivo* studies conducted in Malawi from 1994 to 1998 reported *P. falciparum* sensitivity to SP ranging from 81～88%[5)～7)]. In 1998 the *in vitro* sensitivity to SP and to chloroquine was 38% and 66%, respectively, on isolates from symptomatic malaria patients[7], which suggested a recovery of *in vitro* chloroquine sensitivity and showed a discrepancy of SP sensitivity between *in vivo* and *in vitro* tests.

Mutations in the genes encoding the target enzymes of antifolate drugs have been known for several years to be associated with *in vitro* resistance to these drugs. Pyrimethamine acts by selectively inhibiting dihydrofolate reductase (*dhfr*) in the malaria parasites[8] and *P. falciparum* resistance, *in vivo* and *in vitro*, has been associated with specific point mutations in the *dhfr* gene[9)～13)]. Sulfa drugs act by selectively inhibiting dihydropteroate synthase (*dhps*) earlier in folate pathway of the parasite. The gene encoding *dhps* has been sequenced in *P. falciparum*, and point mutation have been identified that are associated with *in vitro* sulfadoxine resistance under low or no folate testing conditions[14,15].

To investigate these findings further we monitored the current sensitivity of parasites to different antimalarial drugs seven years after Malawi replaced chloroquine with SP. We conducted *in vivo* studies of chloroquine and SP sensitivities in asymptomatic school children and simultaneous *in vitro* tests of chloroquine, pyrimethamine, and SP. Furthermore we studied *dhfr* and *dhps* mutations in *P. falciparum* infections, which are suggested as molecular markers for pyrimethamine and sulfadoxine resistance, respectively[16,17]. In addition, we also examined the *in vitro* efficacy of amodiaquine, quinine and mefloquine as alternative drugs to resistant parasites.

Subjects and Methods

Subjects

Study area

The study was conducted during June and July 2000 in the two primary schools of Maonga and Chimbala villages of Salima District, situated along Lake Malawi. These rural communities are about 20 km away from Salima town. The malaria transmission in this area is perennial, *P. falciparum* is the dominant parasite, and *Anopheles gambiae* is the principal vector[18].

Study population and data collection

School children aged 6～15 years old (yrs) were the main focus of this study and made up 83% of the enrolled population. Some children <6 yrs and adults living in the schools' neighbourhood were also included during the case selection.

The team's clinical officer ascertained the recent malaria history and conducted a physical examination (body temperature and weight and spleen check) of each participant. Thick and thin blood smears were prepared, stained with Giemsa solution and examined under microscopy. If positive, the number of parasites per 100 white blood cells (WBCs) was counted and the parasite density per μL of blood was estimated by assuming a WBC count of 8000 per μL of blood[19]. Only school children with a monoinfection of *P. falciparum* and an asexual parasitaemia of 400～800 parasites per μL of blood were included in the *in vivo* test and those with >800 parasites per μL of blood were included in both *in vitro* and *in vivo* tests. All subjects with >400 parasites per μL of blood were included in genetic analysis. Infected subjects who were not selected for the study were given the standard SP treatment.

Methods

Filter paper blood sampling

Concomitantly with blood smears, finger prick

blood samples were drawn into one or two capillary tubes (75 μL, heparinised, Drummond Scientific Company, USA) and transferred on to chromatography filter paper (31ETCHR, Whatman Limited, England). The dried filter paper samples were stored in small plastic bags at −20 ℃ prior to analyses of parasite genotype by polymerase chain reaction (PCR) and DNA sequencing, and of drug levels by high-performance liquid chromatography (HPLC).

***In vivo* test**

The included children were randomly allocated to either treatment group and were not informed of the specific drug that they received. Either a total amount of 25 mg of chloroquine base per kg body weight over 3 consecutive days or a single dose of SP scaled by body weight to an adult dose of 3 tablets was given. Chloroquine was administered as a tablet containing 250 mg of chloroquine diphosphate (Resochin®, Bayer), corresponding to 150 mg of chloroquine base. SP was given as a tablet containing 500 mg of sulfadoxine and 25 mg of pyrimethamine (Fansidar®, Roche). Each individual was observed for at least 30 minutes after treatment by the principal investigator. If he/she vomited, treatment was repeated.

The follow-up of subjects was conducted in their schools or residences. The parasitological and clinical examinations described above were repeated on days 3, 7, 14, 21, and 28 after the initial treatment. Therapeutic efficacy was classified according to WHO criteria of sensitive (S) and degrees of resistance. RIII, day 3 parasite density (PD) of >25% of day 0 PD; RII, day 3 PD of <25% of day 0 PD and persistent asexual parasitaemia on day 7; RI, no parasites on day 7 followed by recrudescence by day 28; and S, no parasites on day 7 and absence of recrudescence during follow-up.

The trial was terminated if subjects did not complete the scheduled treatment, developed side effects, deteriorated in their clinical conditions or the parasite response was defined as RIII, RII or RI. Under these conditions, a standard treatment of SP or Malarone was alternatively provided. If the subjects developed side effects such as pruritis, skin reactions or severe gastrointestinal-tract symptoms, these events were immediately reported to the study team for prompt actions.

Drug concentrations

Sulfadoxine (SDX) concentrations were determined in a dried filterpaper blood spot (75 μL blood) on day 3 and week 1 in the subjects treated with SP by a HPLC method described elsewhere[20]. Limit of determination using 75 μL of capillary blood spotted on filter paper was 25 μmol/L. Chloroquine and desethylchloroquine concentrations were also determined on day 3 and week 1 in the subjects treated with chloroquine, by a modified HPLC method described elsewhere[21]. Limit of determination using 75 μL of capillary blood spotted on filter paper was 15 nmol/L. In these subjects under *in vivo* tests sulfadoxine, chloroquine and desethylchloroquine concentrations were also determined on day 0 (before treatment).

***In vitro* test**

The *in vitro* sensitivity of *P. falciparum* to SP, pyrimethamine, amodiaquine, quinine, and mefloquine was assessed using WHO microtest kits [Document CTD/MAL/97.20. Instructions of use of the *in vitro* micro-test kit (Mark III), 1997] incubated at 37.5 ℃ for 20∼25 hours in a candle jar. Although we had also assessed sensitivity to chloroquine, we do not present them because there is recent information that the WHO prepared plates distributed during the period of 1999∼2000 were flawed for their CQ levels (Wernsdorfer personal communication) and this would invalidate the results.

After incubation, parasites were harvested and

Giemsa-stained thick smears were prepared. The number of schizonts per 200 asexual parasites was estimated. For amodiaquine, quinine, and mefloquine tests, a schizont was defined as an asexual parasite with ≧3 nuclei. For SP and pyrimethamine tests, a schizont was defined as an asexual parasite having≧8 nuclei. A valid test was defined as a series with growth of≧20 schizonts per 200 asexual parasites in the corresponding control well. The inhibitory effect of the drug was estimated as the difference between the number of schizonts in the control and in the drug dosed well divided by the number of schizonts in the control well. Complete inhibition of schizonts maturation at drug concentrations of≦0.4 μmol/L blood for amodiaquine, ≦2.56 μmol/L BMM for quinine, and≦3.2 μmol/L blood for mefloquine was considered as *in vitro* sensitivity to respective drugs. For pyrimethamine and SP the threshold was set at < 90% of schizont maturation inhibition at 75 nmol/L pyrimethamine of BMM[22].

Parasite genotyping

A quarter of blood spot (19 μL) collected onto filter paper were used as a source of parasite DNA, using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction with some modifications as described elsewhere[23].

The polymerase chain reaction (PCR) and following direct sequence were performed for both *dhfr* and *dhps* genes as described elsewhere[8]. Briefly, nested PCR products of 592 bp for *dhfr* and 727 bp for *dhps* were purified with electrophoresis using 1.2% agarose gel and ethanol (100 % and 70%) precipitation. To minimize sequence artefacts, DNA sequence was determined from both directions using Dye Primer Cycle Sequencing Kit (Perkin Elmer, UK) in an ABI PRISM™ 377 DNA Sequencer. M13 primers for sequencing used in nested PCR were M 13 in 1: 5′-CGCTGTAAAACGACGGCCAGTCTCCTTTTTATGATGGAACAAGTC-3′ and M 13 in 2: 5′-CGGTGTAAAACGACGGCCAGTCATCACATTCATATGTACTATTTATT-3′ for DHFR, M13 ps4: 5′-CCATGTAAAACGACGGCCAGTGGTATTTTTGT-3′ and M13ps3: 5′-ACATGTAAAACGACGGCCAGTATCCAATT-3′ for DHPS. The entire gene was sequenced completely on both strands. Nucleotide sequences were analyzed using GENETIX MAC Ver 8 programs. Two different bases detected in same position were regarded as mixed genotype.

Statistical analysis

Effective concentrations (EC_{50} and EC_{90}) were estimated from the mean percentage maturation of schizonts after standardizing for control growth by the method of Raymond (1983) using the probit software provided by WHO. The chi-square test was used to assess the statistical significance of differences between sensitivity and resistance patterns.

Ethical consideration

The study was approved by the local ethics committees of the Malaria Control Programme of the Malawi Ministry of Health. The village leaders and school teachers were informed of and consented to the study. Parents were briefed on the purpose and procedure of the study and their consent determined their child's participation.

Results

Case selection

We examined a total of 504 subjects Table 1. Among them, 393 (78%) were infected with malaria. Monoinfections of *P. falciparum* and *P. malariae* accounted for 97% and 1% of the positives respectively, while 2% had mixed infections. The overall spleen rate was 55%. All the malariometric parameters were greater in children < 6 years than in those 6〜15 years Table 1.

***In vivo* sensitivity**

A total of 95 children, who fulfilled the inclusion

Table 1 Case selection: age-specific malariometric parameters in Salima, Malawi, 2000

	Age (years)			Total population
	<6	6~15	>15	
Number of individuals examined	57	418	29	504
Number of malaria positive individuals	53 (93%)	328 (78%)	12 (41%)	393 (78%)
P. falciparum	49	321	12	382 (97%)
P. malariae	2	1	0	3 (1%)
Mixed infection	2	6	0	8 (2%)
Parasite density per μL of blood: Mean	2,080	780	400	800
Range	160~33,200	80~25,840	80~5,040	80~33,200
Spleen rate (%)	72	55	31	55

Table 2 *In vivo* tests: characteristics of enrolled population in Salima, Malawi

	Chloroquine	SP
Number of individuals enrolled	54	41
Number of individuals excluded	4	1
Mean age (years)	9.2 [6 ~ 14]	9.5 [6 ~ 15]
Mean body weight (kg)	24.5 [14 ~ 54]	25.3 [13 ~ 46]
Mean body temperature (℃)	36.2 [35.3 ~ 37.2]	36.3 [35.3 ~ 38]
Mean parasite density/μL of blood	1,760 [400 ~ 16,080]	1,840 [400 ~ 26,240]

Ranges shown in brackets.

Table 3 *In vivo* responses of *P. falciparum* infections in school children, Salima, Malawi, 2000

Drug	Treated (n)	Sensitive	RⅢ	RⅡ	RⅠ (days)		
					14	21	28
Chloroquine	50	46	0	1	0	2	1
SP	40	33	0	1	0	2	4

RⅢ: day 3 parasite density (PD) of >25% of day 0 PD,
RⅡ: day 3 PD of <25% of day 0 PD and persistent asexual parasitaemia on day 7,
RⅠ: no parasites on day 7 followed by recrudescence by day 28,
S: no parasites on day 7 and absence of recrudescence during follow-up.

criteria and gave study consent, were enrolled in the *in vivo* study for either chloroquine or SP Table 2. The mean age and clinical and parasitological profiles of both treatment groups were similar. Five of the 95 children were excluded from the results because they did not complete treatment (n = 2) or moved away from Salima district during the follow-up period (n = 2). The fifth excluded (girl, 13 yrs) developed a mild skin reaction one day after receiving SP. She was treated with antihistamines and the skin reaction gradually disappeared within two weeks.

The *in vivo* parasitological responses to chloroquine and SP are shown in Table 3. Overall sensitivity rate of chloroquine and SP were 92% and 83% respectively. Only one case of each group showed RII resistance and the rest were RI (parasite recrudescence on day 21 or 28).

Drug concentrations

None of a total of 90 subjects under *in vivo* tests Table 3 had any detectable concentration of sulfadoxine, chloroquine or desethylchloroquine

Table 4 *In vitro* responses of *P. falciparum* isolates in Salima, Malawi, 2000

Drug	Number of valid isolates	Responses	
		Sensitive (%)	Resistant (%)
SP	52	85	15
Pyrimethamine	52	8	92
Amodiaquine	14	93	7
Quinine	36	100	0
Mefloquine	17	100	0

Sensitive responses: complete schizont inhibition at 3.2 μmol/L blood for amodiaquine, 2.56 μmol/L blood medium mixture (BMM) for quinine, 3.2 μmol/L blood for mefloquine (WHO, 1997); >90% schizont maturation inhibition at 75 nmol/L pyrimethamine of BMM for pyrimethamine and SP (Philipps et al 1998).

on day 0.

Sulfadoxine concentrations (μmol/L) in capillary blood after a single dose SP administration were 205 (107～311) [median (range)] in sensitive subjects (n = 33) and 196 (161～250) in resistant subjects (n = 7) on day 3, and 117 (46～190) and 113 (78～140) μmol/L, respectively, on week 1.

Chloroquine / desethylchloroquine concentrations in capillary blood after initiation of 3-day chloroquine treatment were 1,883 (685～3,780) / 890 (335～1,789) in sensitive subjects (n = 46) and 2,740 (1,904～6,411) / 1,722 (1,361～2,719) in resistant subjects (n = 4) on day 3, and 497 (87～1,280) / 314 (103～900) and 540 (312～1,280) / 536 (291～636), respectively, on week 1.

***In vitro* sensitivity**

P. falciparum isolates from 104 subjects were tested for *in vitro* drug sensitivity. All isolates were assessed for sensitivities to SP, pyrimethamine, and either amodiaquine (24), quinine (53), or mefloquine (27). In total 55% (171/312) of the isolate test series were valid Table 4. In 37% of the tests control schizont counts did not reach ≧20 per 200 asexual parasites. Other reasons for test failures included bacterial contamination (6 %) and staining failure (1%). A significantly lower proportion of antifolate (pyrimethamine and SP) series relative to those of other antimalarials were valid (50% vs 65%, p = 0.003), presumably due to the different definitions of a mature schizont (≧8 vs ≧3 nuclei).

The *in vitro* responses of *P. falciparum* isolates to different antimalarial drugs based on minimum inhibitory concentration are shown in Table 4. The mean percentage maturation of schizonts by different drugs and the EC_{50} and EC_{90} estimated by using probit software[24] are shown in Figure. Although 92% of the isolates were resistant to pyrimethamine, 85% showed complete inhibition at the cut-off concentration of SP Table 4. Amodiaquine completely inhibited 93% of the isolates at the cut-off concentration. All isolates assessed for quinine and mefloquine were completely inhibited at the respective cut-off point concentrations.

Parasite *dhfr* and *dhps* genotypes

The direct sequencing of *dhfr* and *dhps* genes from nested PCR amplifications gave nucleic acid and presumptive amino acid sequences for a total of 173 *P. falciparum* isolates. Among them, *dhfr* codons 16, 51, 59, 108, and 164 were readable in 172 isolates, and *dhps* codons 436, 437, 540, 581 and 613 were readable in 168 isolates Table 5.

In *dhfr* genes no mutations were detected at codons 16 and 164. Out of 172 isolates, a total of 158 isolates possessed *dhfr* gene with 3 mutations (Ile-51, Arg-59, and Asn-108), 11 isolates had 2 mutations (Arg-59 and Asn-108/Ile-51 and Asn-108),

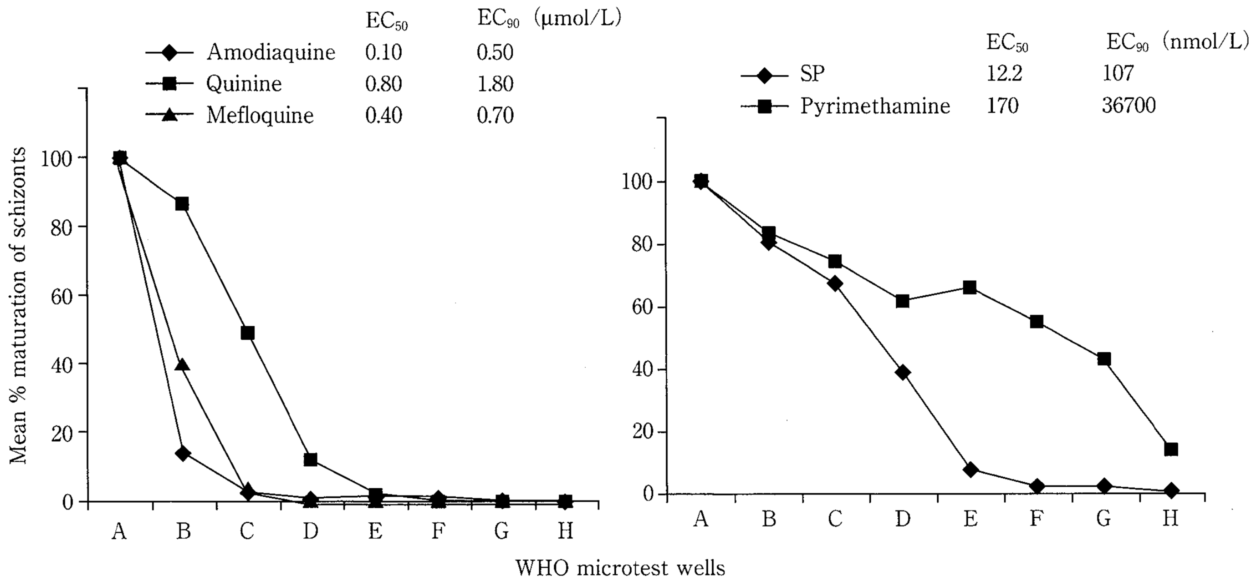

Figure *In vitro* test: mean percentage maturation of schizonts and EC_{50} and EC_{90} estimated by using software provided by WHO (Raymond, 1983). EC_{50} and EC_{90} of SP expressed as pyrimethamine concentration. Well A is the control.

Drugs	Concentrations [Wells B to H]
Amodiaquine (μmol/L blood);	0.05; 0.1; 0.2; 0.4; 0.8; 1.6 and 3.2
Quinine (μmol/L BMM);	0.08; 0.16; 0.32; 0.64; 1.28; 2.56 and 5.12
Mefloquine (μmol/L blood);	0.4; 0.8; 1.6; 3.2; 6.4; 12.8 and 25.6
Pyrimethamine (nmol/L BMM);	2.5; 7.5; 25; 75; 250; 750 and 2500
SP-pyrimethamine;	as above
-sulfadoxine (nmol/L BMM);	200; 600; 2000; 6000; 20000; 60000; 200000

BMM: blood-medium mixture.

and only 3 isolates had wild-type *dhfr* gene.

In *dhps* genes no mutations were detected at codons 436, 581, and 613. Out of 168 isolates a total of 148 possessed *dhps* gene with 2 mutations (Gly-437 and Glu-540), 13 isolates had one mutation (Glu-540), and only 7 isolates had wild-type *dhps* gene.

Among 7 *in vivo* SP resistant cases, 4 cases including one RII possessed 3 mutations in *dhfr* and 2 mutations in *dhps* as mentioned above. The other 3 cases consisted of Arg-59 and Asn-108 in *dhfr*/2 mutations in *dhps* (n = 1), 3 mutations in *dhfr*/Glu-540 in *dhps* (n = 1), and Arg-59 and Asn-108 in *dhfr*/Glu-540 in *dhps* (n = 1). We could not see any statistical significant association between parasite genotypes (*dhfr*/*dhps*/combination) and efficacy phenotypes (*in vivo* SP/*in vitro* SP or pyrimethamine).

Discussion

Although chloroquine resistance was suspected in the early 1980s in Malawi[25], the first case of chloroquine resistant *P. falciparum* assessed according to the WHO standards was confirmed in 1983[26]. A hospital based *in vivo* study which was conducted around the same time in children $<$ 5 yrs using a 7-days assessment, reported chloroquine resistance of 41%[1]. The highest level of resistance (RIII/RII $>$ 80%) was reported in the beginning of 1990s when chloroquine was not able neither to produce a durable clinical and parasitological recovery nor produce an optimal haematological recovery (Hb) in very

Table 5 Point mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes of *P. falciparum* isolates in Salima, Malawi, 2000

Isolates (n)	*dhfr* Amino acids residue					*In vitro* responses*
	16	51	59	108	164	to pyrimethamine
3	Ala	Asn	Cys	Ser	Ile	sensitive
6	Ala	Asn	Arg	Asn	Ile	moderate resistant
5	Ala	Ile	Cys	Asn	Ile	moderate resistant
1	Ala	Asn/Ile	Arg	Asn	Ile	moderate resistant
1	Ala	Ile	Cys/Arg	Asn	Ile	moderate resistant
156	Ala	Ile	Arg	Asn	Ile	moderate resistant

Isolates (n)	*dhps* Amino acids residue					*In vitro* responses*
	436	437	540	581	613	to sulfadoxine
7	Ser	Ala	Lys	Ala	Ala	sensitive
11	Ser	Ala	Glu	Ala	Ala	moderate resistant
6	Ser	Ala/Gly	Glu	Ala	Ala	moderate resistant
2	Ser	Ala	Lys/Glu	Ala	Ala	moderate resistant
142	Ser	Gly	Glu	Ala	Ala	moderate resistant

Underline indicate amino acid changes.
*: Plowe et al 1997.

young paediatric patients[2]. Based on these findings, the country replaced chloroquine with SP as first line drug treatment for uncomplicated malaria [4].

In this study seven years after the policy change we found *in vivo* chloroquine resistance were 2% and 2% of 7 and 14-day assessments respectively in the asymptomatic school children, compared to 20% and 32% in Tanzania, a neighboring country with ongoing chloroquine use[27]. These *in vivo* results are consistent with the previous *in vitro* results conducted in the same area in 1998, which suggested recovery of chloroquine sensitivity[7]. Recently, the K76T mutation in *pfcrt* gene was suggested to convey chloroquine resistance in *P. falciparum*[28]. In a separate study we found a remarkably low prevalence (7%) of the *pfcrt* K76T mutation in *P. falcipatum* isolates in Malawi relative to neighbouring African countries (40~80%) with ongoing chloroquine selection pressure (submitted). These data suggest that the reduced drug pressure accompanying the policy change resulted in a significantly lower prevalence of the *pfcrt* K76T mutation and consequently recovery of chloroquine sensitivity in the parasites.

These findings in Malawi are consistent with earlier suggestions of preserving the antimalarial efficacy of chloroquine by the recovery of sensitivity after removing the selective pressure[29]. Similar findings have also been reported from other areas. In Thailand, chloroquine-resistant *P. falciparum* cases were first described in 1961[30]. Chloroquine resistant Thai strains increased rapidly in terms of numbers and levels of resistance and by mid 1960s a standard dose of chloroquine produced little or no improvement. In 1972 chloroquine was replaced with SP and later with the combination of SP and mefloquine in 1985 because of widespread SP resistance. While chloroquine was being discontinued, chloroquine EC_{50} values decreased from 3.3×10^{-6} M in 1982[31] to

0.8×10^{-6} M in 1990[32]. In Gabon, *in vitro* chloroquine resistance was first documented in 1983[33]. After the change of the antimalarial drug policy in 1992, the *in vitro* chloroquine sensitivity rate increased from 6% in 1994[34] to 55% in 1998 [35].

In genetic analyses we found majority of parasites possess 3 mutations in *dhfr* gene (the codons 108 with 51 and/or 59 and 2 mutations in *dhps* gene, which convey high resistance to pyrimethamine and moderate resistance to sulfadoxine, respectively[17][36]. The former result was consistent with the observed high prevalence of *in vitro* pyrimethamine resistance. Nevertheless the observed high efficacy of both *in vitro* and *in vivo* SP confirms the important role of synergism between pyrimethamine and sulfadoxine in the treatment of moderately pyrimethamine-resistant parasites. Recently, the primary basis for this synergy is suggested to arise from pyrimethamine targeting site (or sites) in addition to *dhfr*, which restores *dhps* as a relevant target for sulfadoxine by blocking folate uptake and/or utilization of parasites[37]. Plowe et al (1997)[17] suggested that the *dhfr* Leu-164 mutation appears late in the course of development of SP resistance and is likely to play an important role in therapeutic failure.

In a follow-up of 14 days, we found only one of 40 asymptomatic school children showed RII parasitological resistance to SP, in contrast to nine of 65 symptomatic children under five showed RII/RIII in the previous study conducted in the same area in 1998[7]. The difference of these two studies may be partially explained by the degrees of semi-immunity in the populations. In case selection we observed a clear age-related pattern in malaria prevalence that suggests the development of a high degree of semi-immunity in this holo-endemic population. These results are consistent with previous reports suggesting the degree of age-related immunity is an important factor influencing the effectiveness of antimalarial treatment[38][39]. Small children with minimal acquired immunity are likely to be affected most severely as the prevalence of resistant strains of *P. falciparum* increase.

Recently SP has become the first-line drug for uncomplicated malaria in many African countries burdened by chloroquine resistant *P. falciparum* strains. Although low levels of *in vivo P. falciparum* sensitivity to SP have been reported in neighbouring Tanzania (26%)[40] and Zambia (42%)[41], our results indicate that SP is still effective in the semi-immune population of Malawi.

All *P. falciparum* isolates tested for quinine and mefloquine were sensitive and the isolates tested for amodiaquine also showed high sensitivity. In Malawi, quinine is used as the second line treatment for SP-resistant malaria as well as for severe and complicated cases. Mefloquine has been used sparingly for chemoprophylaxis mainly in the private sector and among expatriates. Amodiaquine is a potential component of combination treatments. Our *in vitro* findings confirm that these alternative medicines to SP-resistant parasites are still effective in the study area. These results are compatible with those from other African countries[22].

When we compare our *in vitro* study with the previous one conducted in the same locality in 1998[7], our study showed higher *in vitro* sensitivities to all tested drugs (SP, mefloquine, and quinine). This can partially be explained by difference of case selection, that is, isolates in our study were selected from asymptomatic parasite carriers, while those of the previous study were from symptomatic patients seeking treatment at the health centre.

Although pyrimethamine concentrations are more critical than sulfadoxine in clinical efficacy of SP treatment, the former can not be determined from filter paper at present. However cor-

responding correlation between plasma concentrations of pyrimethamine and sulfadoxine is found to be high[20]. We found sufficient drug levels on day 3 and week 1 in capillary blood in the patients who showed parasite recrudescence during follow-up, suggesting these cases are true resistance to either SP or chloroquine. We could detect neither chloroquine nor sulfadoxine at all in blood on day 0 of the studied school children, suggesting community use of antimalarial drugs is generally low even though high malaria endemicity in the study area. In some African communities with high malaria endemicity, antimalarial drug use for symptoms is quite high, e.g. a study in Ghana has shown a high rate (78%) of detectable chloroquine in children before treatment[42]. In Vanuatu, the Southeast Pacific, the relatively low rates of detectable sulfadoxine (8%) and chloroquine (20%) were observed in capillary blood in the patients before treatment.

The results of *in vivo* tests measure directly the real treatment efficacy in patients by incorporating host factors such as acquired immunity to parasites and drug bioavailability. However, it is often difficult to distinguish between reinfection and late recrudescence in the *in vivo* trials. This difficulty highlights the importance of simultaneously carrying out both *in vivo* and *in vitro* tests. Although *in vitro* assays are complementary to *in vivo* tests, *in vitro* results are more directly associated with drug resistance in parasites because they eliminate host factors[43]. *In vitro* tests also allow multiple drug challenges (including new compounds) of individual isolates, investigation of cross-resistance patterns, and comparisons of degrees of drug resistance of different areas at different times. Thus, both *in vivo* and *in vitro* tests have their advantages and are complimentary. Isolates investigated in symptomatic patients or asymptomatic parasite carriers have both provided the necessary foundation for designing efficient trials and preventive strategies against malaria in holoendemic areas[44]. Molecular epidemiological surveys of resistant parasite genotypes may strengthen effectiveness of the existing *in vivo*–*in vitro* test system in fields.

Our study in Malawi clearly shows the recovery of chloroquine sensitivity. The combination of sulfadoxine and pyrimethamine was effective in the presence of pyrimethamine-resistance isolates. The findings also confirm that the possible alternative drugs to SP-resistant parasites in Sub-Saharan Africa (quinine, mefloquine and amodiaquine) are highly effective in the study area.

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References

1) **Heymann DL, Khoromana CO, Wirima JJ et al**: Comparative efficacy of alternative primary therapy for *Plasmodium falciparum* infections in Malawi. Trans R Soc Trop Med Hyg **81**: 722–724, 1987
2) **Bloland PB, Lackritz EM, Kazembe PN et al**: Beyond chloroquine: implications of drug resistance for evaluating malaria therapy efficacy and treatment policy in Africa. J Infect Dis **167**: 932–937, 1993
3) **Slutsker LM, Khoromana CO, Payne D et al**: Mefloquine therapy for *Plasmodium falciparum* malaria in children under 5 years of age in

Malawi: *in vivo/in vitro* efficacy with parasitological outcome. Bull World Health Organ **88**: 53–59, 1990

4) **Ministry of Health Malawi**: Guidelines for the Management of Malaria. Ministry of Health Malawi, Lilongwe (1992)
5) **Nwanyanwu OC, Ziba C, Kazembe P et al**: Efficacy of sulphadoxine/pyrimethamine for *Plasmodium falciparum* malaria in Malawian children under five years of age. Trop Med Int Health **1**: 231–235, 1996
6) **Nwanyanwu OC, Ziba C, Macheso A et al**: Efficacy of sulphadoxine-pyrimethamine for acute uncomplicated malaria due to *Plasmodium falciparum* in Malawian children under five years old. Trop Med Int Health **5**: 355–358, 2000
7) **Takechi M, Matsuo M, Ziba C et al**: Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility *in vitro* of *P. falciparum* isolates to sulphadoxine-pyrimethamine and other antimalarial drugs in Malawian children. Trop Med Int Health **6**: 429–434, 2001
8) **Bzik DJ, Li WB, Horii T et al**: Molecular cloning and sequence analysis of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc Natl Acad Sci USA **84**: 8360–8364, 1987
9) **Peterson DS, Walliker D, Wellems E**: Evidence that a point mutation in dehydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA **85**: 9114–9118, 1988
10) **Cowman AF, Morry MJ, Biggs BA et al**: Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA **85**: 9109–9113, 1988
11) **Zolg JW, Plitt JR, Chen GX et al**: Point mutations in the dihydrofolate reductase-thymidylate synthase gene as the molecular basis for pyrimethamine resistance in *Plasmodium falciparum*. Mol Biochem Parasitol **36**: 253–262, 1989
12) **Peterson DS, Milhous WK, Wellems TE**: Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA **87**: 3018–3022, 1990
13) **Foote SJ, Galatis D, Cowman AF**: Amino acids in the dihydrofolate reductase-thymidylated synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. Proc Natl Acad Sci USA **87**: 3014–3017, 1990
14) **Triglia T, Cowman AF**: Primary structure and expression of the dihydropteroate synthase gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA **91**: 7149–7153, 1994
15) **Brooks DR, Wang P, Read M et al**: Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Palsmodium falciparum*, with differing resistance to sulfadoxine. Eur J Biochem **224**: 397–405, 1994
16) **Reeder J C, Rieckmann K H, Genton B et al**: Point mutations in the dihydrofolate reductase and dihydropteroate synthetase genes and *in vitro* susceptibility to pyrimethamine and cycloguanil of *Plasmodium falciparum* isolates from Papua New Guinea. Am J Trop Med Hyg **55**: 209–213, 1996
17) **Plowe CV, Cortese JF, Djimbe A et al**: Mutation in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis **176**: 1590–1596, 1997
18) **Khoromana CO, Campbell CC, Wirima JJ et al**: *In vivo* efficacy of chloroquine treatment for *Plasmodium falciparum* in Malawian children under five years of age. Am J Trop Med Hyg **35**: 465–471, 1986
19) **Gilles HM, Warrel DA**: Epidemiology of malaria. *In* Bruce-Chwatt's Essential Malariology. 3rd ed (Gilles HM ed), pp124–163, Edward Arnold, London (1993)
20) **Bergqvist Y, Hjelm E, Rombo L**: Sulfadoxine assay using capillary blood samples dried on filter paper-suitable for monitoring of blood concentrations in the field. Ther Drug Monit **9**: 203–207, 1987
21) **Bergqvist Y, Frisk-Holmberg M**: Sensitive method for the determination of chloroquine and its metabolite desethyl-chloroquine in human plasma and urine by high-performance liquid chromatography. J Chromatogr **221**: 119–127, 1980
22) **Philipps J, Radloff PD, Wernsdorfer W et al**: Follow-up of the susceptibility of *Plasmodium falciparum* to antimalarials in Gabon. Am J Trop Med Hyg **58**: 612–618, 1998
23) **Sakihama N, Kaneko A, Hattori T et al**: Limited recombination events in merozoite surface protein-1 alleles of *Plasmodium falciparum* on islands. Gene **14**: 41–48, 2001
24) **Raymond M**: Presentation d'un programme d'analyse log-probit pour micro-ordinnateur. Ser Ent

Med et Parasitol **22**: 117–121, 1985

25) **Slatter MJ, Pettengel K, Taylor RKM**: Chloroquine resistant malaria. S Afr Med J **63**: 838, 1983

26) **Overbosch D, van Den W, Bake AWL et al**: Chloroquine-resistant *falciparum* malaria from Malawi. Trop Geogr Med **36**: 71–72, 1984

27) **Mshinda H, Font F, Hirt R et al**: A comparative study of the efficacy of chloroquine and pyrimethamine-dapsone combination in clearing *Plasmodium falciparum* parasitaemia in school children in Tanzania. Trop Med Int Health **6**: 797–801, 1996

28) **Djimde A, Doumbo OK, Cortese JF et al**: A molecular marker for chloroquine-resistant *falciparum malaria*. N Engl J Med **344**: 257, 2001

29) **White NJ**: Antimalarial drug resistance: the pace quickens. J Antimicrob Chemother **30**: 571–585, 1992

30) **Harinasuta T, Migasen S, Boonag D**: Chloroquine resistance in *Plasmodium falciparum* in Thailand. In UNESCO 1st Regional Symposium on Scientific Knowledge of Tropical Parasites Singapore, pp148–153, UNESCO, Paris (1962)

31) **Thaithong S, Suebsaeng L, Rooney W et al**: Evidence of increasing chloroquine sensitivity in Thai isolates of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg **82**: 37–38, 1998

32) **Wongsrichanalai C, Webster HK, Wimonwattrawatee T et al**: *In vitro* sensitivity of *Plasmodium falciparum* isolates in Thailand to quinine and chloroquine, 1984–1990. Southeast Asian J Trop Med Public Health **23**: 533–536, 1992

33) **Buchard GD, Hortsmann RD, Wernsdorfer WH et al**: *Plasmodium falciparum* malaria: resistance to chloroquine, but sensitive to mefloquine in the Gabon. A prospective *in-vitro* study. Trop Med Parasitol **35**: 1–4, 1984

34) **Brandts CH, Wernsdorfer WH, Kremsner PG**: Decreasing chloroquine resistance in *Plasmodium falciparum* isolates from Gabon. Trans R Soc Trop Med Hyg **94**: 54–556, 2000

35) **Schwenke A, Brandts C, Phillips J et al**: Declining chloroquine resistance of *Plasmodium falciparum* in Lambrene, Gabon from 1992 to 1998. Wien Klin Wochenschr **113**: 63–64, 2001

36) **Triglia T, Cowman AF**: The mechanism of resistance to sulfa drugs in *Plasmodium falciparum*. Drug Resistance Updates **2**: 15–19, 1999

37) **Wang P, Brobey RK, Horii T et al**: Utilization of exogenous folate in the human malaria parasite *Plasmodium falciparum* and its critical role in antifolate drug synergy. Mol Microbiol **32**: 1254–1262, 1999

38) **Kaneko A, Taleo GK, Shirakawa C**: Island malaria control in eastern Melanesia: 2) age-specific manifestation in islanders of drug resistance in *Plasmodium falciparum* and *Plasmodium vivax*. Jpn J Parasitol **43**: 371–383, 1994

39) **Kamya MR, Dorsey G, Gasasira A et al**: The comparative efficacy of chloroquine and sulfadoxine-pyrimethamine for the treatment of uncomplicated malaria in Kampala, Uganda. Trans R Soc Trop Med Hyg **95**: 50–53, 2001

40) **Rϕnn AM, Msangeni HA, Mhina J et al**: High level of resistance of *Plasmodium falciparum* to sulfadoxine-pyrimethamine in children in Tanzania. Trans R Soc Trop Med Hyg **90**: 179–181, 1996

41) **Bijl HM, Kager J, Koetsier DW et al**: Chloroquine and sulfadoxine-pyrimethamine-resistant falciparum in vivo—a pilot study in rural Zambia. Trop Med Int Health **5**: 692–695, 2000

42) **Ofori-Adjei D, Commey JO, Adjepon-Yamoah KK**: Serum chloroquine levels in children before treatment for malaria. Lancet **1**: 1246, 1984

43) **Wernsdorfer WH**: Epidemiology of drug resistance in Malaria. Acta Trop **56**: 143–156, 1994

44) **Owusu-Agyei S, Koram KA, Baird JK et al**: Incidence of symptomatic and asymptomatic *Plasmodium falciparum* infection following curative therapy in adults residents of Northern Ghana. Am J Trop Med Hyg **65**: 197–203, 200

マラウイ国における熱帯熱マラリア感染に対する抗マラリア薬剤効果
—chloroquine より sulfadoxine/pyrimethamine への変更7年後の経過—

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マラウイでは，熱帯熱マラリア患者の chloroquine 治療失敗例の増加に伴い，1993 年から sulfadoxine/pyrimethamine (SP) が chloroquine に代り導入された．この変更から 7 年後，我々はサリマ地区の無症候性熱帯熱マラリア感染学童において *in vitro* および *in vivo* 抗マラリア剤効果，またそれぞれ pyrimethamine および sulfadoxine 耐性と関連する原虫 dihydrofolate reductase 遺伝子（*dhfr*）および dihydropteroate reductase 遺伝子（*dhps*）変異について検討した．

対象学童は無作為に chloroquine 3 日間の標準投与群（n＝50）ないしは SP 一回投与群（n＝40）に分けられ，治療後 28 日間にわたり経過が追跡された．*In vivo* における chloroquine および SP 感受性率はそれぞれ 92% および 83% であった．分離熱帯熱マラリア原虫株の *in vitro* 薬剤感受性は SP（n＝52），pyrimethamine (52)，quinine (36)，mefloquine (17) および amodiaquine (14) に対して検討された．分離株の 92% が pyrimethamine 耐性を示したにも関わらず，85% は SP 感受性であった．Quinine および mefloquine に対して検討したすべて，および amodiaquine に対する 93% の分離株は *in vitro* 感受性であった．173 例の熱帯熱マラリア感染において，3 重変異 Asn-108/Ile-51/Arg-59 *dhfr* および 2 重変異 Gly-437/Glu-540 *dhps* を持つ原虫が高頻度（78%）で認められた．

これらの結果は治療薬剤変更に伴う薬剤圧の減少が原虫 chloroquine 感受性の回復をもたらしたことを示唆した．高度の pyrimethamine に対する *in vitro* 耐性は高頻度に *dhfr3* 重変異が見られたことと一致した．それにもかかわらず観察された高い SP の効果は，高度 pyrimethamine 耐性原虫における sulfadoxine および pyrimethamine 間の相乗作用の重要性を示唆した．