

Original Article

Rifampicin Antagonizes the Effect of Chloroquine on Chloroquine-Resistant *Plasmodium berghei* in Mice

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SUMMARY: Chloroquine (CQ)-resistant *Plasmodium falciparum* appears to decrease CQ accumulation in its food vacuole by enhancing its efflux via an active membrane pump, which has been reported to be a P-glycoprotein-like transporter. Rifampicin (RIF) is a P-glycoprotein inhibitor and also has some antimalarial activity. It is hoped that a combination of chloroquine-rifampicin (CQ + RIF) would be advantageous in the treatment of CQ-resistant malaria. Swiss albino mice were inoculated with CQ-resistant *P. berghei* intraperitoneally, and studied for the effect of CQ versus the combination of CQ + RIF at various doses on the clearance of parasitemia, the survival of the mice, and the recrudescence of malaria. Paradoxically, RIF decreased the survival rate and rate of clearance of parasitemia and increased the rate of recrudescence significantly when combined with various doses of CQ. Our results indicated that RIF worsened the course of the disease, and we concluded that RIF should not be combined with CQ in the treatment of malaria.

INTRODUCTION

Chloroquine (CQ) has been one of the most successful and widely prescribed drugs for malaria due to its efficacy and safety. It is believed that CQ interferes with the heme polymerisation process of plasmodia, which converts heme into non-toxic hemozoin in the food vacuole by forming toxic heme-CQ complexes (1-5). CQ-resistant *Plasmodium falciparum*-infected erythrocytes are known to accumulate less CQ than their sensitive counterparts (6).

P-glycoprotein (P-gp) functions as an efflux pump of many chemotherapeutic agents and causes multidrug resistance (MDR) (7). CQ is a known substrate for the P-gp pump, and it is recognized that other membrane transporters in the ATP-binding cassette family (which includes P-gp) share a considerable amount of common substrates and inhibitors. Pgh1, a homologue of P-gp, is present on the food vacuolar membrane of plasmodia (8). Therefore, it was postulated that CQ-resistant *P. falciparum* expels CQ from the food vacuole via a Pgh1 pump similar to that of MDR cancer cells. Subsequently, *pfmdr1* Y86 (encoded for Pgh1) showed substantial correlation with a high level of drug resistance (9,10).

In principle, P-gp-mediated drug resistance can be circumvented by P-gp inhibitory agents. Several P-gp inhibitors could reverse MDR in cancer in vitro and in vivo. Similarly, CQ resistance was successfully reversed by several drugs including phenothiazines and some calcium channel blockers that are known as P-gp inhibitors (11). These drugs (e.g., verapamil) showed effectiveness in vitro and in vivo against CQ-resistant *P. falciparum* by increasing CQ accumulation inside the food vacuole (12,13). Like verapamil, other P-gp inhibitors such as desipramine and trifluoperazine were also able to reverse CQ resistance at high concentrations, though

they invariably produced undesirable effects (14). Hence, there is a need to find a P-gp inhibitor that can potentially reverse CQ resistance without producing undesirable effects.

Rifampicin (RIF), an anti-tuberculous drug, was reported to be a P-gp inhibitor at therapeutic concentrations in MDR cancer cell lines (15,16). RIF is effective against *Plasmodium vivax* in humans (17), and in *Plasmodium chabaudi* in rodents and CQ-resistant *P. falciparum* in vitro (18). RIF, in combination with isoniazid and co-trimoxazole, was found to be very effective on patients with falciparum malaria in Kenya (19). This dual effect of RIF may lead to improved efficacy of malaria chemotherapy and perhaps new combination treatment strategies to prevent or reverse CQ resistance in malaria. However, the effect of RIF in combination with CQ has not been studied against CQ-resistant *Plasmodium* spp. either in vitro (cell line) or in vivo. Therefore, we studied the effect of this combination on CQ-resistant *Plasmodium* spp., using a murine model of *Plasmodium berghei* infection.

MATERIALS AND METHODS

Animals: Experiments were carried out on Swiss albino mice (6-7 weeks old), weighing 20-25 g. Animals were housed under a light-dark cycle with mice pellets and tap water available ad libitum. No *p*-aminobenzoic acid (PABA) was added to the diet.

Malaria parasites: The cryopreserved CQ-resistant NK65 strain of *P. berghei* was obtained from the Department of Parasitology, Gunma University School of Medicine, Maebashi, Japan, and stored in liquid nitrogen.

Experimental design: Cryopreserved parasites were thawed and mice were infected via the intraperitoneal route as suggested by Kimbi et al. (20). Mice were first allowed to develop parasitemias of more than 20%. Blood was then collected directly from the heart after surgical opening of the thorax under ether anaesthesia. The blood was heparinized, diluted in physiological saline at a ratio of 3:7, and 0.2 ml of this suspension was injected intraperitoneally into the next batch of mice. After the virulence was stabilized

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(after a few batches) and mice developed an average parasitemia of more than 10% (by day 3 or day 4 after infection), they were divided into several groups ($n = 6-8$). Drugs were then administered intraperitoneally for 4 consecutive days (standard antimalarial drug screening) using various doses of CQ alone (5, 10, 20, and 30 mg/kg/day) or CQ in combination with RIF (10, 20, and 40 mg/kg/day). Saline and DMSO controls were included. Following treatment, daily parasitemia values were assessed through microscopic examination of thin smears of the blood obtained from the tail until day 7 (day 0 being the first day of treatment). Mice that managed to clear the parasite from the bloodstream were reassessed by thick smears for confirmation of the recovery. Subsequently, mice were assessed every other day for a month to determine whether recrudescence occurred. The day of death was also noted.

Drugs: CQ and RIF were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Aqueous solution of CQ was prepared. RIF, which is poorly water soluble, was dissolved in 1% DMSO for intraperitoneal administration.

Statistical analysis: Chi-square and Student's *t* test were used to analyze the results. $P \leq 0.05$ was considered as statistically significant.

RESULTS

CQ at a dose of 10 or 20 mg/kg/day on CQ-resistant parasites produced grossly similar rates of parasitemia decline. However, CQ at 5 mg/kg/day showed either no recovery or early recrudescence, and CQ at 2.5 mg/kg/day showed a 'RIII-like' resistance pattern based on the World Health Organization grading criteria of resistance (results not shown). Untreated mice and mice given 1% DMSO/saline as controls became severely anemic and lethargic within a few days, and all mice died within a week of monitoring (Fig. 1). The difference in parasitemia clearance rates between the 5 mg/kg/day and 10 mg/kg/day groups (23.5 and 86.7%, respectively) was highly significant ($P < 0.001$), which suggested that 5 mg/kg/day CQ could be considered a suboptimal dosage.

Co-administration of RIF with 5 mg/kg/day CQ resulted in earlier recrudescence and a rapid rise in parasitemia. No difference in rates of parasitemia decline was noted (Fig. 2). Combining RIF with 10 mg/kg/day CQ showed no significant difference in rates of parasitemia decline and no early recrudescence. There appeared to be a slight trend in decreased rate of parasitemia decline when RIF 40 mg/kg/day was added to CQ 10 mg/kg/day (Fig. 3). This trend was more prominent when RIF 40 mg/kg/day was added to CQ 20 mg/kg/day (Fig.

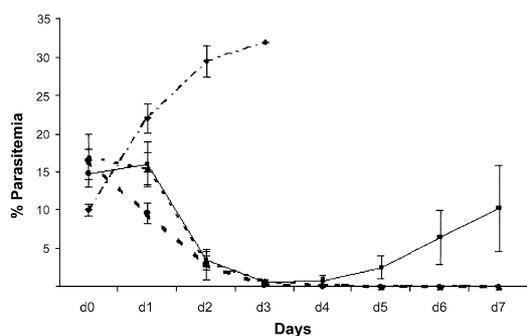


Fig. 1. Rate of parasitemia decline with various chloroquine doses.
 ◆ Control, ■ CQ 5 mg/kg/day, ● CQ 10 mg/kg/day,
 ▲ CQ 20 mg/kg/day.

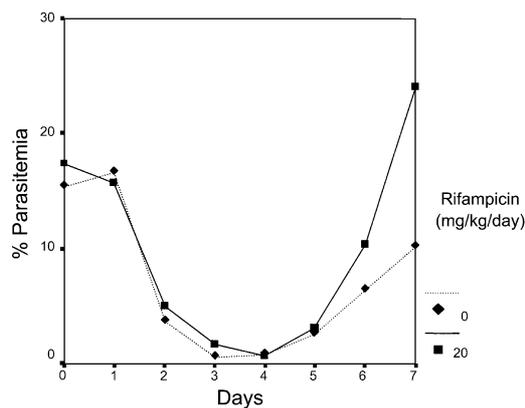


Fig. 2. Effect of rifampicin on parasitemia in mice receiving chloroquine 5 mg/kg/day.

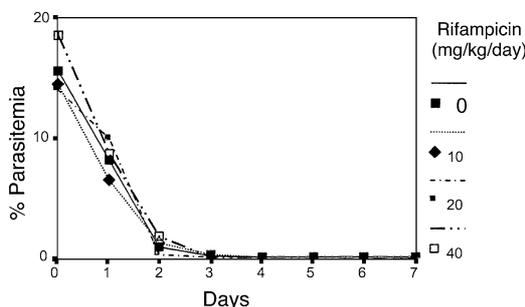


Fig. 3. Effect of rifampicin on parasitemia in mice receiving chloroquine 10 mg/kg/day.

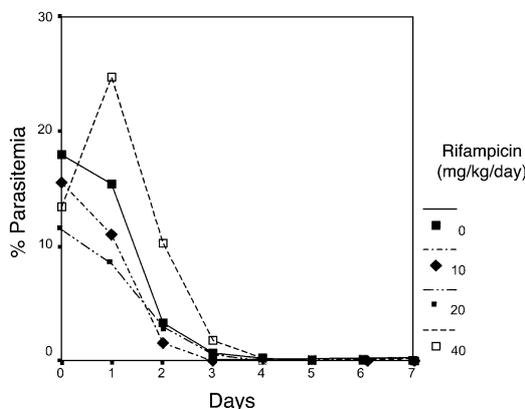


Fig. 4. Effect of rifampicin on parasitemia in mice receiving chloroquine 20 mg/kg/day.

4). Combining RIF with CQ adversely affected the day 7 survival (Table 1).

Recrudescence occurred in almost all cases (96.5%), and those mice ultimately developed lethal levels of parasitemia after the initial stage of recovery. The few completely cured mice were in the 20 mg/kg/day and 30 mg/kg/day groups. The average time interval for recrudescence was longer with higher CQ doses ($P = 0.111$) and shorter for mice receiving RIF concurrently (Table 2). In the CQ 20 mg/kg/day group, a *t*-test comparing the average day of recrudescence between mice receiving (3.0) and not receiving (8.3) RIF was significant ($P < 0.001$), showing that those receiving RIF developed earlier recrudescence.

Table 1. Effect of chloroquine (CQ) and rifampicin (RIF) on day 7 survival

CQ dose (mg/kg/day)	Survival rate on day 7 (%)	
	CQ alone	CQ + RIF (10, 20, 40mg/kg/day)
0	0	0
2.5	0	0
5	85.7	60
10	88.9	71.4
20	100	85.7
30	100	100

Table 2. Effect of chloroquine (CQ) and rifampicin (RIF) on recrudescence

CQ dose (mg/kg/day)	Day of recrudescence	
	CQ alone	CQ + RIF (10, 20, 40 mg/kg/day)
5	3.3	–
10	6.8	5.2
20	8.3	3.0*
30	–	9.0

* $P < 0.001$ when compared to CQ alone.

DISCUSSION

We found that RIF significantly decreases chances of recovery in cases in which the dose of CQ is marginal for cure, shortens the time for recrudescence to appear, and may even slow the initial rate of fall of parasitemia when given at a higher dose of 40 mg/kg/day. This is contrary to what was expected.

P. berghei has been shown to have an *mdr*-like gene that is basically similar to the *pfmdr1* gene in *P. falciparum*, which encodes for MDR (21). In addition, a BLAST performed using the NCBI database reveals that *P. berghei* and other species of murine malaria parasites express putative membrane transporter CG10, which is grossly similar to the putative vacuolar transmembrane CQ resistance transporter encoded by *pfert* in *P. falciparum*. In earlier studies, P-gp inhibitors like verapamil and several anti histamine compounds have been shown to reverse CQ resistance in both *P. berghei* and in *P. falciparum* (13,22,23). Therefore, a CQ-resistant murine malaria model of *P. berghei* infection is an acceptable animal model of CQ-resistant *P. falciparum* malaria.

Recently, RIF has been found to induce as well as to inhibit P-gp (24,25) and also to act as a double-edged sword against MRP-mediated MDR (26). When RIF is given with marginal doses of CQ, it may induce a small subset of resistant parasites to express more Pgh1, enabling them to reduce CQ accumulation in the food vacuole more effectively. Yet other findings showed that Pgh1, if over expressed, may confer relative sensitivity to CQ, simultaneous with resistance to mefloquine, halofantrine, and quinine (5). However, field reports have mostly linked mutant *pfmdr* with increased resistance albeit with incomplete correlation. In short, because RIF is reported to be both a P-gp inhibitor and inducer, it is possible that RIF antagonizes CQ either by inhibiting wild type Pgh1 or by inducing mutant Pgh1.

It has also been postulated that Pgh1 may mediate CQ influx into the food vacuole instead of efflux (27,28). If this mechanism is correct, RIF may antagonize CQ by inhibiting

Pgh1 and therefore decreasing CQ accumulation inside the food vacuole. However, this postulation is unable to explain how verapamil and other reversal agents are able to increase CQ inside the food vacuole and counter resistance. Another factor to consider is the fact that RIF is a known CYP3A4 inducer, which is documented to affect metabolism of quinine and mefloquine (29). Although CQ is also metabolized by CYP3A4, its metabolites are active and it does not undergo such an extensive hepatic metabolism as mefloquine. While RIF is unlikely to reduce chloroquine effectiveness through this mechanism in humans, it is unclear whether this applies equally to mice.

Dosing for RIF was restricted in the sense that it is not soluble in water and DMSO had to be used as a solvent. DMSO is a known membrane irritant but had no effect on mortality or course of parasitemia. Examples of substances dissolved in 2% DMSO and above for antimalarial drug testing in mice via intraperitoneal route have been recorded in the literature (30,31).

RIF is a common antituberculous drug. There may be a substantial proportion of tuberculosis patients who develop malaria in the tropics where RIF, given its antagonism to CQ in terms of the parameters discussed above, may in some way affect or contribute to the resistance in these areas. It appears that RIF use and emergence of CQ resistance in tropical countries coincide. Our findings suggest that RIF antagonizes the effect of CQ. Thus, further studies should be undertaken to explore whether RIF is a culprit in the development of CQ resistance.

Recent literature has put much importance on *pfert* as the main cause of CQ resistance (32,33). Point mutations such as K76T in the *pfert* vacuolar transport protein (which is postulated to be a chloride transporter) (34) are a favored explanation for CQ resistance, and this reasoning is backed firmly by data from genetic crosses, analysis of laboratory-adapted field isolates, and analysis of cases of CQ treatment failure. However, it was also shown that replacement of mutant *pfmdr* genes with normal ones in the presence of a mutant *pfert* gene cuts EC50 values of CQ by half. This seems to suggest that *pfmdr1* still plays a modulatory role in CQ resistance (10,35). Interestingly, verapamil-induced inhibition of a greater pH gradient, and reduced H⁺-ATPase activity was seen in inside-out plasma membrane vesicle preparations prepared from yeast cells expressing *pfert* (34). Regardless of the exact mechanism of resistance, and whether it involves Pgh1, *pfert*, and/or other plasmodial vacuolar transporters, the fact remains that chemosensitizers have been used to excellent effect in reversing CQ resistance, and combinations of such chemosensitizers have been attempted in an effort to reduce the dosage of each drug and thereby minimize their respective adverse effects (14). However, given that our study does not show a beneficial or additive effect of RIF in combination with CQ on CQ-resistant murine malaria and that its effect is even antagonistic in terms of the above parameters, we conclude that RIF should not be given with CQ in combination therapy for malaria, and that RIF's role in the emergence of CQ resistance warrants further exploration.

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REFERENCES

- Slater, A. F., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A. and Henderson, G. B. (1991): An iron-carboxylate bond links the heme units of malaria pigment. *Proc. Natl. Acad. Sci. USA*, 88, 325-329.
- Slater, A. F. (1992): Malaria pigment. *Exp. Parasitol.*, 74, 362-365.
- Slater, A. F. and Cerami, A. (1992): Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature*, 355, 167-169.
- Dorn, A., Stoffel, R., Matile, H., Bubendorf, A. and Ridley, R. G. (1995): Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. *Nature*, 374, 269-271.
- Tracy, J. W. and Webster, L. T., Jr. (2001): Drugs used in the chemotherapy of protozoal infections: malaria. p. 1069-1092. *In* J. G. Hardman, L. E. Limbird and A. G. Gilman (eds.), *Goodman and Gilman's the Pharmacological Basis of Therapeutics*. 10th ed. McGraw-Hill, New York.
- Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. and Schlesinger, P. H. (1987): Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, 238, 1283-1285.
- Salmon, S. E. and Dalton, W. S. (1996): Relevance of multidrug resistance to rheumatoid arthritis: development of a new therapeutic hypothesis. *J. Rheumatol. Suppl.*, 44, 97-101.
- Cowman, A. F., Karcz, S., Galatis, D. and Culvenor J. G. (1991): A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. *J. Cell Biol.*, 113, 1033-1042.
- Djimde, A., Doumbo, O. K., Cortese, J. F., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X. Z., Nomura, T., Fidock, D. A., Wellems, T. E., Plowe, C. V. and Coulbaly, D. (2001): A molecular marker for chloroquine-resistant falciparum malaria. *N. Engl. J. Med.*, 344, 257-263.
- Babiker, H. A., Pringle, S. J., Abdel-Muhsin, A., Mackinnon, M., Hunt, P. and Walliker, D. (2001): High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfert* and the multidrug resistance gene *pfmdr1*. *J. Infect. Dis.*, 183, 1535-1538.
- Martin, S. K., Oduola, A. M. J. and Milhous, W. K. (1987): Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science*, 235, 899-901.
- Goldsmith, R. S. (1995): Antiprotozoal drugs. p. 780-803. *In* B. G. Katzung (ed.), *Basic & Clinical Pharmacology*. 6th ed. Prentice-Hall International Inc., London.
- Rabinovich, S. A., Orlov, V. S., Dadasheva, N. R., Bukhtin, B. A., Maksakovskaia, E. V., Shcherbakov, A. M., Nguyen, D. S. and Vu, T. T. (1996): Ca⁺⁺ ion transport blockers as reversants of drug resistance of malarial parasites. 1. The effect of verapamil on resistance to chloroquine in vivo of *Plasmodium berghei* and in vitro of *Plasmodium falciparum*. *Med. Parazitol. (Mosk)*, 1, 18-22.
- van Schalkwyk, D. A., Walden, J. C. and Smith, P. J. (2001): Reversal of chloroquine resistance in *Plasmodium falciparum* using combinations of chemosensitizers. *Antimicrob. Agents Chemother.*, 45, 3171-3174.
- Fardel, O., Lecreur, V., Loyer, P. and Guillouzo, A. (1995): Rifampicin enhances anti-cancer drug accumulation and activity in multidrug-resistant cells. *Biochem. Pharmacol.*, 49, 1255-1260.
- Furusawa, S., Nakano, S., Wu, J., Sasaki, K., Takayanagi, M. and Takayanagi, Y. (1997): Potentiation of pirarubicin activity in multidrug resistant cells by rifampicin. *Biol. Pharm. Bull.*, 20, 303-306.
- Pukrittayakamee, S., Viravan, C., Charoenlarp, P., Yeamput, C., Wilson, R. J. and White, N. J. (1994): Antimalarial effects of rifampicin in *Plasmodium vivax* malaria. *Antimicrob. Agents Chemother.*, 38, 511-514.
- Strath, M., Scott-Finnigan, T., Gardener, M., Williamson, D. and Wilson, I. (1993): Antimalarial activity of rifampicin in vitro and in rodent models. *Trans. R. Soc. Trop. Med. Hyg.*, 87, 211-216.
- Goerg, H., Ochola, S. A. and Goerg, R. (1999): Treatment of malaria tropica with a fixed combination of rifampicin, co-trimoxazole and isoniazide: a clinical study. *Chemotherapy*, 45, 68-76.
- Kimbi, H. K. and Fagbenro-Beyioku, A. F. (1996): Efficacy of cymbopogon giganteus and enantia chrantha against chloroquine resistant *Plasmodium yoelii nigeriensis*. *East Afr. Med. J.*, 73, 636-637.
- Gervais, G. W., Trujillo, K., Robinson, B. L., Peters, W. and Serrano, A. E. (1999): *Plasmodium berghei*: identification of an *mdr*-like gene associated with drug resistance. *Exp. Parasitol.*, 91, 86-92.
- Peters, W., Ekong, R., Robinson, B. L., Warhurst, D. C. and Pan, X. Q. (1990): The chemotherapy of rodent malaria. XLV. Reversal of chloroquine resistance in rodent and human *Plasmodium* by antihistaminic agents. *Ann. Trop. Med. Parasitol.*, 84, 541-551.
- Valecha, N., Biswas, S., Dewan, S. and Bhambhani, S. (1992): Reversal of chloroquine resistance with verapamil in *P. berghei* in vivo. *Indian J. Malariol.*, 29, 47-53.
- Asghar, A., Gorski, J. C., Haehner-Daniels, B. and Hall, S. D. (2002): Induction of multidrug resistance-1 and cytochrome P450 mRNAs in human mononuclear cells by rifampin. *Drug Metab. Dispos.*, 30, 20-26.
- Finch, C. K., Chrisman, C. R. and Baciewicz, A. M. (2002): Rifampin and rifabutin drug interactions: an update. *Arch. Intern. Med.*, 162, 985-992.
- Kim, H. S., Min Y. D. and Choi, C. H. (2001): Double-edged sword of chemosensitizer: increase of multidrug resistance protein (MRP) in leukemic cells by an MRP inhibitor probenecid. *Biochem. Biophys. Res. Commun.*, 283, 64-71.
- Ginsburg, H. and Krugliak, M. (1999): Chloroquine - some open questions on its antimalarial mode of action and resistance. *Drug Resist. Updates*, 2, 180-187.
- Sanchez, C. P. and Lanzer, M. (2000): Changing ideas on chloroquine in *Plasmodium falciparum*. *Curr. Opin. Infect. Dis.*, 13, 653-658.
- Riditid, W., Wongnawa, M., Mahatthanatrakul, W., Chaipol, P. and Sunbhanich, M. (2000): Effect of rifampin on plasma concentrations of mefloquine in healthy volunteers. *J. Pharm. Pharmacol.*, 52, 1265-1269.
- Hindley, S., Ward, R., Storr, C., Searle, N. L., Bray, P. G., Park, B. K., Davies, J. and O'Neill, P. M. (2002):

- Mechanism-based design of parasite-targeted artemisinin derivatives: synthesis and antimalarial activity of new diamine containing analogues. *J. Med. Chem.*, 45, 1052-1063.
31. Delarue, S., Girault, S., Maes, L., Debreu-Fontaine, M. A., Labaeid, M., Grellier, P. and Sergheraert, C. (2001): Synthesis and in vitro and in vivo antimalarial activity of new 4-anilinoquinolines. *J. Med. Chem.*, 44, 2827-2833.
 32. Sidhu, A. B., Verdier-Pinard, D. and Fidock, D. A. (2002): Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcr* mutations. *Science*, 298, 210-213.
 33. Pillai, D. R., Labbé, A. C., Vanisaveth, V., Hongvangthong, B., Pomphida, S., Inkathone, S., Zhong, K. and Kain, K. C. (2001): *Plasmodium falciparum* malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. *J. Infect. Dis.*, 183, 789-795.
 34. Zhang, H., Howard, E. M. and Roepe, P. D. (2002): Analysis of the antimalarial drug resistance protein *pfcr* expressed in yeast. *J. Biol. Chem.*, 277, 49767-49775.
 35. Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. and Cowman, A. F. (2000): Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, 403, 906-909.