FLUNARIZINE AND VERAPAMIL INHIBIT CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* GROWTH *IN VITRO*

JUTAMAAD SATAYAVIVAD, ORAPIN WONGSAWATKUL, DANAI BUNNAG*
PEERAPAN TAN-ARIYA and CHARIYA R. BROCKELMAN

Departments of Pharmacology and Microbiology, Faculty of Science, Mahidol University, Bangkok 10400 and *Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

INTRODUCTION

Malaria is still a major public health problem in the developing countries. In Thailand alone, 300,000 to 500,000 malaria cases are reported annually. Approximately six out of ten cases are caused by *Plasmodium falciparum* (Harinasuta *et al.*, 1982), among which cerebral involvement can reach 7%. Mortality due to cerebral malaria occurs in 40% of cases with complications, which often involve drug resistant *P. falciparum*, Therefore, there is an urgent need for development of effective antimalarials. To meet this demand, a screening programme was launched to detect the antimalarial activity of the drugs presently available in the market. The criterion for the selection of the drugs to be tested is based on their pharmacological properties in relation to the biochemistry of *P. falciparum* and of the infected erythrocytes.

It has been well documented that Ca^{++} is essential for cell development (Jaffe, 1980). Recently, Ca^{++} was shown to be indispensable for normal growth of *Plasmodium falciparum in vitro* (Wasserman *et al.*, 1982), Tanabe *et al.*, (1982) demonstrated that rat erythrocytes infected with *P. chabaudi* contained higher levels of Ca^{++} as compared with normal uninfected erythrocytes. The enhanced Ca^{++} levels were observed in all stages of parasite development. Ethylene glycol bis (β-aminoethylether) N, N'-tetra-acetic acid (EGTA) had been used to lower Ca^{++} in media *in vitro*, and this condition resulted in the inhibition of *P. falciparum* growth (Wasserman *et al.*, 1982). Vanadate and procaine putative inhibitors of erythrocyte transmembrane Ca^{++} transport were found to inhibit *P. falciparum in vitro* (McAlister and Mishra, 1983). This study was carried out to explore the inhibitory effects of two calcium blockers, verapamil and flunarizine, whose sites of action are different from that of chlorpromazine, a calmodulin inhibitor.

MATERIALS AND METHODS

The chloroquine-resistant *Plasmodium falciparum* ($T_{9/94}$) was obtained by cloning isolated T_9 from a Thai patient from Mae Sod, Tak province, Thailand by Dr. Sodsri Thaithong, Biology Department, Chulalongkorn University, Bangkok. This parasite strain was kindly given to us and it has been kept in the continuous culture in type 0 human erythrocyte at the Microbiology Department, Mahidol University, since 1983.

The chloroquine-resistant *Plasmodium falciparum* ($T_{9/94}$) was continuously cultured at 37.5°C in RPMI 1640 (GIBCO, USA) with L-glutamine containing 25mM HEPES and 0.2% W/V of $NaHCO_3$ and supplemented with 10% (V/V) of pooled non-immune human serum heat-inactivated before use. Blood group "0" obtained from healthy donors was used. The parasites were started at the ring stage using the synchronization method of Lambros and Vanderberg's method (1979). Parasites were maintained in continuous culture as described by Trager and Jen-

sen (1976). The culture medium was changed manually every 24 h. Parasitemia was determined microscopically at 48 h by counting the infected cells against 5,000 erythrocytes on thin smears stained with Giemsa's stain.

Flunarizine and verapamil were kindly donated by Janssen Pharmaceutica and Knoll A.G. respectively. The stock solutions of flunarizine was made by using 16% dimethyl sulfoxide (DMSO) and sterilized through millipore filter membrane, pore size 0.22 μm. Chlorpramazine HCl and Quinine 2HCl sterile solution were purchased from the Thai Government Pharmaceutical Organization, whereas mefloquine solution (0.01 Molar) was from Hoffmann-La Roche, Basel.

The sterile solution of verapamil hydrochloride in distilled water was used as a stock solution. The drugs were diluted with complete RPMI medium to varying concentrations to be tested for the inhibitory effect on parasite growth. Each experiment had control (medium with corresponding volume of DMSO or distilled water but without drug) and two replicates for each drug concentration. The parasite cultures were allowed to expose to drug for 48 h. Experiments on each drug were repeated 4 times in replicates.

The 50% inhibitory concentration (IC_{50}) was the concentration of a drug which could reduce parasite population growth during one schizogonic cycle to be only one half of the control (without drug), whereas IC_{99} was the concentration of a drug which completely inhibited parasite multiplications. Both IC_{50} and IC_{99} were calculated from linear regression analysis ($Y = a + bx$, where $Y = 50$ and 99, respectively). Y is the percent inhibition on parasite population growth, while x is the concentration of the drug.

$$\text{Percent of inhibition} = \frac{100 \times (a-b)}{a}$$

a = the number of parasites in control

b = the number of parasites in medium with test drug.

RESULTS

Verapamil (5×10^{-7} to 5×10^{-5} M) and flunarizine (1.05×10^{-6} to 1.05×10^{-4} M) were tested for their inhibitory effects on chloroquine-resistant *Plasmodium falciparum* growth *in vitro* after 48 h of drug exposure. These drugs inhibited the parasite population growth in a concentration dependent pattern (Fig. 1). The IC_{50} and IC_{99} calculated from linear regression analysis are illustrated in Table 1.

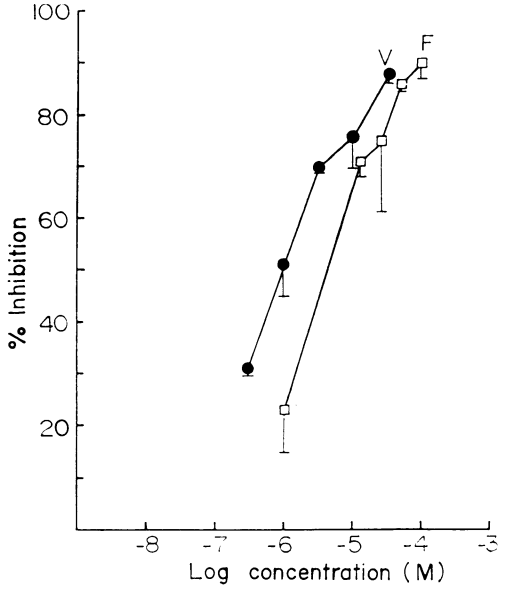

Fig. 1—The concentration-dependent inhibitory effect of verapamil (●——●) and flunarizine (□——□) on chloroquine-resistant *P. falciparum* growth *in vitro*.

Our common practice to use synchronous parasites all in the ring stage as starting material has made possible further detailed observations on how effectively the drug interferes with parasite development. As shown in Fig. 2, the differential count of each stage of parasite revealed that flunarizine at the concentration of 1.31×10^{-5} M arrested one half of the parasites at the early and late schizont stages. Only a few underwent

complete schizogony to merozoites which re-invaded new erythrocytes. Hence, the number of resultant rings at 48 h of incubation was only 5 as compared with 52 in cultures receiving flunarizine at the lower concentration of 1.05×10^{-6} M. A concentration of flunarizine at 5.24×10^{-5} M arrested parasite development almost completely. Ten out of 25 rings of the first generation (from T_0) died and nine rings developed only to the trophozoite stage, leaving the remaining five as morphologically abnormal rings.

Inhibition of parasite multiplication by verapamil was comparable in magnitude to that achieved with flunarizine (Fig. 3) or chlorpromazine (Table 1 & 2). These three calcium blockers are, however, less potent when compared with the IC_{50} of quinine of mefloquine (Table 2).

Table 1

The calculation of linear regression equations and calculated IC_{50} and IC_{99} of various test drugs on *P. falciparum* growth *in vitro*.

Drugs	Equations	Inhibitory Concentration(M)	
		50% inhibitory concentration (IC_{50})	Complete inhibitory concentration (IC_{99})
Verapamil	$Y = 207.85 - 26.84\times$	1.32×10^{-6}	8.71×10^{-5}
Flunarizine	$Y = 231.33 - 34.28\times$	5.13×10^{-6}	1.38×10^{-4}

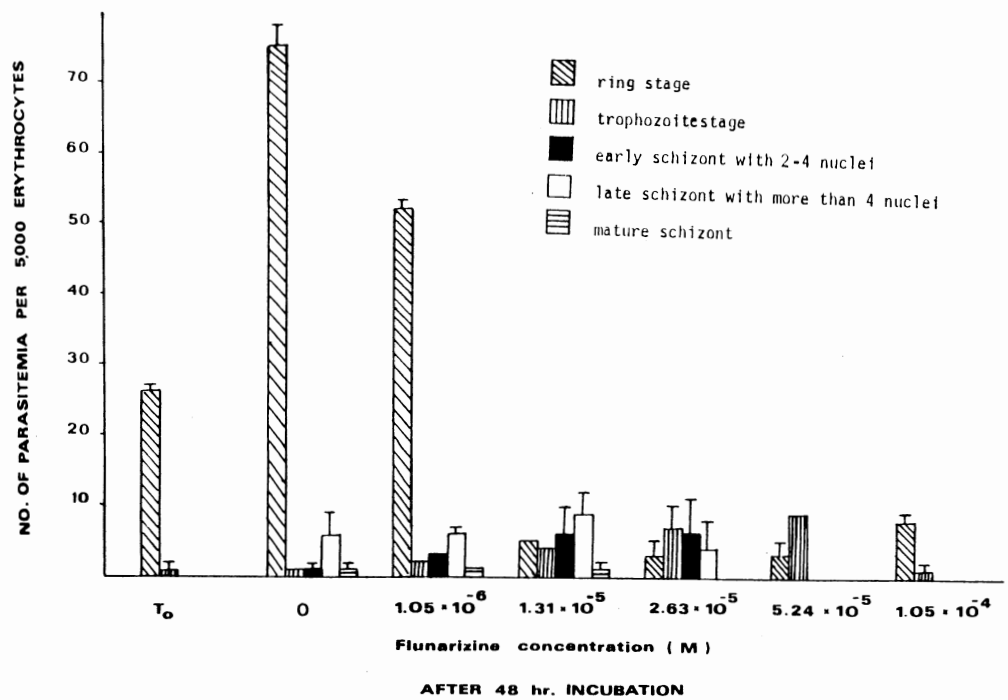

Fig. 2—Growth-distribution of asexual state of *P. falciparum* after flunarizine exposure at time 0 and 48 h. Each bar represents the mean ± standard error (X ± S.E.) of parasite counts per 5000 erythrocytes (N = 4).

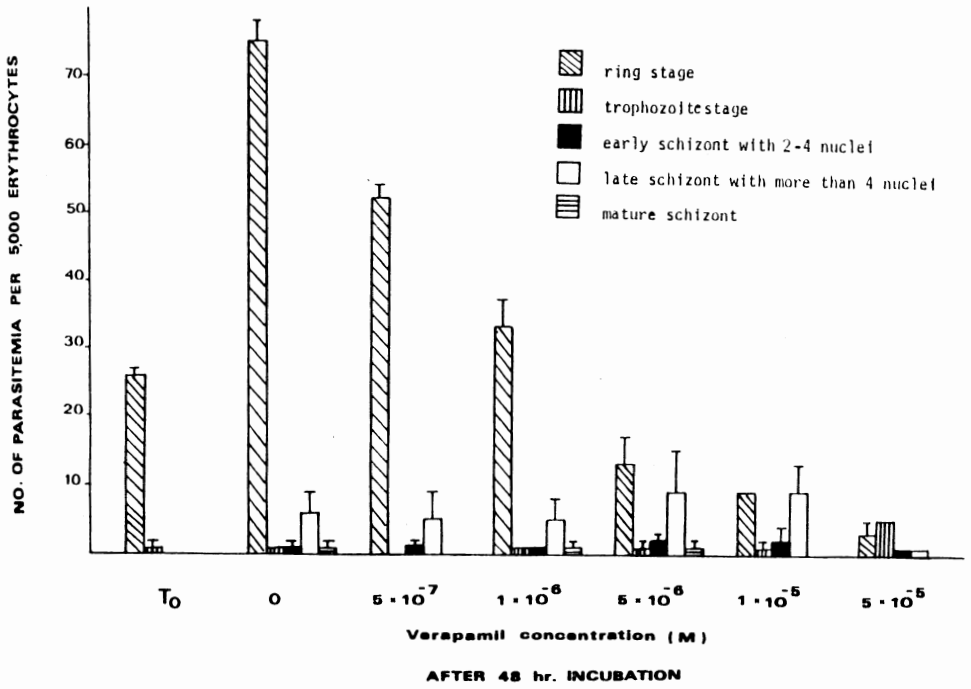

Fig. 3—Growth-distribution of asexual state of *P. falciparum* after verapamil exposure at time 0 and 48 h. Each bar represents the mean ± standard error (X ± S.E.) of parasite counts per 5000 erythrocytes (N = 4).

Table 2

The calculated IC_{50} and IC_{99} of various test drugs on *P. falciparum* growth *in vitro*.

Drug	Inhibitory concentration (M)	
	50% inhibitory conentration (IC_{50})	Complete inhibitory concentration (IC_{99})
Chlorpromazine	3.55×10^{-6}	2.69×10^{-5}
Quinine	3.98×10^{-8}	1.41×10^{-7}
Mefloquine	6.31×10^{-11}	9.55×10^{-8}

DISCUSSION

Results from this study have clearly shown that the Ca^{2+} blockers, flunarizine and verapamil inhibited *P. falciparum* growth *in vitro* at the same magnitude as chlorpromazine. The latter compound has been reported to possess antimalarial activity *in vitro* by Panijpan and Kantakanit (1983) and by Wongsawatkul (1986). The former group suggested that a hemin-mediated mechanism might be the basis of the activity. Calmodulin is present in red blood cells (Schatzmann, 1983) and chlorpromazine is known to be a calmodulin

inhibitor (Prozialeck and Weiss, 1982). A calmodulin-calcium complex is reported to enhance the influx of Ca^{++} into the cytoplasm (Larsen and Vincenzi, 1979). It has been suggested (Wongsawatkul, 1986), pers comm. that calmodulin inhibition may be one of the mechanisms for the antimalarial action of chlorpromazine. Calcium entry blockers have been used for the treatment of ischemia both in the heart and other tissues (Vanhoutte, 1981; Fleckenstein, 1984). Furthermore, flunarizine has been found to suppress echinocyte formation (Flameng *et al.*, 1979; De Clerck *et al.*, 1981) and the protective effect of flunarizine on the brain has been demonstrated in a variety of animal models of anoxia, hypoxia, and ischemia (cited from Borger *et al.*, 1984). Some of the cardinal features of cerebral malaria are hyperpyrexia, convulsions, hypoglycemia, severe anemia, dehydration and pulmonary oedema (Harinasuta *et al.*, 1985). These manifestations make patients very prone to brain ischemia and we anticipate that if calcium blockers have antimalarial action, they should also have some therapeutic use in the management of cerebral malaria. In this study ,both verapamil and flunarizine were shown to have inhibitory effects on *P. falciparum* growth *in vitro* at concentrations similar those for chlorpromazine inhibition. Verapamil is a calcium blocker used in the treatment of cardiovascular disorders and its pharmacodynamics and pharmacokinetics have been studied extensively (Fleckenstein, 1984, Hamann *et al.*, 1984). Flunarizine also has been studied thoroughly (Holmes *et al.*, 1984). However, there are questions as to whether the antimalarial activity of these drugs will occur at acceptable plasma concentrations that are devoid of serious adverse effects but provide desirable hemodynamic alterations. Further studies in mice infected with *P. berghei* are being performed to find the correlation between the doses that exhibit antimalarial activity and those that have effect in anoxic animals.

In summary, this study reports for the first time that three calcium blockers (verapamil, flunarizine and chlorpromazine) with presumably different sites of action that have antimalarial activity against *P. falciparum in vitro*. Since it is apparent from the literature that calcium blockers are a heterogenous population of molecular structures with differing properties (Chin, 1986), it is interesting to evaluate the antimalarial activity of this group of drugs thoroughly in order to search for one with potent antimalarial activity and favorable hemodynamic effects but with few side effects.

SUMMARY

Using pharmacological properties in relation to the biochemistry of *P. falciparum*, verapamil, flunarizine, and chlorpromazine which are calcium blockers were selected to test for their antimalarial activity against *P. falciparum in vitro*. Results revealed that the drugs inhibited parasite population growth in the following order of IC_{50}: verapamil 1×10^{-6}M, chlorpromazine 3.5×10^{-6}M, and flunarizine 5×10^{-6} M. These three calcium blockers have antimalarial effects on chloroquine resistant parasite (alone $T_{9/94}$) but are less potent when compared with the efficacy of quinine or mefloquine *in vitro*.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Khunying Tranakchit Harinasuta for valuable suggestions, Janssen Pharmaceutica, Knoll A.G. and F. Hoffmann La Roche, Co. Ltd. for drugs used in this study, Dr. T.W. Flegel for improving the manuscript.

REFERENCES

BORGERS, M., DE CLERCK, F., VAN REEMPTS, J., XHONNEUX R. and VAN NUETEN, J., (1984). Selective blockade of cellular Ca^{2+} – overload by flunarizine. *Inter. Angio.* (Suppl. to No. 2), *3* : 25.

CHIN J.H., (1986). Differential sensitivity of calcium channels to dihydropyridines. The modulated receptor hypothesis. *Biochem. Pharmacol., 35* : 4115.

DE CLERCK, F., BEERENS, M., THONE', F. and BORGERS, M., (1981). Effect of flunarizine on the human red cell shape changes and calcium deposition induced by A 23187. *Thrombosis Res., 24* : 1.

FLAMENG, W., VERHEYEN, F., BORGERS, M., DE CLERCK, F. and BRUGMANS, J., (1979). The effect of flunarizine treatment on human red blood cells. *Angio., 30*: 516.

FLECKENSTEIN, A., (1984). History and prospects for a multifaceted pharmacodynamic principle In: *Calcium Antagonists and Cardiovascular Disease*, p. 9, L.H. Opie (ed.) Raven Press, New York.

HAMANN, S.R., BLOUIN, R.A. and MCALLISTER, R.G. JR., (1984). Clinical pharmacokinetics of verapamil. *Clin. Pharmacokinetics., 9 :* 26.

HARINASUTA, T., DIXON, K.E., WARRELL, D.A. and DOBERSTYN, E.B., (1982). Recent advances in malaria with special reference to Southeast Asia. *Southeast Asian J. Trop. Med. Pub. Hlth., 13* : 1.

HARINASUTA, T., LOOAREESUWAN, S. and BUNNAG, D., (1985). Cerebral malaria in Thailand and clinical manifestations and the management. *Mosq-Borne Dis. Bull., 1* : 49.

HOLMES, B., BROGDEN, R.N., HEEL, R.C., SPEIGHT, T.M. and AVERY, G.S., (1984). Flunarizine : A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use. *Drugs,27* : 6.

JAFFE, L.F., (1980). Calcium explosions as triggers of development. *Ann. N. Y. Acad. Sci., 339* : 86.

LAMBROS, C. and VANDERBERG, J.P., (1979). Synchronization of *Plasmodium falciparum* erythrocytic stage in culture. *J. Parasitol., 65* : 418.

LARSEN, F.L. and VINCENZI, F.F., (1979). Calcium transport across the plasma membrane : Stimulation by calmodulin. *Science, 204* : 306.

MCALISTER, R.O. and MISHRA, G.C., (1983). Putative inhibitors of erythrocyte transmembrane Ca^{++} kill *Plasmodium falciparum in vitro. J. Parasitol., 69* : 977.

PANIJPAN, B. and KANTAKANIT, N., (1983). Chlorpromazine enhances hemolysis induced by haemin. *J. Pharm. Pharmacol., 35* : 473.

PROZIALECK, W.C. and WEISS, B., (1982). Inhibition of calmodulin by phenothiazine and related drugs : structure-activity relationships. *J. Pharm. Exp. Ther., 222* : 509.

SCHATZMANN, H.J., (1983). The red cell calcium pump. *Ann. Rev. Physiol., 45* : 303.

TANABE, K., MIKKELSEN, R.B. and WALLACH, D.F.H., (1982). Calcium transport of *Plasmodium chabaudi*-infected erythrocytes. *J. Cell. Biol., 93* : 680.

TRAGER, W. and JENSEN, J.B., (1976). Human malaria parasites in continuous culture. *Science., 193* : 673.

VANHOUTTE, P.M., (1981). Calcium entry blockers and cardiovascular failure. *Fed. Proc., 40* : 2882.

WASSERMAN, M., ALARCON C. and MENDOZA, P.M., (1982). Effects of Ca^{++} depletion on the asexual cell cycle of *Plasmodium falciparum. Am. J. Trop. Med. Hyg., 31* : 711.