Biochemical Pharmacology, Vol. 55, pp. 433–440, 1998.



ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00482-6

# Synergy between Two Calcium Channel Blockers, Verapamil and Fantofarone (SR33557), in Reversing Chloroquine Resistance in *Plasmodium falciparum*

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**ABSTRACT.** This study describes the synergistic interaction of two calcium channel blockers, verapamil (VR) and SR33557 or fantofarone (SR), in reversing chloroquine resistance in *Plasmodium falciparum*, the causative agent of human malaria. The two calcium channel blockers exhibited an intrinsic antimalarial activity at 10 and 1 μM for verapamil and fantofarone, respectively. Isobolograms revealed that chloroquine and verapamil, and chloroquine and fantofarone, acted synergistically against chloroquine-resistant strains of *P. falciparum*. When used at subinhibitory concentrations, verapamil appeared 2 to 3 times more potent than fantofarone in reversing chloroquine resistance. Indeed, verapamil completely reversed the chloroquine resistance in *P. falciparum*, while fantofarone did so only partially. In the highly chloroquine-resistant strain FcB1, VR and SR acted synergistically to reverse CQ resistance, and the concentrations of VR used in these combinations could be reduced 10- or 100-fold (e.g. 100 nM and 10 nM) those required when this drug was used alone. In the moderately chloroquine-resistant strain K1, a combination of VR and SR for CQ resistance reversal allowed us to reduce the concentration of these chemosensitizers 1000- and 100-fold, respectively. The maximum tolerable plasma level beyond which side-effects occurred when using verapamil is 2.5 μM. Thus, the approach described, which allowed us to lower the doses of chemosensitizers, could well prevent toxic effects in humans and enlighten the advantages of polychemotherapy. BIOCHEM PHARMACOL 55;4:433–440, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** *Plasmodium falciparum;* drug resistance reversal; chloroquine; calcium channel blockers; verapamil; fantofarone (SR33557); malaria

Chloroquine resistance (CQR)§ in *Plasmodium falciparum* is one of the major causes of the malaria recrudescence in the world. CQR phenotypes in *P. falciparum* were first compared to the multidrug resistance (MDR) status in some mammalian tumor cells [1, 2]. Altered pharmacology of drugs in MDR cells is related to the P-glycoprotein (P-gp) [3, 4], and many drugs including calcium channel blockers were shown to interact with this protein [5] and to restore drug concentrations in resistant strains to levels found in their drug-sensitive parentage [6, 7]. In CQR *P. falciparum* phenotypes, it was suggested that a chloroquine efflux mechanism was acquired by mutating or amplifying P-glycoprotein homologue (Pf.mdr-like) genes in the parasites [8–10], but no linkage between the CQR phenotype and the Pf.mdr genes was found in genetic cross [11]. However, as in some MDR mammalian tumor cells, many drugs including calcium channel blockers such as verapamil [12] Ro 11.2933/001 [13] (verapamil analog), diltiazem, nifedipine, nicardipine [14] and amlodipine [15] were shown to restore chloroquine sensitivity to CQR *P. falciparum* strains.

Many cellular processes are regulated by calcium ions acting as intracellular messengers [for review see refs. 16 and 17], and the major pathway for calcium entry is controlled by different types of channels with fundamental differences in the mechanisms governing their opening and closing [for review see ref. 16]. Recently, indolizinesulfones have been described as a new family of molecules acting as L-type $Ca^{2+}$ channel blocker, and fantofarone (SR 33557) has its own binding site on the α1 subunit of the 1,4, dihydropyridine (DHP) binding protein [18–21], which conferred a structurally unrelated status in comparison to the other L-type $Ca^{2+}$ channel blockers such as DHPs, phenylalkylamines and benzothiazepines. Furthermore, fantofarone did not act on the P-glycoprotein in cancer cells as did verapamil [5], but on another 65-kDa plasma membrane protein, and it was shown that fantofarone was 4.5 times more potent than verapamil in restoring drug

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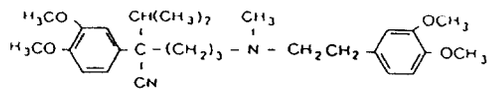
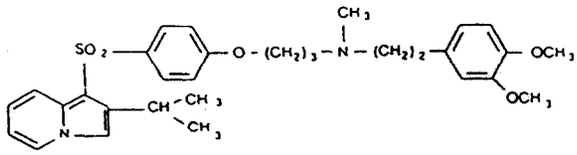
§ *Abbreviations:* CQ, chloroquine; CQR, chloroquine-resistant; CQS, chloroquine-sensitive; DOP, degree of potentialisation; MDR, multidrug resistance; P-gp, glycoprotein P; SFIC, sum of the fractional inhibitory concentrations; SR, fantofarone; VR, verapamil.

Received 7 March 1997; accepted 5 August 1997.



*Verapamil*



*SR 33557*

**FIG. 1. Chemical structure of verapamil and SR 33557.**

sensitivity in the murine leukemia P388/ADR cell line (resistant to doxorubicin) [22]. As verapamil was shown to reverse CQR in several *P. falciparum* strains [12] and not to use the route of the verapamil-sensitive $Ca^{2+}$ channel [23], it was of interest to compare the effects of these two calcium channel blockers on *P. falciparum* chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) strains. The major limiting factor for achieving adequate serum concentrations of chemosensitizers to reverse the MDR status are their intrinsic side-effects. However, by combining several chemosensitizing agents with non-overlapping toxicities, one may achieve an overall anti-MDR effect greater than that possible with individual agents at higher doses, as investigated in cancer chemotherapy [24, 25]. To test such a hypothesis, isobolograms were realized in order to determine if a synergy exists between the two chemosensitizers VR and SR. In the present study, we have shown that these drugs exhibit intrinsic antimalarial activity, with a slightly higher efficiency of SR, and a synergistic effect with CQ. In addition, we have found that VR and SR act synergistically to reverse CQ resistance in the highly CQ-resistant strain FcB1 and that the concentration of VR used in these combinations can be reduced 10- or 100-fold those required when this drug is used alone. Finally, we have confirmed all these results in the moderately CQ-resistant *P. falciparum* strain K1, and interestingly, found that in this case, the VR and SR concentrations useful for CQ resistance reversal can be reduced 1,000- and 100-fold, respectively.

## MATERIALS AND METHODS

### *Chemicals*

Fantofarone (see Fig. 1) was a generous gift from Professor Michel Lazdunski (Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Auripolis, France). Verapamil (Fig. 1) and chloroquine phosphate were obtained from Sigma. [$^3$H]Hypoxanthine (1–5 Ci/mol; 1 Ci = 37 GBq) was supplied by Amersham.

### In vitro P. falciparum Culture

Three strains of *P. falciparum* were chosen: the chloroquine-sensitive F32/Tanzania strain (a generous gift from Dr. S. Jepsen, Staten Serum Institute, Copenhagen, Denmark); the chloroquine-resistant FcB1/Colombia strains, kindly donated by Dr. H.G. Heidrich (Max-Planck Institute für Biochemie, Martinsried bei Munich, Germany) and K1 (a generous gift from Professor Marcel Hommel, Liverpool School of Medicine, UK). The parasites were maintained in a continuous culture, according to the method described by Trager and Jensen [26]. Human erythrocytes (type $O^+$) were used at 3–5% hematocrit in an RPMI 1640 medium at pH 7.5, supplemented with 5% human $O^+$ serum, 11 mM glucose, 25 mM HEPES, 44 mM sodium bicarbonate, 60 $\mu g/mL^{-1}$ gentamicin. The culture was performed at 37° in an atmosphere of 91% $N_2$, 6% $O_2$ and 3% $CO_2$.

### *Effects of Calcium Channel Inhibitors on Chloroquine Resistance*

ANTIMALARIAL ACTIVITIES OF CQ, VR AND SR. Chloroquine diphosphate (CQ) was dissolved in RPMI 1640 medium without serum, VR and SR in dimethylsulfoxide (DMSO) to obtain a stock solution of $10^{-2}$ M. After sterilization by filtration through a 0.22 µM cellulose acetate membrane filter, serial dilutions of the drugs (ranging from $10^{-3}$ to $10^{-12}$ molar concentrations) were made in complete medium of RPMI 1640.

Dose-response data of antimalarial activity were assessed by the method of Desjardins et al. on asynchronous cultures [27]. Incorporation of radiolabeled hypoxanthine was used to measure growth of the parasites. Experiments were conducted under a hematocrit of 2% and a parasitemia of 1%. Incubation of cells was performed in 96-well microtitration plates, in a candle jar for 24 hr, at 37°. Then 0.5–1 $\mu Ci/mL^{-1}$ [$^3$H]hypoxanthine were added to each well and the cells were then incubated for an additional 18 hr. At the end of the incubation period, the contents of each well were harvested onto fiber glass filters using a Skatron semi-automated cell harvester. Then, the filters were placed in a scintillation vial with 2 mL of liquid scintillation fluid. Radioactivity was counted with a 1214 Rackbeta Scintillation Counter (LKB).

REVERSAL OF CHLOROQUINE RESISTANCE BY VR AND SR. After establishing the antimalarial activity of VR and SR, their effect on the CQR strains was tested by measuring the $IC_{50}$ of CQ in the presence of a constant subinhibitory concentration of the calcium channel inhibitors, according to Martin et al. [12]. These concentrations are lower than those giving 10% inhibition of the growth of the parasites.

**TABLE 1. Fifty percent inhibition concentration ($IC_{50}$) and the standard deviation of chloroquine (CQ), verapamil (VR), and SR 33557 (SR)***

| Strains | CQ (nM) | VR (μM) | SR (μM) |
|---|---|---|---|
| $F_{32}$ | 14 ± 1.63 | 36 ± 2.05 | 3.1 ± 0.14 |
| FcB1 | 109 ± 4.08 | 13.18 ± 1.38 | 1.03 ± 0.02 |

*Serial dilutions of the drugs were made in complete medium. Thereafter, incorporation of radiolabeled hypoxanthine was used to measure growth of the parasites. The $IC_{50}$ values are the means of three independent experiments and were obtained from dose-response data of the antimalarial activity of the different drugs on asynchrous cultures.

COMBINED USE OF VR AND SR IN MODULATING CHLOROQUINE RESISTANCE. In a series of experiments, we tested the ability of VR and SR to reverse chloroquine resistance when used in combination. For this purpose, dose-response data of the antimalarial activity of CQ were assessed by the method of Desjardins et al. on asynchronous cultures [27] in presence of constant subinhibitory concentrations of VR and SR used in combination. Serial dilutions of CQ ranging from $10^{-3}$ to $10^{-12}$ were achieved in presence of 0.1 μM SR + 0.1 μM VR; 0.1 μM SR + 10 nM VR; 10 nM SR + 10 nM VR; 1 nM SR + 1 nM VR and 3 nM VR + 1 nM SR.

INTERACTIONS BETWEEN CQ AND THE CALCIUM CHANNEL BLOCKERS VR AND SR. The interactions (synergism, antagonism or additive effects) between CQ and the calcium channel blockers VR and SR were assessed by isobolograms [12, 28]. Three concentrations of CQ (300 nM; 150 nM; 75 nM) were mixed with 12.5 μM, 25 μM and 50 μM verapamil, respectively. As the antimalarial activity of SR was 10 times more potent than that of verapamil, the concentrations of this drug used in combination with CQ were 10 times lower than those of verapamil. The $IC_{50}$ values obtained were compared to the $IC_{50}$ value of each drug when used alone. Then the corresponding ratios were plotted on an isobologram.

INTERACTIONS BETWEEN VR AND SR. To determine the nature of the interactions between VR and SR, three combinations of the VR/SR ratio established from their respective $IC_{50}$ values were used. Indeed, SR was combined to VR as follow: 30 μM VR + 1 μM SR; 10 μM VR + 1 μM SR; 10 μM VR + 3 μM SR. From these combinations, serial dilutions were achieved, and $IC_{50}$ values determined for each drug. Then, the sums of the fractional inhibitory concentrations (SFIC) were determined according to Berenbaum [28].

## RESULTS

$IC_{50}$ values obtained for CQ, VR and SR are summarized in Table 1. As expected, these results show that the F32 strain of *P. falciparum* was 7 to 8 times more susceptible to CQ than the FcB1 strain. It can be seen that the calcium channel blockers VR and SR possessed a weak intrinsic antimalarial property compared to CQ, and both appeared slightly more potent on the CQ-resistant than on the CQ-sensitive parasites. Interestingly, SR was *ca.* 10 times more potent than verapamil.

When using subinhibitory concentrations of each calcium channel blocker, (concentration approximately 1/10 of their $IC_{50}$), neither verapamil nor fantofarone exhibited a significant effect on the CQS strain F32 (data not shown). In contrast, Fig. 2 and Table 2 clearly show that at these concentrations, verapamil completely restored the CQ sensitivity of the CQR FcB1 strain ($IC_{50}$ now 17 nM), whereas fantofarone only partially restored CQ sensitivity, decreasing the $IC_{50}$ value for CQ to approximately one-third (35 nM) that of the $IC_{50}$ for CQ of the sensitive F32 *P. falciparum* strains (15 nM, Table 1). The degree of potentialisation (DOP) revealed that VR was 2 to 3 times more potent than SR in reversing CQ resistance.

Below 1 μM, VR did not reverse CQ resistance. As SR only partially reversed the CQ resistance of the FcB1 strain, it was interesting to evaluate the ability of these compounds to modulate drug resistance when used in combination. To do so, we chose combinations in which the SR concentration was constant at 0.1 μM (the concentration necessary to reduce the $IC_{50}$ of CQ on FcB1 to one-third) and associated with verapamil concentrations ranging from 0.1 μM to 10 nM (concentrations unable to reverse CQ resistance when used alone) (Table 3). In a first combination (0.1 μM SR + 0.1 μM VR), CQ resistance was completely reversed in the FcB1 strain. In addition, the $IC_{50}$ value of CQ fell to 4.88 nM, a value 3-fold lower than that obtained on the CQ-sensitive strain F32. In a second combination (0.1 μM SR + 10 nM VR), CQ resistance was again reversed, and the $IC_{50}$ of CQ became equal to that of the CQS strain F32. Thus, although SR appeared unable to completely reverse CQ resistance when used alone at 0.1 μM, the CQ sensitivity of the resistant parasite was completely restored when this drug was used in combination with very low concentrations of verapamil.

In another series of experiments, we determined the nature of the interactions between each calcium channel blocker and chloroquine. For this purpose, isobolograms were drawn according to Berenbaum [28]. Experiments were performed on the FcB1 strain (Table 4). Three concentrations of CQ (300 nM, 150 nM and 75 mM) were mixed with 12.5 μM, 25 μM or 50 μM VR or with 1.25 μM, 2.5 μM and 5 μM SR. The $IC_{50}$ values of the different drugs were determined by using serial dilutions. The sums of the fractional inhibitory concentrations (SFIC) were lower than 1, ranging from 0.095 to 0.366. These results indicated a synergistic effect of each calcium channel blocker on chloroquine activity. Furthermore, the concave shape exhibited by the isobolograms in Fig. 3 confirmed these results. The degree of potentialisation (DOP) corresponding to the ratio of the $IC_{50}$ of CQ alone to the $IC_{50}$ of CQ in presence of VR or SR showed that verapamil was ca. 2

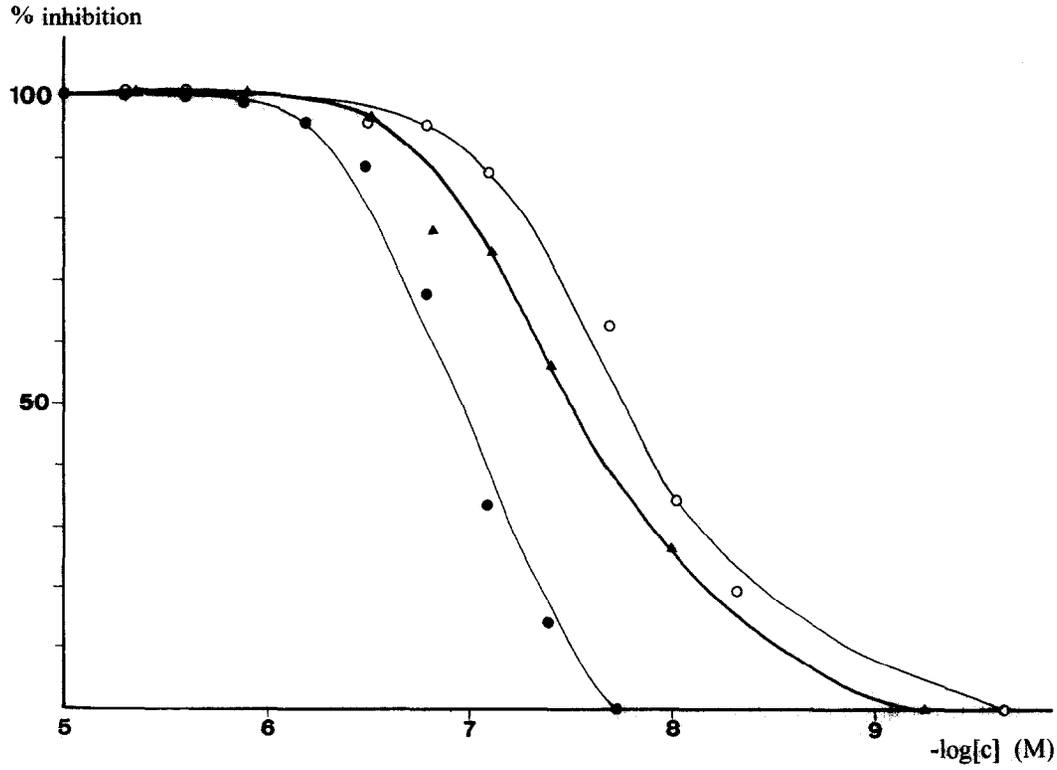



**FIG. 2. Dose-response curves of chloroquine and activities of the chemosensitizers on the CQ-resistant P. *falciparum* strain FcB1. These curves were generated by using a serial dilution of chloroquine alone (●), a serial dilution of chloroquine in the presence of a constant concentration of SR: 0.1 μM (◇), and a serial dilution of chloroquine in presence of a constant concentration of VR: 1 μM VR (○). Results represent the means of three independent experiments.**

times more effective than fantofarone in potentiating the effect of chloroquine.

The interactions between SR and VR are shown in Table 5. Three combinations of these two drugs were achieved in the absence of CQ as described in "Materials and Methods," and the SFIC calculated. As the values obtained were lower than 1, these experiments demonstrated that SR and VR acted synergistically on the FcB1 strain.

In another series of experiments, we evaluated the CQR reversal properties of SR and VR on K1 (clone B1), a moderately CQR strain of *P. falciparum* (Table 6). The $IC_{50}$ values of CQ, VR and SR on this strain were 48.5 nM, 5.42 μM and 1.37 μM, respectively. It appeared that the K1 strain was twofold less resistant to CQ than the FcB1 strain. Fantofarone, when used at 0.1 μM, modulated CQ resistance with the $IC_{50}$ of the drug falling to 17.79 nM, a value close to that obtained on the CQS strain. In addition, we found that very low concentrations of VR and SR used in combination (10 nM SR + 10 nM VR; 3 nM VR + 1 nM SR) in presence of CQ were able to completely reverse CQ resistance.

**TABLE 2. $IC_{50}$ and the standard deviation of chloroquine against the CQR P. *falciparum* strain FcB1 in the presence of subinhibitory concentrations of VR (1 μM and 2 μM) and subinhibitory concentrations of SR (0.1 μM and 0.25 μM)***

| | CQ + 1 μ VR | CQ + 2 μM VR | CQ + 0,1 μM SR | CQ + 0,25 μM SR |
|---|---|---|---|---|
| $IC_{50}$ (nM) | 17 ± 1.5 | 13 ± 1.3 | 35 ± 1.0 | 35 ± 1.0 |
| DOP | 6.4 | 8.3 | 3.1 | 3.1 |

*Values are the means of three independent experiments. DOP is defined as the degree potentiation and is calculated by $IC_{50}$ CQ alone/$IC_{50}$ CQ + chemosensitizer. It appeared that VR was 2 to 3 times more potent than SR in modulating CQ resistance.

## DISCUSSION

The FcB1 strain of *P. falciparum* appeared 7 to 8 times more resistant to chloroquine than the sensitive F32 strain. The

**TABLE 3. Combined use of VR and SR in modulating CQ resistance***

| Combinations | 0.1 μM SR + 0.1 μM VR | 0.1 μM SR + 10 nM VR |
|---|---|---|
| $IC_{50}$/CQ | 4.88 ± 0.27nM | 16.39 ± 0.81nM |
| DOP | 22.3 | 6.6 |

*Dose-response data of the antimalarial activity of CQ were assessed on asynchronous cultures (FcB1 strain) in presence of constant subinhibitory concentrations of VR and SR used in combination (0.1 μM SR + 0.1 μM VR; 0.1 μM SR + 10 nM VR). As revealed by the DOP values, it appears that these combinations potentiated the antimalarial activity of CQ 22- and 6-fold, respectively. Results represent the means of three independent experiments.

**TABLE 4. $IC_{50}$ and the standard deviation of CQ, VR and SR in drug combinations on asynchronous cultures (FcB1 strain)***

| Combinations | 300 nM CQ + 12.5 μM VR | 300 nM CQ + 1.25 μM SR | 150 nM CQ + 25 μM VR | 150 nM CQ + 2.5 μM SR | 75 nM CQ + 50 μM VR | 75 nM CQ + 5 μM SR |
|---|---|---|---|---|---|---|
| $IC_{50}$ | 9.06 ± 0.42 nM CQ<br>0.38 ± 0.11 μM VR | 13.12 ± 0.61 nM CQ<br>0.05 ± 0.01 μM SR | 4.37 ± 0.13 nM CQ<br>0.74 ± 0.08 μM VR | 9.37 ± 0.68 nM CQ<br>0.15 ± 0.01 μM SR | 2.11 ± 0.08 nM CQ<br>1.41 ± 0.21 μM VR | 5 ± 0.13 nM CQ<br>0.33 ± 0.04 μM SR |
| SFIC | 0.108 ± 0.011 | 0.168 ± 0.016 | 0.095 ± 0.004 | 0.236 ± 0.018 | 0.125 ± 0.017 | 0.366 ± 0.091 |
| DOP | 12 | 8 | 25 | 11 | 51 | 21 |

*CQ was mixed with VR or SR in a fixed ratio of their $IC_{50}$ values when used alone. The $IC_{50}$ values of the different drugs in combination were determined by using serial dilutions. Then, the sums of the fractional inhibitory concentrations (SFIC) were calculated. As shown in this table, these sums were lower than 1, indicating a synergistic effect of each chemosensitizer on CQ activity. Results represent the means of three independent experiments.

indolizinesulfone SR33557 or fantofarone (SR) was 10 times more potent than the phenylalkylamine verapamil (VR) on the two *P. falciparum* strains. As revealed by the isobolograms, the two calcium channel blockers potentiated the CQ sensitivity activity on the CQ-resistant *P. falciparum* strain, verapamil appearing 2 to 3 times more potent than fantofarone. Furthermore, when used at similar subinhibitory fractions of their $IC_{50}$, VR was 2 to 3 times more potent than SR in decreasing CQ resistance.

There is much controversy concerning the mode of antimalarial action of CQ as well as the mechanism of CQ resistance in *P. falciparum* [29]. The model based on the observation that verapamil reverses CQ resistance by preventing CQ efflux from resistant cells [12, 30] is similar to the overexpressed P-glycoprotein in MDR tumor cell lines which functions as an ATP-dependent pump, expeling many anticancer drugs [31, 32]. However, in addition to the absence of relation between the resistance to CQ and the overexpression of the Pf.MDR1 glycoprotein [11], it was shown that verapamil did not modulate the efflux of CQ in resistant strains or even under conditions of full reversion of drug resistance by verapamil [33, 34]. A second model, the so-called weak base hypothesis, proposes that CQR *P. falciparum* strains have a weakened vacuolar protein pump so the CQ may not accumulate in the food vacuoles [35]. These two models purport that CQ import is realized by simple diffusion, but recent data from Lanzer and coworkers demonstrated the presence of a chloroquine importer, probably a plasmodial $Na^+/H^+$ exchanger, with a lower transport activity and a reduced affinity for CQ in the CQR parasite isolates [36].

In *P. falciparum*, neither the cytotoxic targets nor the

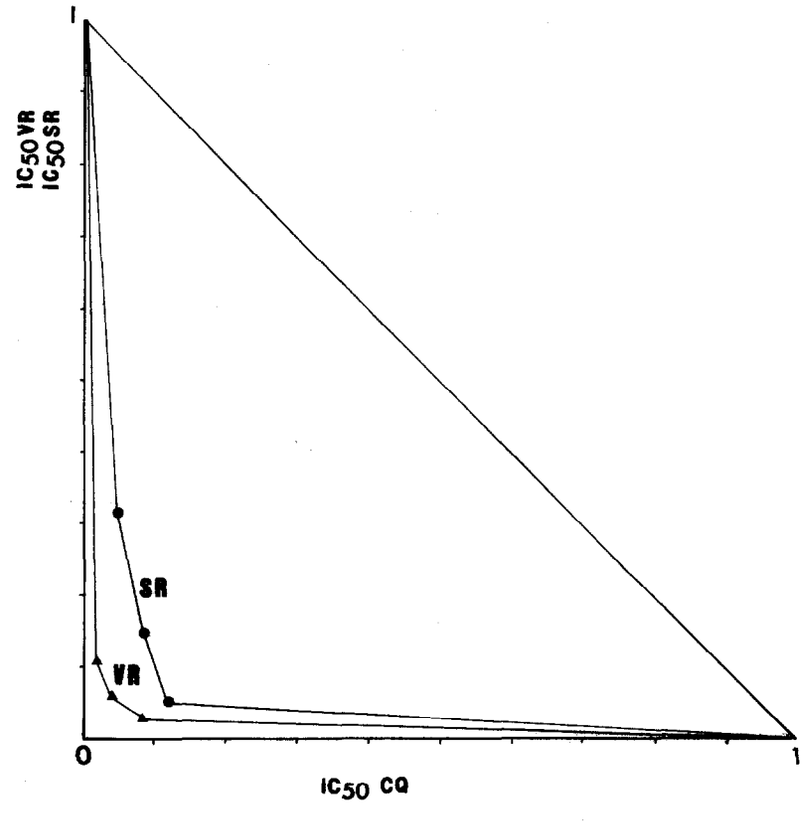



**FIG. 3. Isobolograms constructed from $IC_{50}$ values in Table 4. Control $IC_{50}$ normalized to one unit refers to CQ alone, VR or SR alone. In each drug combination, $IC_{50}$ was plotted as fraction of control $IC_{50}$ values. Isobolic curves are concave and reveal synergistic effects of CQ and VR, CQ and SR on the CQR *P. falciparum* strain FcB1. Results represent the means of three independent experiments.**

**TABLE 5. $IC_{50}$ and the standard deviation of VR and SR when used in combination on asynchronous cultures (FcB1 strain)***

| Combinations | 30 μM VR + 1 μM SR | 10 μM VR + 3 μM SR | 20 μM VR + 2 μM SR |
|---|---|---|---|
| $IC_{50}$ | VR: 1.29 ± 0.16<br>SR: 0.04 ± 0.01 | VR: 0.44 ± 0.12<br>SR: 0.86 ± 0.11 | VR: 1.17 ± 0.21<br>SR: 0.17 ± 0.09 |
| SFIC | 0.13 ± 0.03 | 0.86 ± 0.11 | 0.24 ± 0.10 |

*VR and SR were mixed in three different combinations and their $IC_{50}$ values calculated as in Table 4. The sums of the fractional inhibitory concentrations (SFIC) were lower than 1 and thereby reveal that the two calcium channel blockers acted synergistically. Results represent the means of three independent experiments.

reversal mechanism of the chloroquine resistance of VR have been elucitated. Recently, we have demonstrated that the inhibitory effect of VR on the parasite's maturation does not depend on a change in its $Ca^{2+}$ content [23]. Ye and Van Dyke [37] have also indicated that the reversal of CQ resistance by VR and its derivatives is not linked to an inhibition of the calcium channel. A fluorescent derivative of VR has been shown to accumulate in the lysosomes of drug-sensitive NIH/3T3 cells and to be rapidly transported out in MDR cells [38]. In *P. falciparum*, the target of the cytotoxicity of VR could be the food vacuole, an organelle with lysosomal features, and the accumulation of VR could disrupt the functioning of a number of lysosomal enzymes as observed in cancer cells [39]. Such a situation could explain that reversion of CQ resistance by verapamil could be achieved, although the CQ concentration did not reach levels observed in sensitive strains [33]. Fantofarone does not interact with P-glycoprotein of the leukemia P 388/ADR, resistant to doxorubicin but with a 65-kDa protein [22]. In contrast to fantofarone's greater efficiency than verapamil in restoring drug sensitivity in this leukemia cell line, fantofarone only partially restores the CQ-sensitive of resistant *P. falciparum* strains. The more spectacular effect of fantofarone is its synergistic effect with verapamil, probably by an independant mechanism, which enlightens the advantages of polychemotherapy in malaria, as in cancer or viral therapy.

A major difficulty for medical applications of the drug resistance reversal strategy is that the doses of chemosensitizers needed to reverse the drug resistance status are often toxic [for review see ref. 40]. For example, in terms of the potential clinical utility for reversing MDR *in vivo*, verapamil is limited by its cardiovascular effects in humans at plasma concentrations in the 2 to 6 μM range needed to antagonize MDR *in vitro* [41]. Indeed, the maximum tolerable plasma level beyond which side-effects become unacceptably frequent is 2.5 μM. Therefore, approaches which will help to lower the doses of chemosensitizers and prevent toxic effects need to be investigated. Thus, we have examined the combined use of VR and SR in order to determine whether lower doses of each agent could be used to overcome drug resistance. Using the highly CQ-resistant strain FcB1, we found that VR and SR acted synergistically to reverse CQ resistance. In addition, the concentrations of VR used in these combinations were reduced 10- to 100-fold (e.g., 100 to 10 nM) those needed when the drug is used alone. These results were confirmed in the moderately CQ-resistant strain K1. Interestingly, the concentrations of VR and SR useful for CQ resistance reversal were reduced 1,000- and 100-fold, respectively. As the chemosensitizer concentrations needed to reverse CQ resistance are considerably reduced when used in combination, one could efficiently apply this approach in human with no significant cardiovascular effects.

The strategy described in this paper is based on the combination of chemosensitizers for drug resistance circumvention and confirms the results obtained by many investigators in cancer chemotherapy. Indeed, Ford et al. [42] have explored this strategy *in vitro* by analyzing the combined effect of the calmodulin (CaM) antagonist trans-flupenthixol plus verapamil for the reversal of MDR in the human MDR MCF-7 breast cancer cell line. Using the

**TABLE 6. Summary of the $IC_{50}$ and the standard deviation of CQ, VR and SR used alone or in combination on the moderately CQ-resistant K1 strain***

| Drugs | CQ | VR | SR | CQ + 1 μM VR | CQ + 0.1 μM SR | CQ 10 nM SR + 10 nM VR | CQ 1 nM SR + 1 nM VR | CQ 1 nM SR +3 nM VR |
|---|---|---|---|---|---|---|---|---|
| $IC_{50}$ | 48.50 ± 1.32 nM | 5.42 ± 0.45 μM | 1.37 ± 0.14 μM | 14.65 ± 0.37 nM (CQ) | 17.79 ± 0.32 nM (CQ) | 10.81 ± 0.32 nM (CQ) | 45.14 ± 1.65 nM(CQ) | 12.71 ± 0.41 nM (CQ) |
| DOP | — | — | — | 3.3 | 2.7 | 2.5 | 1.0 | 3.8 |

*The $IC_{50}$ and the standard deviation of CQ, VR and SR when used alone on the moderately CQ-resistant K1 strain. On the other hand, the $IC_{50}$ of CQ was determined in presence of the chemosensitizers used alone at a subinhibitory concentration, or in presence of the chemosensitizers used in combination. It appeared that when used in combination, concentrations of chemosensitizers ranging from 10 nM to 1 nM reversed CQ resistance. Results represent the means of three independent experiments.

isobologram method, they found these two chemosensitizers to be additive in their ability to reverse doxorubicin resistance. Similarly, other investigators have studied the effects of VR plus cyclosporine A (CsA) *in vitro*. Hu et al. [43] have shown that these two chemosensitizers at doses of approximately 1 μM synergistically reverse vinblastine resistance in MDR human leukemia cells. Slater's group found CsA and VR to be highly synergistic in their effect on daunorubicin in the human MDR ALL cell line [44]. Another study reported a synergistic interaction between VR and quinine for potentiation of doxorubicin and vinblastine cytotoxicity in MDR human myeloma cells [45].

The observations described in this paper need to be explored *in vivo* to confirm these *in vitro* experimental data. Future studies may reveal that the malaria parasites contain a multiplicity of genes which upon transcriptional activation can function to alter drug transport processes and thus contribute to the development of chloroquine resistance. Identifying and characterizing these genes will be important for the screening of new chemosensitizers. The exact identity of proteins which contribute to chloroquine resistance remains to be determined. We are currently trying to identify and to characterize overexpressed proteins in different CQR *P. falciparum* strains, particularly a homologue of the 65-kDa protein described in MDR cancer cells.

*We thank Dr. Y. Boulard for fruitful discussion and Ms. N. Caillaud and R. Tchéprakoff (CNRS) for technical assistance. This work was supported by the Centre National de la Recherche Scientifique (GDR 1077) and in part by the European Economic Community (TS3-CT 92-0145).*

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