Reversal of Chloroquine Resistance in *Plasmodium falciparum* by Verapamil

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The parasite *Plasmodium falciparum*, like neoplastic cells, develops resistance to multiple structurally unrelated drugs. If the mechanisms by which *P. falciparum* and neoplastic cells become resistant are similar, then it may be possible to reverse the resistance in the two types of cells by the same pharmacological agents. Verapamil, a calcium channel blocker, completely reversed chloroquine resistance in two chloroquine-resistant *P. falciparum* clones from Southeast Asia and Brazil. Verapamil reversed chloroquine resistance at the same concentration ($1 \times 10^{-6} M$) as that at which it reversed resistance in multidrug-resistant cultured neoplastic cells. This same concentration of verapamil had no effect on chloroquine-sensitive parasites. Hence, chloroquine resistance in *P. falciparum* may fit the criteria for the multidrug-resistant phenotype.

CHLOROQUINE, A 4-AMINOQUINOline, was developed as a substitute for quinine in the treatment of malaria during World War II (*1*). Its initial success promised a global eradication of malaria, but such optimism was dampened by 1960 with the reports of chloroquine-resistant malaria from Thailand and Colombia (*2, 3*). Chloroquine-resistant malaria is now spreading rapidly across malaria endemic areas. By 1984 chloroquine-resistant *Plasmodium falciparum* had spread to 15 countries in eastern Asia and Oceania, 10 in South America, and 15 in Africa south of the Sahara. Mefloquine, a 4-quinolinemethanol, is the only drug proved to be effective against multiple drug–resistant malaria (*4*), but failures with mefloquine treatment were reported before its licensure was completed (*5*). An isolate of *P. falciparum* from Thailand was shown in vitro to be resistant to mefloquine, chloroquine, and quinine (*6*). Similar patterns of cross-resistance were also observed during laboratory induction of drug resistance in cloned strains of *P. falciparum*. With the acquisition of resistance to the sesquiterpene lactone Qing hao su (artemisinin), there was concomitant decreased susceptibility to mefloquine and halofantrine (*7*). This resistance to multiple drugs by an isolate of *P. falciparum* and, in particular, a change in the susceptibility profile to other structurally unrelated drugs upon induction of resistance with a single drug is reminiscent of multidrug resistance in cancer chemotherapy.

Multidrug resistance is encountered in cancer chemotherapy when treated neoplastic cells become resistant not only to the drug used in treatment, but also to other drugs (*8*). An in vitro correlate of this phenomenon occurs when cross-resistance to anthracyclines, *Vinca* alkaloids, protein synthesis inhibitors, and other structurally unrelated drugs is established by selecting cultured cells for resistance to a single drug (*9*). Studies of the molecular basis for this kind of resistance suggest that enhanced active efflux of the drug prevents its accumulation to toxic levels within the cytosol of the resistant cells (*10–12*). If this active efflux is inhibited, the drug then accumulates and the resistant cells again become sensitive (*10–12*). Verapamil, a calcium channel blocker, reverses drug resistance in cultured neoplastic cells by inhibiting the active efflux of these drugs from the resistant cells (*10–12*).

If chloroquine resistance in *P. falciparum* malaria were due to a similar failure of the drug to reach toxic levels within the parasite because of an acquired enhanced active efflux, one might also be able to reverse this resistance with verapamil. We tested this hypothesis by evaluating the susceptibilities of cloned chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*. Incorporation of radiolabeled hypoxanthine was used to measure asynchronous growth of the parasites, and inhibition of incorporation was the measured parameter of the response of the parasites to the drugs. The semiautomated microdilution technique (*13*) was used to determine in vitro the susceptibilities of the chloroquine-sensitive West African clone (D-6) and two chloroquine-resistant clones from Indochina (W-2) and Brazil (IEC-306). Preliminary studies with verapamil suggested that verapamil alone had weak intrinsic antimalarial properties in vitro. Although 1000-fold less potent than chloroquine or mefloquine, the drug showed an inhibitory concentration (IC_{50}) of 6 to 8 μg/ml, similar to that of tetracycline. The interaction of verapamil and chloroquine was therefore assessed by means of two different methods. In the first method, the drugs were diluted in fixed ratios of starting concentrations predetermined to generate well-defined concentration response curves over a 64-fold range. In the second method, concentration response data for the chloroquine-sensitive and chloroquine-resistant clones were generated by

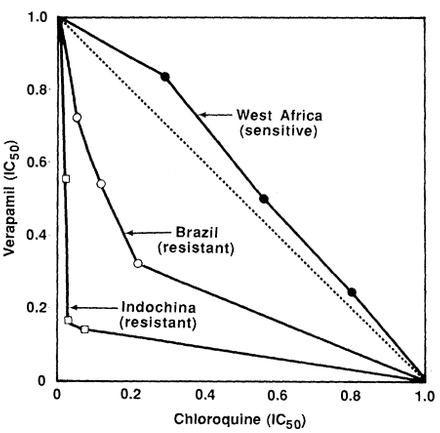

Fig. 1. Isobologram showing a selective marked synergistic effect of chloroquine and verapamil on resistant malaria parasites. Control IC_{50} normalized to one unit of IC_{50} refers to chloroquine alone and verapamil alone. In each drug combination IC_{50} was plotted as fractions of control IC_{50}'s.

Table 1. Fifty percent inhibitory concentration (IC_{50}) of chloroquine (CQ) and verapamil (VER) for each drug alone and in combination. Twofold dilutions of drug or drug combination at fixed ratios of their IC_{50} were added to parasite cultures, and inhibition of incorporation of radiolabeled hypoxanthine was determined by a semiautomated microdilution technique (*13*). All drug concentrations and combinations were run simultaneously in duplicate with the same batch of parasites.

Clone	Single drugs		Drug combinations		
	CQ (n*M*)	VER (μ*M*)	50 n*M* CQ 50 μ*M* VER	25 n*M* CQ 75 μ*M* VER	75 n*M* CQ 25 μ*M* VER
West Africa (D-6)	14.1	15.7	8.0 n*M* CQ 8.0 μ*M* VER	4.4 n*M* CQ 13.2 μ*M* VER	11.4 n*M* CQ 3.8 μ*M* VER
Indochina (W-2)	41.9	8.0	1.3 n*M* CQ 1.3 μ*M* VER	1.5 n*M* CQ 4.4 μ*M* VER	3.8 n*M* CQ 1.3 μ*M* VER
Brazil (IEC-306)	52.3	11.5	6.3 n*M* CQ 6.3 μ*M* VER	2.8 n*M* CQ 8.3 μ*M* VER	11.7 n*M* CQ 3.9 μ*M* VER

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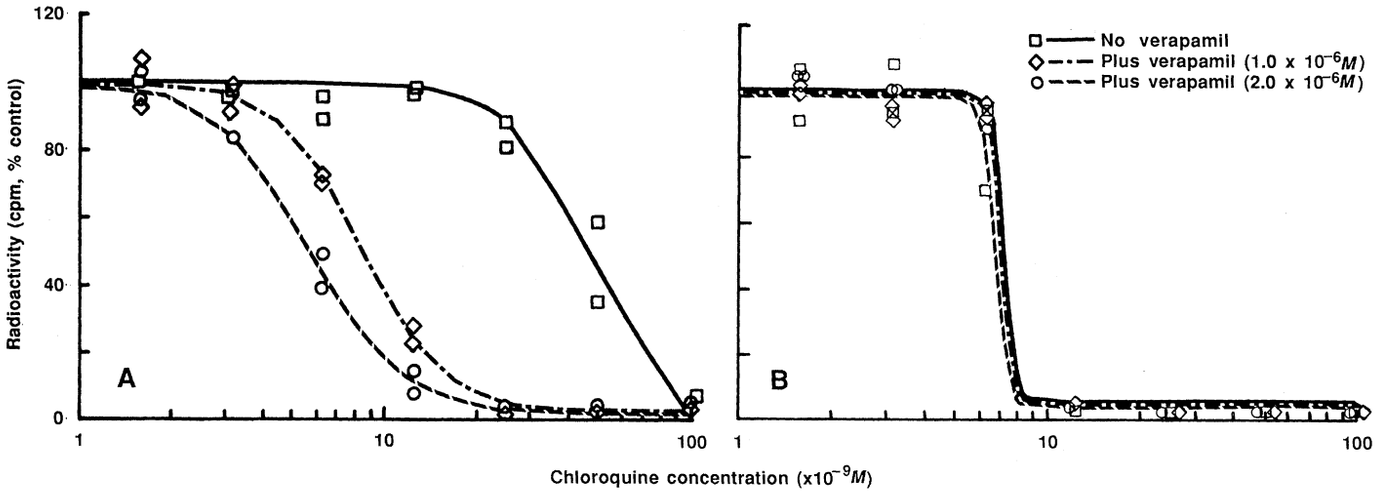

Fig. 2. Dose-response curves of (**A**) chloroquine-resistant *P. falciparum* clone (W-2) from Indochina and (**B**) chloroquine-sensitive *P. falciparum* clone (D-6) from West Africa in the absence or presence of a constant concentration of verapamil. The two concentrations of verapamil used had been previously determined to have no significant effect on parasite growth.

using chloroquine alone or chloroquine in the presence of a constant subinhibitory concentration of verapamil.

Table 1 shows the IC_{50} of chloroquine and verapamil for each drug alone and in three fixed ratio combinations. An isobologram (Fig. 1) constructed from these data shows the marked synergistic effect of the two drugs against the resistant clones (W-2 and IEC-306) but not against the sensitive clone (D-6). The concave curves in Fig. 1 depict synergism between the two drugs (*14*). Table 2 shows that verapamil at constant subinhibitory concentrations reduces the IC_{50} of the resistant clone to that of the sensitive clone, whereas the IC_{50} of the sensitive clone remained unchanged in the presence of similar concentrations of verapamil. Figure 2 shows this specificity of the chloroquine potentiating effect of verapamil for the resistant clone.

The antimalarial activity of chloroquine is related to its ability to reach high concentrations within the parasite food vacuole (*15*). The mechanism by which this concentration effect is achieved is unclear, but it is believed to involve binding to a putative chloroquine receptor (*16*). In spite of our lack of understanding of the mechanism of action of chloroquine as an antimalarial agent, it has consistently been shown with *P. berghei* in mice (*17*), *P. falciparum* in *Aotus* monkeys (*18*), and *P. falciparum* in vitro (*19*) that erythrocytes infected with chloroquine-resistant parasites accumulate less chloroquine than those with sensitive parasites. Although our findings with verapamil may be indicative of a quantitative or qualitative restoration of the effective binding properties of the chloroquine receptors, such a mechanism of action has not been described for verapamil in other biological systems. Indeed, verapamil is known to reverse drug resistance in cultured neoplastic cells by inhibiting active efflux of the drug from resistant cells (*10–12*).

The mechanism by which verapamil inhibits efflux of anticancer drugs from resistant cancer cells is not completely understood but may involve its ability to bind to a 150K to 170K membrane-associated glycoprotein. Verapamil inhibits the binding of photoaffinity-labeled vinblastine to a similar 150K to 170K membrane protein present only on resistant KB carcinoma cells (*20*). There is no direct evidence for the presence of such a protein in resistant malaria parasites, but our findings with verapamil provide the rationale for looking at similarities in acquisition of drug resistance between plasmodia and neoplastic cells. A 140K to 170K membrane glycoprotein (P-glycoprotein) has been identified as the product of the *mdr*1 gene (*21*). This gene is amplified or overexpressed in human cell lines of diverse origin selected for multidrug resistance with various lipophilic drugs (*22*). The possibility that a parallel gene and gene product is involved in resistant plasmodia, although highly speculative, is intriguing. The observation that chloroquine reverses multidrug resistance in human KB carcinoma cells (*23*) is consistent with our proposed analogy.

In cardiac tissues verapamil binds to the inner surface of the plasmalemma and stereospecifically inhibits a calcium-activated adenosinetriphosphatase (*24*). We know of no evidence that the verapamil-associated calcium pump and the efflux pump involved in drug resistance reversal are one and the same. Hence the calcium channel–blocking properties of verapamil may or may not be the same as its chloroquine potentiating actions.

The idea that a cell can protect itself from the toxic effects of a drug by preventing the drug's accumulation within its cytosol is appealing. Unicellular organisms eliminate some of their toxic wastes by excretion across the plasma membrane. Thus, the capacity for active efflux of cytotoxic drugs may not be an attribute of cultured, resistant, mammalian neoplastic cells alone, but also of resistant parasitic and free-living organisms. Additional studies are required to document these findings in vivo. If reversal of drug resistance is validated in a clinical setting, this observation may suggest a new approach for the potential management of infections caused by resistant organisms.

Table 2. Fifty percent inhibitory concentration ($\times 10^{-9}M$) of chloroquine against a chloroquine-sensitive and a chloroquine-resistant clone of *P. falciparum*. Data were generated from concentration-response of parasites to chloroquine alone and in the presence of a constant concentration of verapamil that had been determined to have no significant effect on parasite growth. All drug concentrations and combinations were run simultaneously in duplicate with the same batch of parasites; CQ, chloroquine.

Clone	(IC_{50} + 1 SD)		
	CQ alone	CQ plus $1 \times 10^{-6}M$ VER	CQ plus $2 \times 10^{-6}M$ VER
West Africa (D-6), CQ-sensitive	8.6 ± 0.3	7.8 ± 0.6	8.8 ± 0.3
Indochina (W-2), CQ-resistant	46.5 ± 2.5	8.4 ± 0.4	5.6 ± 0.3

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U3 Sequences from HTLV-I and -II LTRs Confer *px* Protein Response to a Murine Leukemia Virus LTR

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Human T-cell leukemia virus (HTLV) types I and II are unusual among replication-competent retroviruses in that they contain a fourth gene (*x*) necessary for replication. The *x* gene product, *px*, transcriptionally transactivates the viral long terminal repeat (LTR), and is thus a positive regulator. To investigate *px* transactivation, sequences from the U3 regions of the LTRs of HTLV-I and -II were inserted into the Moloney murine leukemia virus (M-MuLV) LTR by recombinant DNA techniques. Transient expression assays of the chimeric LTRs indicated that the HTLV sequences conferred to the M-MuLV LTR responsiveness to HTLV *px* protein. M-MuLV enhancers were not required for function of the chimeric LTRs. Infectious recombinant M-MuLVs containing chimeric LTRs were also generated. These viruses showed higher infectivity when assayed in mouse cells expressing HTLV-II *px* protein compared to normal mouse cells. Thus the HTLV sequences were able to confer *px* responsiveness to infectious M-MuLV. The generation of a virus dependent on a transactivating protein for its replication has implications for the evolution of the human T-cell leukemia viruses.

HUMAN T-LYMPHOTROPIC VIRUSES types I and II (HTLV-I and -II) are replication-competent retroviruses that induce two forms of T-lymphoma in humans (*1*). In addition to the three standard retroviral genes *gag, pol*, and *env*, these viruses contain an additional gene called *x, lor*, or *tat* (*2*). This gene encodes proteins px^{I} and px^{II} of 40 kD and 37 kD, respectively (*3*); these proteins transcriptionally transactivate the HTLV-I or -II long terminal repeats (LTRs) (*4–7*). As a result, transcription from the HTLV LTRs is much more efficient in HTLV-infected cells that express *px* compared with matching uninfected cells (*8*). Also, deletions in the *x* gene of HTLV-II destroy infectivity of the virus (*9*). Thus *px* protein is a positive regulator of HTLV-I and -II expression, and is required for viral replication. The sequences in the HTLV LTRs that respond to the *px* protein have also been investigated. The presence of three copies of conserved 21-bp repeats in the U3 regions of both of these viruses suggested that these repeats might have important enhancer or regulatory function (*2, 10*). The HTLV LTRs also contain regions with transcriptional enhancer activity, as well as sequences that respond to *px* transactivation (*11–13*). In the experiments reported here, U3 sequences from the HTLV-I and -II LTRs were inserted into the U3 region of the LTRs of Moloney murine leukemia virus (M-MuLV). This resulted in the formation of chimeric LTRs that responded to *px*. In addition, replication-competent M-MuLVs containing HTLV-II U3 sequences were responsive to *px* and required it for efficient replication.

The regions of the HTLV-I and -II LTRs used for insertion into the M-MuLV LTR are indicated in Fig. 1A by the cross-hatching. These sequences contained the three copies of the 21-bp repeats but lacked the HTLV TATA sequences at −30. Thus insertion of these sequences into an M-MuLV LTR would not be expected to introduce an additional start site for transcription. In the wild-type M-MuLV LTR, the enhancer sequences are indicated by the stippled box; in addition, another LTR, ΔMo, which lacks the M-MuLV enhancer sequences (*14*), is shown. The HTLV-I and -II U3 sequences were inserted into the wild-type M-MuLV and ΔMo LTRs by means of Xba I linkers, resulting in the recombinant constructs indicated (Fig. 1A). After the chimeric LTRs were generated they were introduced into a plasmid for expressing the chloramphenicol acetyltransferase (CAT) gene and used in transient expression assays (*15*). The LTRs were also used to generate plasmids containing a complete M-MuLV proviral organization with the chimeric LTRs at both ends (Fig. 1A).

The effect of placing the HTLV sequences into the wild-type and ΔMo M-MuLV LTRs was first investigated by transient expression assays in mouse NIH 3T3 cells. Insertion of either HTLV-I or -II sequences into the wild-type M-MuLV LTR did not significantly affect the LTR transcriptional activity (Fig. 1B). In addition, when the HTLV sequences were inserted in either orientation into the ΔMo LTR (which shows no transcriptional activity because it lacks the enhancer sequences), weak but detectable activity was observed. This indicated that the inserted HTLV-I and -II sequences have weak enhancer activity in NIH 3T3 cells, even in the absence of *px* protein.

To test the effect of HTLV-I *px* on the chimeric LTRs, we transfected the CAT expression plasmids into human osteosarcoma (HOS) and HTLV-I–infected HOS (HOS/PL) cells (*16*). In these experiments,

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