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Reversal of Chloroquine Resistance In Falciparum Malaria Independent of Calcium Channels

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Racemic verapamil and close structural derivatives gallopamil and devapamil completely reverse chloroquine-resistance in falciparum malaria at 1-2 micromolar concentrations. If the R-(+) isomers of these calcium channel inhibitors are used, chloroquine-resistance is again completely reversed at similar doses. However, these R-(+) isomers do not bind to cardiovascular calcium channels which are stereospecific for the S-(-) isomer of the drugs. Further since calcium channel inhibition is not involved, toxicity associated with this activity can be avoided. Therefore it is possible that a series of R-(+) isomers could be found that alter the resistant state without possessing significant toxicity. It is postulated that these lipophilic drugs are interacting with the mechanism of resistance, possibly a multidrug resistance glycoprotein pump.

Malaria is implicated in the death of approximately 1 million individuals each year (1). It is a disease that affects children to a much greater extent than adults since immunity is rather slow to develop. A major problem in many tropical regions is that most useful drugs against malaria (e.g., chloroquine, chloroquine congeners, etc.) are losing their effectiveness (2). The spread of this drug resistance is becoming more rapid and appears to be expanding into new areas where the carrier Anopheles or similar mosquito exists. Chloroquine-resistant strains of falciparum malaria originally were recognized in South America and Southeast Asia around the time of the Vietnam war. Similar strains are now found in East Asia and Subsaharan Africa (3).

Until recently there were mainly two approaches used to counteract the problem of drug resistance. Either new drugs were sought that would inhibit the growth of the parasite or vaccines could be developed which would help prevent infection (4). Resistance to the newly developed drugs, such as mefloquine, appears to be occuring almost before there is widespread distribution of the drug (5). Vaccines seem to be directed to a short term protection of the immunologically-naive individual. Since the disease is mostly a problem for people in the third world, that is, to those who have already been exposed to the parasite, protection by vaccines would seem to be an impractical solution to the drug resistance problem.

A new approach was taken by Martin and associates (6) when they found that verapamil can reverse the resistance to chloroquine in several strains of chloroquine-resistant falciparum malaria (CRFM). This work was patterned after a similar observation in cases of multiple drug resistance in cancer (7). However, a major difficulty in this approach to treatment of

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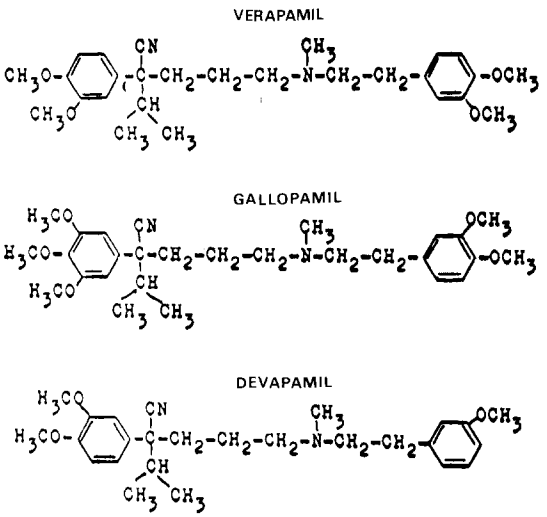

Figure 1. Structures of Verapamil and derivatives Gallopamil and Devapamil.

both diseases, and probably also to the treatment of multiple drug resistance in bacteria, is that the doses of ± verapamil needed to reverse the drug resistance often are toxic (8). Therefore this use of verapamil may be impractical. Originally verapamil was used in the treatment of angina pectoris and, more recently, in hypertension and cardiac arrhythmias (9). It has been recognized that verapamil probably exerts its therapeutic effects by blocking cardiovascular calcium channels (10).

Are there less toxic derivatives of verapamil that can reverse resistance to chloroquine? Could alternative factors play a role? Closer examination of this problem reveals that verapamil is a racemic mixture consisting of S-(-) and R-(+) isomers (11). Further, it has been shown that it is the S-(-) rather than the R-(+) isomer that binds to cardiovascular calcium channels (12), i.e. the calcium channel appears to be sterospecific (13). This observation raises the following questions. Would the R(+) isomer reverse the resistance to chloroquine exhibited by the falciparum malaria strains? Is resistance related to the calcium channels or is the effect of verapamil related to a hydrophobic interaction with the mechanism of resistance? If the R-(+) isomer were effective would this separate the reversal of resistance from the cardiovascular toxicity caused by the S-(-) isomer which binds to the calcium channel? The purpose of this research effort is an attempt at answering these important questions.

Since preliminary work indicated that reversal of chloroquine resistance is probably related to structural differences among the isomers and derivatives of verapamil our initial work was done with the racemic mixtures and R-(+) isomers of verapamil, gallopamil, and devapamil. The structure of these compounds is given in figure one.

MATERIALS AND METHODS

The drugs ± verapamil, ± gallopamil and ± devapamil and their R-(+) isomers were kindly supplied by Knoll AG Pharmaceutical Co., Ludwigshafen, Federal Republic of Germany. The absolute configuration of R-(+) verapamil was established by Ramuz (14). The drugs were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution ($1x10^{-2}$ M) and later diluted

appropriately for assay. The final concentration of DMSO was less than 0.1% which has been shown to exert no inhibitory effect on the growth of the parasite.

Chloroquine (CQ) diphosphate, a gift from the Winthrop-Sterling Drug Company, Renssalaer, New York, U.S.A., also was utilized in these experients and was dissolved in phosphate buffer (pH 7.2).

FCMSU1/Sudan strain and cloned Indochina (W-2) strain of P. falciparum were kindly donated by Dr. J.B. Jensen and Dr. W.K. Milhous respectively. The former is sensitive to CQ and the latter is resistant to CQ. Both strains have been maintained in our laboratory for almost one year. The two strains of the parasite were cultured according to the candle jar method of Trager and Jensen (15). In a given experiment, 4-day-old Petri dish cultures (approx. 10% parasitemia) were diluted with medium containing an amount of noninfected type A human erythrocytes to obtain a culture with a final hematocrit of 1.5% and parasitemia of 0.5-1.0%. The resulting culture was ready for addition to microtitration plates with 96 flat-bottom wells.

The testing procedure used was similar to that described by Desjardins et al. (16). Briefly, the final volume added to each of the 96-well microtitration plates was 250 μl and consisted of 25 μl of complete medium with or without CQ, 175 μl of either the parasitized culture or a nonparasitized human erythrocyte control, and 25 μl of complete medium with or without verapamil or derivative; and 25 μl (0.5μCi) [2,8-^{3}H]adenosine. The microtitration plates were incubated in a candle jar for 24 hr at 37°C.

As the malaria parasite grows ^{3}H-adenosine is metabolized and incorporates into polymeric RNA and DNA (18). The labeled polymers are trapped on glass fiber filters and unincorporated material is washed away. In the absence of drug there is 100% incorporation of the labeled material. When drugs interfere (directly or indirectly), an inhibitory dose of 50% (IC_{50}) can be calulated (19). This formed the basis for the initial screening system for antimalarial drugs (20-24). The experiments were repeated three times except where noted. Statistical analysis was done using Student's t test for significance (19).

RESULTS AND DISCUSSION

Initially the direct toxic effects of ± verapamil ± gallopamil and ± devapamil in 1-2 micromolar doses was assessed and no apparent toxicity was found (data not presented). In Table I the reversal of chloroquine resistance of Plasmodium falciparum (malaria) was studied at 1-2 micromolar doses of racemic verapamil (V), gallopamil (G) or devapamil (D). Complete reversal of chloroquine resistance was observed (R/S ratio approximately 1) when any of these drugs were included in the culture medium, ± V was included to confirm the original work of Martin et al. (6) who studied the drug in the same parasite strain.

Our data now extends this observation to the derivatives G and D. Since it is known that cardiovascular tissue exhibits a stereospecificity for the S-(-) isomer of verapamil and derivatives (13), the R-(+) isomer was tried in hopes of escaping the acute toxicity associated with high dose ± V. In Table II the R-(+) isomers of V, G and D are used to completely reverse resistance to chloroquine in falciparum malaria (R/S ratio approximately 1). These compounds reportedly have 1/10 the acute toxicity of ± verapamil (12). Although all of these isomers reverse chloroquine resistance, G appears to be about twice as potent as the other two drugs. Similar potency differences have been reported for the cardiovascular effects of (±) gallopamil (25).

If R-(+) isomers of verapamil and derivatives reverse resistance to chloroquine it seems likely we are dealing with a non-stereospecific

Table I

REVERSAL OF CHLOROQUINE-RESISTANT STRAIN OF P.FALCIPARUM BY (±)-VERAPAMIL/DERIVATIVES

DRUGS**	IC_{50} OF CQ VS P.FALCIPARUM (MEANS ± S.D. nM)*		RATIO
	SENSITIVE STRAIN	RESISTANT STRAIN	(R/S)
CQ^+	15.1	56.6	3.8
CQ + V_{1+}	21.4	12.4	0.5
CQ + V_{2+}	20.5	6.8	0.3
CQ	22.4 ± 5.8#	111.8 ± 19.0	5.0
CQ + G_1	27.3 ± 5.5	40.2 ± 7.0[a]	1.5
CQ + G_2	25.8 ± 7.5	25.0 ± 8.1[a]	1.0
CQ	18.0 ± 2.1#	138.2 ± 2.6	7.7
CQ + D_1	26.9 ± 4.5	49.2 ± 8.1[a]	1.8
CQ + D_2	22.4 ± 3.8	20.7 ± 3.2[a,b]	0.9

* The data in the table result from 3 trials except where noted with mark + (n=2).
** V_1 and V_2, G_1 and G_2, and D_1 and D_2: $1x10^{-6}$ and $2x10^{-6}$ M of (±)-verapamil, (±)-gallopamil and (±)-devapamil respectively; CQ=chloroquine.
\# Significant difference ($P<0.05$) between sensitive and resistant strain.
[a] Significant difference ($P<0.05$) between resistant strain with and without the drug, as compared with data of CQ alone.
[b] Significant difference ($P<0.05$) between resistant strain with different doses of the drug.

interaction not associated with a binding to cardiovascular calcium channels. We postulate that this may be a hydrophobic interaction with the mechanism of resistance itself. Such an interaction would not display a stereospecific preference for either S-(-) or R-(+) isomers. Since the mechanisms involved in chloroquine and mefloquine resistance appear to be similar, drug resistance in malaria may be related to the multiple drug resistance found in cancer chemotherapy. The latter resistance involves a membrane glycoprotein of 150-170Kd (7) which is intertwined with the membrane of the resistant cancer cell (26). It is believed that the anti-cancer drug enters the resistant cell, but is immediately pumped out of the cell by this glycoprotein with ATPase activity (27). Racemic verapamil innibits the pump and resistance to multiple anticancer drugs is thereby reversed. If the pump in multiple drug resistant cancer cells is similar to the one in malaria, then R-(+) verapamil and similar derivatives should be useful in treating both of these diseases.

Ryall (28) stated that it is unclear whether reversal of drug resistance by verapamil in malaria is a consequence of effects on calcium homeostasis, an altered lysosomal function or results from change in membrane permeability. It would seem that altered cellular permeability is the likely option but only further work will completely clear the issue.

Since R-(+) lipophilic drugs with verapamil-like activity can reverse chloroquine resistance, surely within the 3000 or so verapamil derivatives that have been synthesized there is a compound that produces a reversal of chloroquine resistance without having major toxic effects to humans.

Table II

REVERSAL OF CHLOROQUINE-RESISTANT STRAIN OF P.FALCIPARUM BY ($R^{@}$)-(+)-VERAPAMIL/DERIVATIVES

DRUGS**	IC_{50} OF CQ VS P.FALCIPARUM (MEAN ± S.D. nM)*		RATIO (R/S)
	SENSITIVE STRAIN	RESISTANT STRAIN	
CQ	32.3 ± 10.5#	157.5 ± 59.4	4.9
CQ + V_1	29.3 ± 7.3	72.0 ± 22.4[a]	2.5
CQ + V_2	26.7 ± 6.0	39.7 ± 14.3[a]	1.5
CQ	20.3 ± 3.4#	119.5 ± 15.0	5.9
CQ + G_1	25.4 ± 7.7	35.2 ± 5.6[a]	1.4
CQ + G_2	23.1 ± 9.8	23.9 ± 8.1[a]	1.0
CQ	24.6 ± 7.7#	137.8 ± 12.2	5.6
CQ + D_1	24.6 ± 6.2#	36.5 ± 3.8[a]	1.5
CQ + D_2	22.4 ± 5.6	30.8 ± 2.8[a,b]	1.4
CQ + D_4	22.0 ± 2.1	23.7 ± 6.2[a,b]	1.1

@ R is from R, S convention of organic chemistry
* All of the data in the table results from 3 trials.
** V_1 and V_2, G_1 and G_2, and D_1, D_2 and D_4 1 x 10^{-6}, 2 x 10^{-6} and 4 x 10^{-6} M of (R)-(+)-verapamil, (R)-(+)-gallopamil and (R)-(+)-devapamil Respectively; CQ = chloroquine.
\# Significant difference ($P<0.05$) between sensitive and resistant strain.
a Significant difference (<0.05) between resistant strain with and without the drug, as compared with the data of CQ alone.
b Significant difference ($P<0.05$) between resistant strain with different concentrations of the drug.

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REFERENCES

1. Greenwood, B.M., (1985) Parasite Immunology 7, 191-199.
2. Trenholme, G.M., (1975) Science 190, 792-794.
3. Payne, D. (1987) Parasitology Today 3, 241-246.
4. Nussenzweig, V. and Nussenzweig R.S. (1985) Annals of the Institute Pasteur Immunology 136D 301-306.

5. Boudreau, E.F., Webster, K., Pavanek, K. and Thosinga, L. (1982) Lancet, II, 1335.
6. Martin, S.K., Oduola, A.M., and Milhous W.K. (1987) Science 235, 899-901.
7. Safa, A.R., Glover C.J., Sewell, J.L., Meyers, M.B., Biedler, J.L., and Felsted, R.L. (1987) J. Biol. Chem. 262, 7884-7888.
8. Moscow, J.A., and Cowan, K.H. (1988) J. of National Cancer Institute 80, 14-20.
9. Clayman, C.B. (1988) Guide to Prescription and Over the Counter Drugs Random House, New York, 530.
10. Bigger, T.J. and Hoffman, B.F. (1983) In Goodman and Gilman's The Pharmacological Basis of Therapeutics, MacMillan and Co., NY, 77.
11. Bayer, R., Hennekes, R., Kauffman R., Mannhold, R., (1975) Naunnyn-Schmiedeberg and Arch Pharmacol 290, 49-68.
12. Bayer, R., Kalusche, D., Kaufman, R., and Mannhold, R., (1975) Naunyn-Schmiedeberg's Arch. Pharmacol. 290, 81-97.
13. Bayer, R., Kaufmann, R., and Mannhold, R. (1975) Naunyn-Schmiedeberg's Arch Pharmacol 290, 69-80.
14. Ramuz, H., (1975) Helvetica Chim. Acta, 58, 2050-2060.
15. Trager, W., and Jensen J.B. (1976) Science 193 673-675.
16. Desjardins, R.E., Canfield, C.J., Haynes, J.C. and Chubay, J.D. (1979) Antimicrobial Agents and Chemotherapy 16, 710-718.
17. Carter, G. and Van Dyke, K. (1972) Helm. Soc. of Wash. D.C. 39 244-249.
18. Tallarida, R.J., and Murray R.B. (1987) Manual of Pharmacological Calculations with Computer Progams Springer Verlag, N.Y. 85.
19. Lantz, C.H., Van Dyke, K. and Carter, G. (1971) Exp. Parasitol 29, 402-416.
20. Ye, Z., Van Dyke K. and Wimmer, M. (1987) Experimental Parasitology 64, 418-423.
21. Van Dyke, K., Szustkiewicz, C., Cenedella, R.J., and Saxe, L.H. (1970) Chemotherapy 15, 177-188.
22. Manandhar, M. and Van Dyke, K. (1975) Experimental Parasitology 37, 138-146.
23. Van Dyke, K. and Newcomer, C. (1975) Fed Proc. 34, 807.
24. Mannhold, R., Steiner, R., Haas, W. and Kaufmann, R. (1978) Naunyn-Schmiedeberg's Arch Pharmacol. 302, 217-226.
25. Shier, W.T. (1987) J. Toxicol - Toxin Reviews 6, 73-97.
26. Cornwell, M.M., Tsuro, T., Gottesman, M.M., and Pastan, I. (1987) FASEB J. 1, 51-54.
27. Ryall, J.C. (1987) Parasitology Today 3, 256.