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THE POTENTIATING ACTION OF TETRANDRINE IN COMBINATION WITH CHLOROQUINE OR QINGHAOSU AGAINST CHLOROQUINE-SENSITIVE AND RESISTANT FALCIPARUM MALARIA

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Summary. Using chloroquine-sensitive (CS) and chloroquine-resistant (CR) strains of *Plasmodium falciparum* *in vitro*, interactions between tetrandrine (TT) and either chloroquine (CQ) or qinghaosu (QHS, artemisinin) were assessed using isobolograms. Sums of the fractional inhibitory concentration for the combination of the two drugs are less than one and therefore, we can conclude that *in vitro* TT and CQ or QHS act synergistically against CS and CR falciparum malaria. Remarkably, using CR malaria, TT can lower the IC_{50} dose of CQ as much as 40 fold. These drug combinations may impair the advantage that the development of CQ resistance conveys on the parasite.

Previously we reported that *in vitro* TT has promise as an antimalarial drug, since it had selective inhibitory activity against the chloroquine-resistant strain of *P.falciparum*. Furthermore, TT increased the antimalarial efficacy of CQ against both the resistant and sensitive strain of *P.falciparum* (1). Important questions remain unresolved, however, such as "what is the nature of the interaction between TT and CQ?" and "Is the interaction synergistic or additive?" In this study, we examined the interaction between the drugs to establish an experimental basis for a rational clinical use of this combination in humans. In addition, the interaction between TT and QHS was tested in this study.

QHS is a new antimalarial drug with a rapid and potent effect on the CQ-resistant strain of *P.falciparum* (2,3). The recrudescence rate has been very high (up to 21%) when QHS was used clinically to treat malaria (4). This is probably due to the fact that QHS has a short half-life in plasma (about 30 minutes) according to the results of a pharmacokinetic study (5). Therefore, QHS has been of limited use. A combination of QHS with a long-lived antimalarial drug such as TT may increase the apparent effectiveness

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of QHS. Based on previous findings with radioactively labeled TT, the half-life of TT was 30 hours in mice, 37-45 hours in rats (unpublished data) and 16-25 hours in humans (6). Therefore, the combined use of TT with QHS has the potential for being a more potent therapeutic regimen against malaria.

Materials and Methods

Owing to its insolubility in water at pH 7, TT was dissolved in an acidic solution and then the pH was adjusted to 6 with 1.0 N NaOH (1). This constituted the stock solution (1×10^{-2}M) of TT. Drug was sterile filtered using a 0.22 micron cellulose acetate membrane. Further serial dilutions of TT were made using the complete RPMI 1640 medium containing 10% pooled human A+ serum, 25 mM HEPES and 25 mM $NaHCO_3$.

QHS was kindly supplied to our laboratory by Dr. D.L. Klayman, Walter Reed Institute of Research. QHS was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 1×10^{-3}M. Before each experiment, the stock of QHS was diluted with RPMI 1640 medium without serum to produce a solution at a concentration of 1×10^{-4}M. After sterilization by filtration, serial dilutions of QHS were made in the complete medium of RPMI 1640. The final DMSO concentrations of 0.2% or less had no detectable effect on parasite growth.

Chloroquine diphosphate (Winthrop-Sterling Drug Company, Renssalaer, New York, USA) was dissolved in phosphate buffer (pH 7.2), diluted and sterilized as described for QHS.

Two strains of falciparum malaria were used in these experiments: one is the CQ-sensitive FCMSU1/Sudan strain donated by Dr. J.B. Jensen and the other is the CQ-resistant Indochina (W2) strain provided by Dr. W.K. Milhous. Both parasite strains have been cultured in our laboratory for over two years using the candle jar method (7). For a given experiment, 4-day-old Petri dish cultures with a 5-10% parasitemia were diluted with medium containing sufficient non-parasitized human erythrocyte type A to obtain a culture with a final hematocrit of 1.5% and a parasitemia of 0.5-1.0%.

Dose-response data of antimalarial activity were assessed using the method of Desjardins and co-workers (8). Briefly, the final volume (250 ul) in each well of a 96-well microtiter plate consisted of: (1) 25 ul complete medium with or without drug or drug combination; and (2) 200 ul of the parasitized cultures or non-parasitized human erythrocytes as a control. After incubation of the plates in a candle jar for 24 hours at 37°C, 25 ul of [2,8-^{3}H] adenosine (0.5 uCi) were added to each well. The plates were then incubated at 37°C for an additional 18 hours.

At the end of the incubation, the contents in each well were harvested onto fiberglass filter disks using a Bellco semi-automated cell harvester. The filters were washed with distilled water five times and each disk placed in a scintillation vial with 10 ml of liquid scintillation fluid (9). Radioactivity was counted using a Packard Tri-Carb scintillation spectrometer Model 2425.

The drugs were mixed for the combination experiments in fixed ratios as described by Martin and colleagues (10). Three concentrations of TT (1×10^{-6}, 2×10^{-6} and 3×10^{-6}M) were mixed with CQ or QHS at concentrations of 3×10^{-7}, and 2×10^{-7} and 1×10^{-7}M, respectively. Ratios of TT/CQ or TT/QHS in concentration were 10:3, 10:1 and 30:1, respectively. IC_{50} values of the drugs were calculated using an Apple IIe computer Isobolograms were constructed from the IC_{50} values of each individual drug and drug combination. Duplicate wells were prepared for each drug concentration. Experiments were repeated a total of three times.

Results and Discussion

IC_{50} values obtained for TT and CQ or QHS are summarized in Tables 1 and 2. Isobolograms were then constructed based on these IC_{50} values. Results were expressed in two ways. One is the sum of the fractional inhibitory concentration (SFIC), and the other is an isobolic picture as described by Berenbaum (11). The interaction between two drugs was determined using the following criteria: when SFIC had a value greater than one, antagonism was said to occur; if the SFIC equaled one, there is a simple additive effect; and if the SFIC value obtained was less than one, there is a synergistic interaction.

As seen in Tables 3 and 4, none of the SFIC data in the tables is greater than one. Therefore, it appears that the drug combinations used in all three different fixed ratios between TT and CQ or QHS act synergistically against both the CQ-sensitive and the CQ-resistant strains of *P.falciparum in vitro*.

Figures 1 and 2 show the shape of the isobolic curves obtained for the combination between TT and CQ or QHS. When the isobolic curve is concave, all combinations between two drugs are synergistic. Conversely, if the isobolic curve is convex all drug combinations produce antagonistic results. The curve shapes of the combinations between TT and CQ or QHS in these figures are concave, regardless of which strain of the malarial parasite was used. Therefore, the drug interactions appear to be acting synergistically.

Study of drug combinations usually can provide indirect evidence for separate mechanisms of antimalarial action (12). Since there is a synergistic interaction between TT and CQ, that implies that each drug possessed its own separate antimalarial action. Currently, there are two major hypotheses concerning the mechanism of antimalarial action of CQ: one is that CQ acts as lysosomotropic agents (13, 14); the other is that CQ

Table 1

IC_{50} (nM) OF TT AND CQ FOR EACH DRUG ALONE AND IN COMBINATION*

MALARIA***	SINGLE DRUG		DRUG COMBINATION**		
	TT	CQ	TT(1.0uM) CQ(0.3uM)	TT(2.0uM) CQ(0.2uM)	TT(3.0uM) CQ(0.1uM)
S STRAIN	498.1±93.7	26.7±3.8	54.9±7.1(TT) 16.5±2.1(CQ)	114.1±23.0(TT) 11.4± 2.3(CQ)	223.3±38.6(TT) 7.4± 1.3(CQ)
R STRAIN	197.5±24.7	185.8±4.9	79.5±13.7(TT) 23.8±4.1(CQ)	79.5±16.1(TT) 8.0± 1.6(CQ)	124.6± 9.6(TT) 4.2± 0.3(CQ)

*The data in the table above are mean values ± S.D. (nM) from three experiments except where noted.

**Ratios of TT/CQ in the drug combinations are 10:3, 10:1, and 30:1, respectively.

***S and R strains represent CQ-sensitive (FCMSU1/Sudan) and resistant (w2) strain of *P. falciparum*, respectively.

Table 2

IC_{50} (nM) OF TT AND QHS FOR EACH DRUG ALONE AND IN COMBINATION*

MALARIA***	SINGLE DRUG		DRUG COMBINATION**		
	TT	QHS	TT(1.0uM) QHS(0.3uM)	TT(2.0uM) QHS(0.2uM)	TT(3.0uM) QHS(0.1uM)
S STRAIN	410.2±69.0	36.7±4.7	71.9±8.9(TT) 21.6±2.7(QHS)	113.5±6.3(TT) 11.4±0.6(QHS)	219.5±35.5(TT) 7.3±1.2(QHS)
R STRAIN	205.6±49.8	47.8±14.5	59.6±13.7(TT) 17.9±4.1(QHS)	71.8±13.8(TT) 7.2±1.4(QHS)	136.9±41.6(TT) 4.6± 1.4(QHS)

*The data in the table above are the mean values ± S.D. (nM) from three experiments except where noted.

**Ratios of TT/QHS in the drug combinations are 10:3, 10:1, and 30:1, respectively.

***S and R strains represent CQ-sensitive (FCMSU1/Sudan) and resistant (w2) strain of P. falciparum, respectively.

forms a lytic or membrane-toxic complex with ferriprotoporphyrin IX (15). The mode of antimalarial activity of TT is unclear. However, using S180 cells in vitro to observe biochemical effects of TT, the results revealed that TT appeared to inhibit the biosynthesis of nucleic acids and protein (16). Presumably, the mode of TT antimalarial action is independent from that of CQ or QHS whose antimalarial activity was characterized by a damage to the membrane system of the parasite (17, 18).

Table 3

EFFECT OF COMBINATION OF TETRANDRINE AND CHLOROQUINE ON P.FALCIPARUM

MALARIA**	TRIAL	SFIC*		
		1.0 uM TT 0.3 uM CQ	2.0 uM TT 0.2 uM CQ	3.0 uM TT 0.1 uM CQ
S STRAIN	1	0.77	0.66	0.73
	2	0.64	0.77	0.70
	3	0.78	0.55	0.75
	MEAN ± S.D	0.73 ± 0.06	0.66 ± 0.09	0.73 ± 0.02
R STRAIN	1	0.60	0.45	0.74
	2	0.68	0.63	0.76
	3	0.36	0.30	0.50
	MEAN ± S.D	0.55 ± 0.14	0.46 ± 0.14	0.67 ± 0.12

*SFIC represents sum of fractional inhibitory concentration as described by Berenbaum (11); SFIC is equal to one in cases of additive effects of the drugs, higher than one in cases of antagonism, and lower than one in synergistic action.

**S and R strains: chloroquine sensitive (FCMSU1/Sudan) and resistant (w2) strains of P. falciparum.

Table 4
EFFECT OF COMBINATION OF TETRANDRINE AND QINGHAOSU ON P.FALCIPARUM

MALARIA** TRIAL		SFIC*		
		1.0 uM TT 0.3 uM QHS	2.0 uM TT 0.2 uM QHS	3.0 uM TT 0.1 uM QHS
S STRAIN	1	0.77	0.68	0.71
	2	0.74	0.49	0.72
	3	0.79	0.62	0.77
	MEAN ± S.D	0.77 ± 0.02	0.60 ± 0.08	0.73 ± 0.03
R STRAIN	1	0.63	0.46	0.71
	2	0.77	0.72	0.74
	3	0.64	0.40	0.81
	MEAN ± S.D	0.68 ±0.06	0.52 ± 0.14	0.75 ± 0.04

*SFIC represents sum of fractional inhibitory concentration as described by Berenbaum (11); SFIC is equal to one in cases of additive effects of the drugs, higher than one in cases of antagonism, and lower than one in synergistic action.

**S and R strains: chloroquine sensitive (FCMSU1/Sudan) and resistant (w2) strains of P. falciparum.

The search for alternative therapeutic regimens for the treatment and prevention or the slowing of the emergence of CQ-resistant strains of falciparum malaria using drug combination (12, 19, 20, 21, 22) has thus far been unsuccessful. We present experimental evidence in this paper supporting the possibility that use of a drug combination of TT and CQ or QHS might become an important weapon against drug-resistant-malaria infections. The use of antimalarial drug combinations has become an accepted therapeutic approach. One of the reasons for that is that the drug combinations may delay the development of resistance in the parasite to an individual

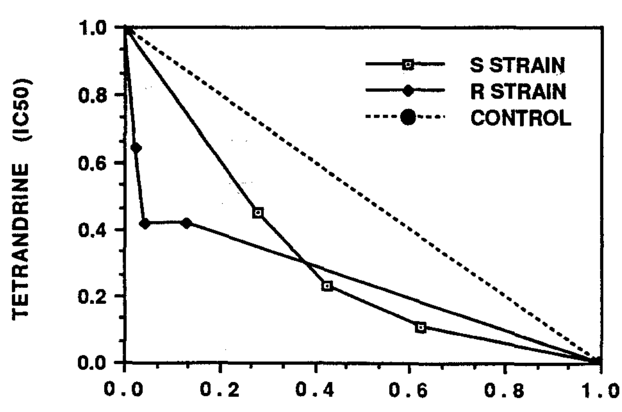

Figure 1. Synergistic effect of tetrandrine and chloroquine on CQ-sensitive (S strain) and CQ-resistant (R strain) P.falciparum in vitro.

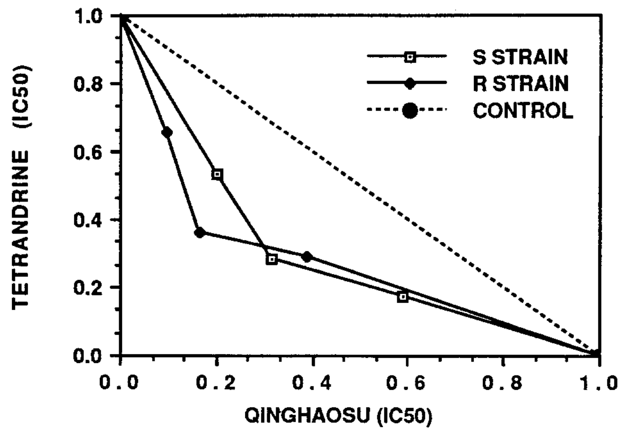

Figure 2. Synergistic effect of tetrandrine and qinghaosu on CQ-sensitive (S strain) and CQ-resistant (R strain) *P.falciparum in vitro*.

drug (20). For example, resistance to mefloquine, a new antimalarial drug against CQ-resistant *P.falciparum* infection, has developed even before its frequent use among malarial patients (23). Therefore, since the combination of TT and CQ exhibits a synergism against the CQ-sensitive strain, its use may retard the emergence of both CQ and TT resistant malaria.

Owing to its high recrudescence in treatment of the malaria, there is limited current use of QHS as a primary antimalarial in therapy. Presently, derivatives of QHS are being developed because their higher potency may prove useful in the inhibition of recrudescence. However, initial trials using artemether, a more potent antimalarial derivative of QHS (24), still showed a very high recrudescence rate in clinical use (25). Therefore, an effective drug combination may prove to be of more importance in inhibiting the recrudescence. Synergistic effects were found between QHS and mefloquine or tetracycline against both CQ-sensitive and resistant strains of *P.falciparum in vitro*, but combinations of QHS with CQ or pyrimethamine were antagonistic (19, 26). We demonstrated a clearly effective synergistic combination in QHS and TT. This combination could eliminate the recrudescence seen when QHS is used alone, possibly due to its short half-life.

It is known that both QHS and TT possess potential antimalarial activity against the CQ-resistant strain of *P.falciparum* (1, 2, 3). Therefore, their synergistic combination against the CQ-resistant falciparum malaria becomes a useful alternative for treatment of CQ-resistant *P.falciparum* infections. Also, the combination might delay the emergence of the parasite resistance to either drug.

As seen in Tables 1 and 2, all IC_{50} values for TT and CQ or QHS in the combinations used against the CQ-resistant malaria are smaller than corresponding values obtained against the CQ-sensitive parasite, except for the combination of TT and CQ in a ratio of 10:3. Apparently, this observation results from the selective antimalarial effect of TT on the CQ-resistant strain of the parasite. In these experiments, TT was 2.0-2.5 times more potent as an antimalarial against the CQ-resistant than against the CQ-sensitive strain based on IC_{50} values in Tables 1 and 2. This accounts for the more potent antimalarial effects in drug combinations against the CQ-resistant falciparum malaria. Apparently, when the proportion of TT to CQ in the combination was decreased to a TT/CQ ratio of 10:3, the selective antimalarial activity of TT in the combination was no longer dominant over the CQ-resistant parasite and, therefore, chloroquine resistance is revealed. Different results were obtained when combinations of TT and QHS were used probably because QHS possesses similar antimalarial activity against both falciparum strains. Unlike the combination of TT and CQ, the IC_{50} values of TT and QHS, even at a combination ratio of 10:3, for the CQ-resistant falciparum malaria were smaller.

A most important effect of synergism between TT and CQ or QHS is that the addition of even a small amount of TT markedly lowered the required dose of CQ or QHS. This is most dramatic when comparing the IC_{50} values of CQ alone with the IC_{50} values of CQ in the combinations for the CQ-resistant strain (seen in Table 1). Even at the lowest dose of TT (the combination of TT and CQ with the ratio of 10:3), there is a 7.7 fold lowering the dose of CQ. At the highest dose of TT (the TT/CQ ratio of 30:1), the CQ dose is lowered 44 fold. Using the CQ-sensitive strain, CQ doses were lowered 1.6 and 3.6 fold at the least and the highest dose of TT, respectively. Clearly the addition of TT is much more effective in the CQ resistant strain. In addition, as shown in Table 1, the IC_{50} of CQ in combination against the CQ-resistant strain was lower than that for CQ alone against the sensitive strain. That indicated that using the standard CQ doses against CQ-sensitive falciparum malaria (i.e., 1.5 g for three days) might cure the CQ-resistant infections when used in combination with an appropriate dose of TT. It may be that this combination will be particularly effective when used in CQ-resistant falciparum malaria. Furthermore, TT was not a toxic compound according to its clinical trials in treatment of silicosis. TT can be given orally at the dose of 200 mg or 300 mg/day for 3 months when used in humans (27). We hope that the combinations of TT with either CQ or QHS will play an important role in prevention and treatment of the CQ-resistant strain of *P.falciparum*.

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References

1. Ye, Z. and Van Dyke, K. (1989). Biochem. Biophys. Res. Commun. 159, 242-248.
2. Ye, Z., Van Dyke, K. and Wimmer, M. (1987). Exptl. Parasitl. 64, 418-423.
3. China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials (1982). J. Tradit. Chin. Med. 2, 45-50.
4. Qinghaosu Antimalarial Coordinating Research Group (1979). Chin. Med. J. 92, 811-816.
5. China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials. (1982). J. Tradit. Chin. Med. 2, 25-30.

6. DeContic, R.C., Muggia, F., Cummings, F.J., Calabres, P. and Greasey, W.A. (1975). Proc. Amer. Assoc. Cancer Res. 16, 96.
7. Jensen, J.B. and Trager, W. (1977). J. Parasitol. 63, 883-886.
8. Desjardins, R.E., Canfield, J.C., Haynes, J.C. and Chubay, J.D. (1979). Antimicrob. Agents Chemother. 16, 710-718.
9. Carter, G.W. and Van Dyke, K. (1971). Clin. Chem. 17, 576-580.
10. Martin, S.K., Oduola, A.M., and Milhous, W.K. (1987). Science 235, 899-901.
11. Berenbaum, M.C. (1978). J. Infect. Dis. 137, 122-130.
12. Geary, T.G., Bonanni, L.C. and Jensen, J.B. (1986). Ann. Trop. Med. Parasitol. 80, 285-291.
13. Homewood, C.A., Warhurst, D.C., Peters, W. and Baggaley, V.C. (1972). Nature Lond. 235, 50-52.
14. Krogstad, D.J. and Schlesinger, P.H. (1986). Biochem. Pharmac. 35, 547-552.
15. Fitch, C.D. (1983). In Malaria and the Red Blood Cell, pp. 222. CIBA Foundation Sympsoium 94, Pitman, London.
16. Greasey, W.A. (1976). Biochem. Pharmac. 25, 1887-1891.
17. Ellis, D.S., Li, Z.L., Gu, H.M., Peters, W., Robisnon, B.L., Tovey, G. and Warhurst, D.C. (1985). Ann. Trop. Med. Parasitol. 79, 367-374.
18. Ye, Z., Li, Z., Li, G., Fu, X., Liu, H. and Gao, M. (1983). J. Tradit. Chin. Med. 3, 95-102.
19. Chawira, A.N. and Warhurst, D.C. (1987). J. Trop. Med. Hyg. 90, 1-8.
20. Peters, W. and Robinson, B.L. (1984). Ann. Trop. Med. Parasitol. 78, 459-466.
21. Gershon, P.D. and Howells, R.E. (1984). Ann. Trop. Med. Parasitol. 78, 1-11.
22. Warhurst, D.C., Robinson, B.L. and Peters, W. (1976). Ann. Trop. Med. Parasitol. 70, 253-258.
23. Boudreau, E.F., Webster, H.K., Pavanand, K. and Thosingha, L. (1982). Lancet. II, 1335.
24. Gu, H., Liu, M., Lu, B., Xu, J., Chen, L., Wang, M., Sun, W., Xu, B. and Ji, R. (1981). Acta Pharmacol. Sinica. 2, 138-140.
25. Myint, P.T., Shwe, T. (1987). Trans. R. Soc. Trop. Med. Hyg. 81, 559-561.
26. Stahel, E., Druilhe, P. and Gentilini, M. (1988) Trans. R. Soc. Trop. Med. Hyg. 82, 221.
27. Cooperative Group of Therapeutic Study of Tetrandrine on Silicosis. (1983). Chin. J. Indust. Hyg. Occupatl. Dis. 1, 136-139.