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Activity of a Combination of Three Cinchona Bark Alkaloids against *Plasmodium falciparum* In Vitro

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In vitro studies with quinine, quinidine, cinchonine, and cinchonidine showed that despite a similarity of chemical structure, the effectiveness of these cinchona bark alkaloids against several culture lines of *Plasmodium falciparum* varied widely. Depending on the strain tested, quinidine and cinchonine were 1 to 10 and 1 to 5 times, respectively, more active than quinine. A combination made of equal parts of quinine, quinidine, and cinchonine was found to have several interesting features; it had activity similar to that of quinine against quinine-susceptible strains but was found to be 2 to 10 times more effective against strains resistant to quinine and had a more consistent effect than any of the alkaloids used singly. The potentiation was found to depend mainly on the presence of cinchonine in the mixtures studied. Synergism was also confirmed in a study of 25 *P. falciparum* strains isolated from Thai patients. Combinations of cinchona bark alkaloids could thus be of interest in areas where *P. falciparum* is becoming less susceptible to quinine.

During the past decades, the number of drugs consistently effective for the treatment and prophylaxis of malaria infection has decreased as a result of the progressive emergence of *Plasmodium falciparum* resistance to each of them, particularly chloroquine, pyrimethamine-sulfadoxine, and quinine (Qn) (6, 9, 11, 12). It is generally agreed, but not well established, that the emergence and spread of resistance has resulted from drug pressure. Among some of the major antimalarial drugs, most of them belonging to the quinoline group, cross resistance has been described, particularly between chloroquine and quinine (5). Cross resistance may also be responsible for the occurrence of parasites resistant to mefloquine, a quinoline methanol, which was recently observed in areas of the world where drug pressure by this new compound has never been exerted (3, 4).

In view of the morbidity-mortality rates prevailing when drugs were still effective, this situation is very puzzling, especially because, in contrast to antibacterial agents, only a very limited number of antimalarial compounds are available. One possible way to face this situation is to use combinations of antimalarial drugs (for example, quinine-tetracyclines [8]), which are now increasingly applied in multiresistant areas.

We report here in vitro results obtained with a combination of chemically related compounds which were found to have a synergistic effect upon resistant parasites.

MATERIALS AND METHODS

In vitro drug susceptibility assay. The in vitro response of either cultured lines of *P. falciparum* or freshly collected isolates to various antimalarial drugs was measured by a one-step in vitro method previously described (10). Solutions containing Qn, quinidine (Qd), cinchonine (Cn), cinchonidine, mefloquine hydrochlorides, and chloroquine sulfate were prepared daily for each experiment. The salts were solubilized in 70% ethanol (20 mg in 2 ml), which ensured sterility and allowed us to avoid filtration on membranes which are known to retain drugs (1). Various combinations of cinchona alkaloids were made from these stock solutions; further dilutions were performed in RPMI 1640 medium and distributed in 50-μl volumes in wells 1 to 11 of 96-well, flat-bottom microdilution plates (Nunc) so as to reach final concentrations of drug ranging from 2,000 to 1.95 ng/ml. Parasitized erythrocytes from stock cultures suspended in RPMI medium supplemented with 10% AB serum and [^{3}H]hypoxanthine were added to each microdilution plate well (except for four control wells, in which were placed noninfected erythrocytes), so that each well contained 6 μl of packed erythrocytes, 194 μl of medium (250 μl including the drug solvent), and 0.4 μCi of [^{3}H]hypoxanthine. The starting parasitemia was 0.2 to 0.3%.

The plates were incubated for 72 h at 37°C in a 5% CO_2–95% air mixture. Cells were collected on glass fiber filters (Whatman GFC) with the use of a cell harvester (Ilacon minimash), and the incorporated radioactivity was counted by liquid scintillation (Econofluor, New England Nuclear Corp.) in a spectrophotometer (Packard Instrument Co.).

The results in counts per minute obtained in duplicate wells for each drug or drug combination tested were plotted on a graph, and the effective concentration (in nanograms per milliliter) inhibiting parasite [^{3}H]hypoxanthine incorporation by 50% (EC_{50}) was calculated (after logarithmic transformation of the data) and used to express isolate susceptibility.

***P. falciparum* isolates and lines.** Several *P. falciparum* strains adapted to in vitro culture conditions were used: FCR3-FMG (Gambia), UPA-PFL3 (Uganda), UPAS (cultivated after passage in a Saimiri monkey), T1 (Thailand), T23 (derived from a Thai isolate described below), FCPS44 (Ivory Coast), and two clones, D3 (derived from FCR3-FMG) and 7G8 (derived from a Brazilian isolate). Since these strains were used after in vitro cultivation under drug pressure for several weeks or months or were found, after unfreezing and recultivation without drug pressure, to change their drug susceptibilities (except for the clones), several substrains or lines were obtained with different

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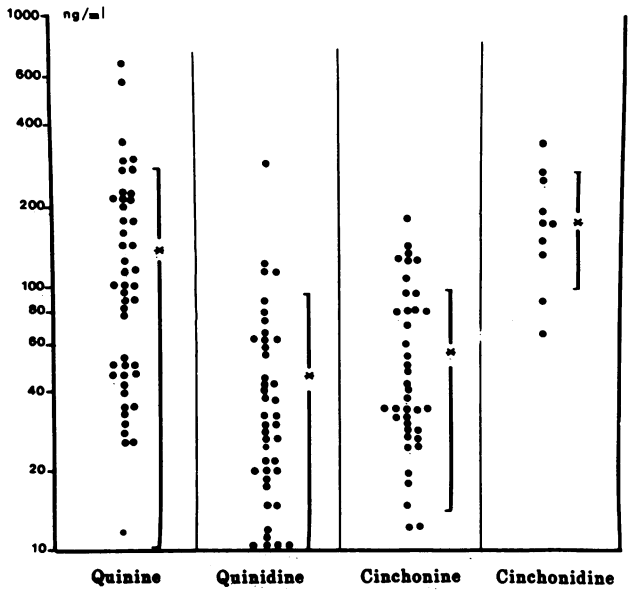

FIG. 1. In vitro response of cultured lines to each of the four alkaloids. Results are expressed as EC_{50}s in nanograms per milliliter. Asterisks and brackets correspond to the means ± standard deviations of the EC_{50}s recorded for each drug.

EC_{50}s. These lines, derived from the strains listed above, are referred to below as x1, 2, 3, etc.

In addition, 25 *P. falciparum* isolates from Thai patients were studied during the summer of 1983 by the same method; 22 of them were collected from patients admitted to the Hospital for Tropical Diseases, Bangkok, Thailand, and were used the same day as blood collection; 3 were cryopreserved isolates tested after unfreezing. All patients came from east and northeast Thailand provinces.

On the basis of comparative results from in vivo and in vitro assays in these areas, the cutoff point of resistance was estimated to be 35 ng/ml for chloroquine and 100 ng/ml for Qn. No such comparison was available for other cinchona alkaloids.

Analysis of results. To evaluate the interaction between drugs, we initially used the isobologram method; thereafter results were expressed by calculating the sum of fractional inhibitory concentrations (ΣFIC) as described by Berembaum (2). ΣFIC = 1 indicates an additive effect between drugs, and ΣFIC < 1 expresses a synergism (7). ΣFIC > 1 indicates the opposite, although a combination can be called antagonistic only when the value is above 4. Student's *t* test was used for statistical comparison of the mean EC_{50}s.

RESULTS

A large range of in vitro susceptibilities to Qn was observed among the strains or the lines studied. All data collected with cultured lines of *P. falciparum* during this study are shown in Fig. 1. The EC_{50}s recorded varied from 12 to 680 ng/ml. The EC_{50}s of Qd and Cn were in most cases lower than those of Qn. Cinchonidine was the least effective of the four alkaloids studied. Depending on the line tested, Qd and Cn were, respectively, 1 to 10 and 1 to 5 times more potent than Qn, whereas cinchonidine activity was similar to or lower than that of Qn. On average, the mean EC_{50}s of Qd and Cn were 3 and 2.5 times, respectively, lower than that of Qn.

A correlation between the responses to cinchona alkaloids was found among Qn-susceptible lines (for Qn and Cn, $r_{20\ df} = 0.4305$ [$P < 0.05$]; for Qn and Qd, $r_{18\ df} = 0.5084$ [$P < 0.05$]; and for Qd and Cn, $r_{17\ df} = 0.9595$ [$P < 0.001$]) but not among Qn-resistant ones. In the resistant lines, a low level of susceptibility to Qn could correspond to a high degree of susceptibility to Qd or Cn (for Qn and Cn, $r_{20\ df} = 0.2757$ [$P > 0.10$]; for Qn and Qd, $r_{20\ df} = 0.3167$ [$P > 0.10$]). However, in Qn-resistant parasites, responses to Qd and Cn were still related (for Qd and Cn, $r_{17\ df} = 0.6329$ [$P < 0.01$]).

Since these results suggested that molecules with great chemical similarities could occasionally behave independently and do not consistently show cross resistance, we investigated the benefit of combining the most effective ones.

The results of such an experiment performed with three lines of parasites and combinations of two and three alkaloids are summarized in Table 1. Against parasites susceptible to each of the three alkaloids (i.e., UPASx1), the combinations tested were no more effective than the most effective alkaloid (ΣFIC values close to 1). Conversely, the results recorded with a *P. falciparum* line highly resistant to Qn and partially resistant to the two other alkaloids (UPASx3) showed the greatest effectiveness of all types of combinations tested: calculated ΣFICs were well below 1, indicating that the drugs acted synergistically. In particular, a combination made of equal parts of Qn, Cn, and Qd was 5.5 times more effective than what would be expected from an additive effect. Against a third parasite line moderately resistant to Qn but highly susceptible to Qd (UPASx2), variable results were recorded with combinations of two drugs, but the combination of three alkaloids was better than other combinations.

Further experiments were performed to investigate the effect of combinations made of various proportions of three drugs, particularly with reduced amounts of Qd. The combination made of equal parts of Qn, Cn, and Qd was compared for 12 lines of *P. falciparum* with other combinations of the same drugs (Qn-Cn-Qd: 60-30-10%, 40-40-20%,

TABLE 1. Effectiveness of alkaloids used alone and in combination

P. falciparum lines	EC_{50} (ng/ml) at indicated alkaloid concn[a]												
	100% Qn	100% Qd	100% Cn	50% Qn-50% Cn	60% Qn-40% Cn	70% Qn-30% Cn	80% Qn-20% Cn	50% Qn-50% Qd	80% Qn-20% Qd	90% Qn-10% Qd	50% Qd-50% Cn	90% Qd-10% Cn	33% Qn-33% Qd-33% Cn
UPASx1	45	22	27	35 (1.03)	45 (1.26)	38 (1.19)	47 (1.18)	31 (1.05)	50 (1.34)	50 (1.22)	35 (1.44)	27 (1.20)	33 (1.15)
UPASx2	100	22	60	80 (1.06)	NA[b]	85 (1.02)	85 (0.96)	50 (1.38)	80 (1.36)	100 (1.35)	NA	NA	36 (0.86)
UPASx3	280	80	130	NA	100 (0.52)	100 (0.48)	60 (0.26)	90 (0.72)	120 (0.64)	140 (0.62)	50 (0.50)	30 (0.36)	25 (0.18)

[a] Numbers in parentheses correspond to ΣFICs.
[b] NA, Not available.

45-45-10%, and 40-20-40%). In all cases except one, the combination made of equal parts was always more effective or as effective as the other combinations studied (data not shown).

In view of the above results, further experiments with this combination against Qn-susceptible and resistant lines (Table 2) were performed. In Qn-susceptible lines, the EC_{50} of the combination was close to the arithmetic mean of the EC_{50} of each drug. In contrast, in Qn-resistant *P. falciparum* lines, the EC_{50} of the mixture was much lower than the arithmetic mean of EC_{50}s. The ΣFIC confirms that synergism is observed mainly when Qn is less effective. Conversely, no synergism was found (ΣFIC close to 1) when parasite lines were susceptible to the three alkaloids.

Table 2 also shows that Qn-susceptible lines were also Qd and Cn susceptible, whereas Qn-resistant ones were not in each instance Qd and Cn resistant. Because some lines were

TABLE 2. Effectiveness of alkaloids against cultured Qn-susceptible and -resistant lines

P. falciparum line[a]	EC_{50} (ng/ml)[b]				
	Qn	Qd	Cn	Qn-Cn-Qd[c] (ΣFIC)	Qn-Cn-Qd/Qn
Qn susceptible					
FCR3x4	52	26	26	15 (0.48)	0.28
FCR3x5	42	43	19	40 (1.32)	0.95
FCR3x6	88	40	72	78 (1.30)	0.88
FCR3x8	80	22	18	15 (0.56)	0.18
FCR3x9	90	32	34	50 (1.19)	0.55
UPAx2	26	20	28	26 (1.06)	1
UPAx3	82	26	34	47 (1.25)	0.57
UPAx4	45	10	15	12 (0.75)	0.26
UPAx5	45	17	22	27 (1.13)	0.60
UPASx1	45	22	27	33 (1.15)	0.73
UPASx1	35	12	29	23 (1.12)	0.65
UPASx2	30	32	34	44 (1.37)	1.46
UPASx1	42	7.4	12.5	15.5 (1.23)	0.36
UPASx2	95	27	35	53 (1.34)	0.55
D3	46	11	25	18 (0.91)	0.39
7G8	27	8	12	14 (1.14)	0.51
FCPS44x1	34	19	34	30 (1.10)	0.85
FCPS44x2	35	19	32	37 (1.39)	1.18
Qn resistant					
FCR3x1	160	120	92	64 (0.53)	0.40
FCR2x2	680	115	92	58 (0.40)	0.085
FCR3x3	540	88	78	93 (0.80)	0.172
FCR3x7	180	64	80	68 (0.76)	0.377
UPAx1	150	58	26	56 (1.15)	0.373
UPASx2	100	22	60	36 (0.86)	0.36
UPASx3	280	80	130	25 (0.19)	0.089
UPASx4	212	31	32	25 (0.55)	0.117
T1x3	105	62	48	39 (0.60)	0.371
T1x4	115	37	40	57 (0.98)	0.495

[a] Qn-susceptible lines were those for which EC_{50} < 100 ng/ml; Qn-resistant lines were those for which EC_{50} ≥ 100 ng/ml.

[b] Means ± standard deviations: for Qn-susceptible lines, 52.1 ± 23.4 (Qn), 21.8 ± 10.4 (Qd), 28.2 ± 13.3 (Cn), 32.0 ± 17.5 (Qn-Cn-Qd), and 0.65 ± 0.33 (Qn-Cn-Qd/Qn); for Qn-resistant lines, 252.2 ± 198.8 (Qn), 67.7 ± 33.4 (Qd), 67.5 ± 33.2 (Cn), 52.1 ± 21.1 (Qn-Cn-Qd), and 0.28 ± 0.15 (Qn-Cn-Qd/Qn).

[c] Combination of equal parts.

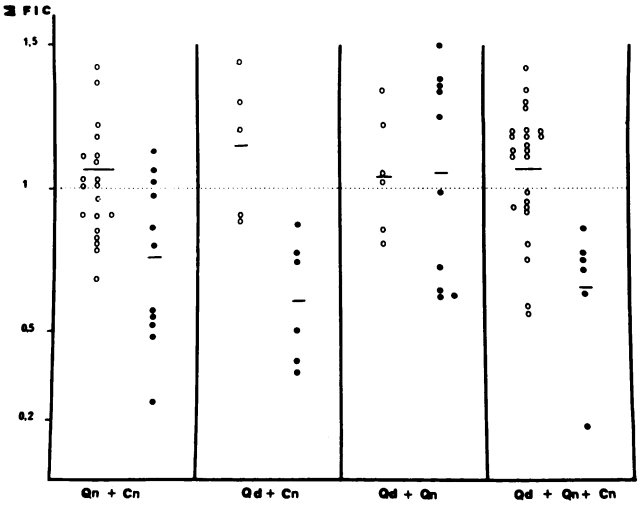

FIG. 2. Distribution of the ΣFICs obtained with different mixtures of cinchona bark alkaloids. Each point corresponds to the ΣFICs obtained in each experiment. Symbols: ○, values for quinine-susceptible *P. falciparum* parasites (EC_{50} < 100 ng/ml); ●, values recorded with quinine-resistant parasites (EC_{50} ≥ 100 ng/ml); ———, mean ΣFICs. ΣFIC < 1 indicates synergism.

more susceptible to Qd or to Cn, no single drug appeared consistently effective. In contrast, combinations appeared to be on average more consistently effective at low concentrations for all lines tested than each drug alone: for Qn-resistant lines, the combination was 5 times more effective than Qn (range 2 to 10 times) and the standard deviation was 10 times smaller than that of Qn. Since all data were obtained by an isotopic method, an experiment was performed in which parasite growth of line FCPS44 was assessed by microscopic examination of Giemsa-stained smears. A greater effect of the combination, compared with the effects of single drugs, was also found (ΣFIC = 0.44).

A summary of all ΣFIC data shows that combination of two and three alkaloids may result in synergism or a simple additive effect (Fig. 2). Synergism was observed mainly on Qn-resistant lines and depended mostly on the presence of Cn in the mixture studied (Fig. 2). Combinations of Cn with either Qn or Qd were more frequently synergistic on Qn-resistant lines, whereas no difference between Qn-resistant and -susceptible lines was found with the combination of Qn-Qd (which does not contain Cn). The differences of the mean ΣFICs between Qn-susceptible and -resistant lines are statistically significant for associations Cn-Qd, Cn-Qn, and Cn-Qn-Qd ($P < 0.01$) but not for the combination of Qn-Qd.

In addition to these results, obtained with laboratory-adapted *P. falciparum* lines, we evaluated the effect of the Qn-Cn-Qd association on freshly collected Thai isolates in comparison with the effects of other antimalarial drugs (Table 3). In vitro, 84% of the 25 Thai isolates were resistant to chloroquine (EC_{50} > 35 ng/ml) and 32% were resistant to Qn (EC_{50} > 100 ng/ml). One isolate was resistant to mefloquine. A correlation was found between the in vitro responses to chloroquine and to Qn ($r = 0.72$ [$P < 0.01$]). The combination of three cinchona alkaloids was found to be two to three times more effective than Qn alone, and its effectiveness was greater in Qn-resistant isolates. On the average, for the nine Qn-resistant isolates, the mean EC_{50} ± standard deviation for Qn was 174 ± 56 ng/ml, and the mean EC_{50} ± standard deviation for the combination of Qn-Cn-Qd was 57 ± 31 ng/ml ($t = 5.47$ [$P < 0.001$]).

TABLE 3. Effectiveness of alkaloids against 25 Thai isolates

Isolate no.[a]	EC_{50} (ng/ml)[b]				
	Qn	Qn-Cn-Qd	Qn-Cn-Qd/Qn	Chloroquine	Mefloquine
Qn susceptible					
1	20	15	0.75	94	<7.8
2	22[c]	28	1.27	15	175
3	27	35	1.3	60	<7.8
4	42	40	0.95	36	<7.8
5	43	16	0.37	33	<7.8
6	43	38	0.88	125	<7.8
7	57	45	0.78	49	<7.8
8	58	15	0.25	43	<7.8
9	59	23	0.38	47	<7.8
10	62	15	0.24	95	<7.8
11	68	15	0.22	62	<7.8
12	70	38	0.54	72	<7.8
13	75	23	0.30	47	<7.8
14	76[c]	27	0.35	20	<7.8
15	78	24	0.30	105	<7.8
16	93	22	0.23	125	<7.8
Qn resistant					
17	100	30	0.3	130	<7.8
18	110	43	0.39	115	9
19	135	19	0.14	20	<7.8
20	160	45	0.28	130	<7.8
21	170	74	0.43	110	<7.8
22	170	94	0.55	270	<7.8
23	220	44	0.2	230	<7.8
24	250[c]	115	0.46	220	<7.8
25	250	50	0.2	150	<7.8

[a] Qn-susceptible isolates were those for which EC_{50} < 100 ng/ml; Qn-resistant isolates were those for which $EC_{50} \geq 100$ ng/ml.

[b] Means ± standard deviations: for Qn-susceptible isolates, 55.81 ± 21.33 (Qn), 26.18 ± 10.17 (Qn-Cn-Qd), 0.56 ± 0.37 (Qn-Cn-Qd/Qn), and 64.18 ± 35.13 (chloroquine); for Qn-resistant isolates, 173.88 ± 55.88 (Qn), 55.11 ± 31.13 (Qn-Cn-Qd), 0.32 ± 0.13 (Qn-Cn-Qd/Qn), and 152.77 ± 75.95 (chloroquine).

[c] Isolates cultured after freezing.

DISCUSSION

Our in vitro study of cinchona bark alkaloids shows that closely related molecules, some being isomers of the others, exhibit important differences in their activity on *P. falciparum*. In some instances, particularly in Qn-resistant parasites, cross resistance among these drugs was not observed. In such cases the combination of several alkaloids was valuable, since it resulted in synergism.

Most *P. falciparum* lines studied were more susceptible to Qd and Cn. Since the initiation of our study, the greater susceptibility of *P. falciparum* to Qd has been confirmed by other in vitro (A. Sabchareon et al., Bull. W.H.O., in press), as well as in vivo (13, 16; P. Suntharasamai, S. Vanijanond, T. Harinasuta, and D. Bunnag, Abstr. 11th Int. Congr. Trop. Med. Malaria, p. 50, 1984), studies which were performed in areas where Qn-resistant parasites are prevalent.

In addition, our results show that Cn is just as promising as Qd in cases of Qn resistance. In about half of the parasite lines studied, Cn had an even greater effect than that of Qd.

It should be stressed that the effect of Cn or Qd is not consistently greater, some isolates being resistant to one or the other and rarely to both. From a practical point of view, these results mean that no single alkaloid would be consistently effective in each patient.

In this respect the combinations of two, but mostly three, alkaloids were found to be of interest. In some instances, the synergistic effect of these compounds could be demonstrated. It was not consistent but was observed mostly in Qn-resistant parasites or more generally when the parasite was not susceptible to one or two of the alkaloids. Cn was found to be the most critical component of the mixture. Cn was able to produce a synergistic effect when combined with either Qn or Qd or both. In contrast, the combination of Qn and Qd was simply additive, possibly because they are only isomers of one another.

The combination made of equal parts of Qn-Cn-Qd was studied in greater detail with several lines, as well as with several isolates. It was found synergistic against Qn-resistant parasites and, moreover, synergistic against parasites resistant to more than one alkaloid (Qn and Qd or Qn and Cn). From a practical point of view, the interest of this drug combination lies in its more consistent effect at low concentrations on various parasite lines, whether adapted to culture or isolated from Thai patients. This feature is important in view of the inconsistent effectiveness of alternative alkaloids used singly.

Our study does not provide an understanding of the reasons for a Qn-resistance-dependent synergistic effect. It is not known why chemically related molecules can behave independently. This phenomenon is reminiscent, however, of similar findings among drugs of the 4-amino-quinolines group, such as amodiaquine and chloroquine (15) or desethylchloroquine and chloroquine (14).

Thus, there is no satisfactory rationale for designing a combination of drugs with optimal effect. Among the ones we studied, the combination made of equal parts of three alkaloids had valuable in vitro features. Obviously the in vivo value of the combination described here will depend greatly on the possible toxicity and on pharmacokinetics of the mixture, especially since it contains Cn, which has been less studied than the two other alkaloids. As an example, it should be recalled that Qd has been reported recently to be more effective than Qn (Suntharasamai et al., 11th Int. Congr. Trop. Med. Malaria), but its greater effectiveness in vitro did not result in much higher efficacy in vivo, since for the same total therapeutic dose, levels of Qd in the blood were lower than those of Qn (Sabchareon et al., in press).

Qn is the oldest antimalarial compound, yet it still occupies a place of choice among the available drugs. Despite the occurrence of resistance, it remains an essential antimalarial compound because its fast action is critical in treating complicated, i.e., cerebral, cases and because resistance has remained at R1 level up to now. Thus, Qn still has a fast and consistent initial effect on clinical symptoms. In this context, it appears to be a valuable goal to optimize its effectiveness. Improved therapies based on precise pharmacokinetic studies are one of the means to reach this goal; a combination of synergistic compounds may be another.

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