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PHARMACOLOGY OF THE MALARIA PARASITE—A STUDY OF DOSE–RESPONSE RELATIONSHIPS IN CHLOROQUINE-INDUCED AUTOPHAGIC VACUOLE FORMATION IN *PLASMODIUM BERGHEI*

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Abstract—Dose–response relationships in chloroquine-induced pigment clumping (CIPC) in erythrocytic *Plasmodium berghei* have been studied *in vitro* and the mode of action of competitive inhibitors has been examined. Antimalarially active cinchona alkaloids inhibit CIPC competitively, presumably by binding to the same receptor sites as chloroquine. The antimalarially inactive 8,9 *threo* diastereomer of quinine, 9-epiquinine, does not affect CIPC. Although the β-adrenergic antagonist dichloro-isoproterenol competes with chloroquine, isoproterenol has no stimulatory and little inhibitory effect on pigment clumping, indicating that a conventional β-adrenergic site is not involved. Several experimental antimalarial drugs were studied as inhibitors of CIPC and the results are discussed. WR 142,490 a substituted quinoline methanol, was the most active competitive inhibitor of CIPC tested, with an affinity for the 'clumping site' 100 times that of quinine. In comparison with Fitch's high affinity site, the clumping site is more structure-specific. Similarities between the drugs studied are discussed, and possible modes of interaction with the receptor in normal and chloroquine-resistant parasites are suggested.

Aggregation of hemozoin pigment which takes place in erythrocytic malaria parasites following chloroquine treatment represents the fusion together of digestive vacuoles and their encirclement by membrane to give an autophagic vacuole [1, 2]. In *Plasmodium berghei* the process requires energy [3, 4] and synthesis of RNA and protein [5]. It has been shown in *P. knowlesi* that degradation of ribosomal RNA occurs after the autophagic vacuole has been formed [6].

It was reported in an earlier paper that the antimalarial drug quinine inhibited chloroquine-induced pigment clumping (CIPC) competitively. The logarithm of the concentration of quinine inhibiting by 50% and the logarithm of the chloroquine concentration were not however directly proportional [7, 8]. We have studied here the chloroquine dose–response curve in more detail, and examined further the relationships of quinine and other inhibitory drugs with chloroquine.

METHODS

Maintenance of parasites. The 'N' strain of *P. berghei*, maintenance, and strains of albino mice were as described previously [5]. Mice were inoculated on day 0 (D.0) and used as donors for incubations *in vitro* on D + 1, +2, or +3. For convenience in counting, donors with fewer than 15% of erythrocytes infected were used in all experiments.

Culture medium and incubation procedures. The cultivation system described earlier [5] was used with minor modifications. Ten ml of concentrated medium 199 with Earle's or Hank's salts ('Flow Labs') were diluted to 100 ml with distilled water. Glucose (0·2 g); 0·148 g $NaHCO_3$, and 11 ml foetal bovine serum (membrane filter sterilized, Flow 4-055) were added. The medium, equilibrated and at pH 7·4, was distributed in 3·8 or 3·9-ml volumes into test tubes fitted with silicone stoppers. After equilibration at 37° for 15 min the culture tubes were ready for addition of infected blood. Heparinised infected blood (0·04 ml) was added to each tube of culture medium. The tubes were then rotated at 12 rev./hr at 37° in a roller tube apparatus.

Studies on drug action. Chloroquine diphosphate was added in 0·1 ml 0·85% NaCl (w/v) at a concentration 40 times the desired final concentration, and allowed to act for 80 min. Saline was added to control tubes. Experiments were carried out using tubes in duplicate.

Inhibitors were added to the incubation system 15 min before chloroquine. In all inhibition experiments a control series was set up with chloroquine alone to confirm that the 50% clumping value fell within the usual range (see Results Section A). All tubes, including controls with and without inhibitor or chloroquine or both, were incubated for the same overall length of time. Drugs were dissolved in saline, or first in ethanol, and added to tubes at 40 times the desired concentration in 0·1 ml saline, 0·1 ml saline being added at the same time to controls. Up to 10% (v/v) of ethanol included in the control saline had no influence on the results (final concentration, 0·25%). 0·1% Tween 80 (BDH) similarly had no effect.

Preparation and interpretation of blood films. On completion of incubation the tubes were centrifuged and thin films prepared as previously described. Unfixed dry films were examined under oil immersion,

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and pigmented cells were classified as having 'fine', 'granular' or 'clumped' pigment [5]. For the purposes of this work, only the percentage of 'clumped' pigment was of interest. Fifty pigmented cells were counted on each film. Mean percentage clumped in control tubes was subtracted from values obtained from experimental tubes to give corrected values. These corrected values were later (see Results Section A) expressed as percentages of 88 to give final values.

Interpretation of competitive-inhibition dose–response curves. The competitive inhibition of CIPC by quinine (for example) is analogous to an orthodox pharmacological system, with an agonist (chloroquine) complexing with a hypothetical receptor leading to a response (clumping) in the tissue studied. The competitive antagonist (quinine) raises the concentration of agonist necessary to produce 50% response (K_m) by a factor termed the dose-ratio (DR).

The results obtained using quinine and other drugs showing competitive effects, were therefore handled as suggested by Paton [9]:
from Gaddum's equation [10]:

$$DR - 1 = \frac{[i]^n}{K_i} \tag{1}$$

(where n is the number of molecules of antagonist interacting with the receptor; [i] is the concentration of the antagonist and K_i is the dissociation constant of the antagonist–receptor complex).

It follows that the slope of the line obtained by plotting log (DR − 1) against log [i] is equal to n:

$$\log (DR - 1) = n.\log [i] - \log K_i. \tag{2}$$

When $n = 1$,

$$\log K_i = \log [i] - \log (DR - 1). \tag{3}$$

When log (DR − 1) = 0, (DR − 1 = 1),

$$\text{then } \log K_i = \log [i], \tag{4}$$

and this is the intercept on the abscissa [9].

When $n = > 1$, n molecules of antagonist compete with 1 molecule of agonist (see equation (1)).

But K_i here is the product of n unknown K_i values:

$$K_i = K_{i_1}.K_{i_2}.K_{i_3}\ldots K_{i_n} \tag{5}$$

$$K_{i_1}.K_{i_2}.K_{i_3}\ldots K_{i_n} = \frac{[i]^n}{DR - 1} \tag{6}$$

when DR − 1 = 1

$$K_{i_1}.K_{i_2}.K_{i_3}\ldots K_{i_n} = [i]^n. \tag{7}$$

The antagonist concentration at this point represents the geometric mean (gm) of the K_i values, for n molecules of the antagonist.

$$(K_{i_1}.K_{i_2}.K_{i_3}\ldots K_{i_n})^{1/n} = [i] = 'gmK_i' \tag{8}$$

and in the log-relationship the intercept on the abscissa represents mean log K_i.

Given DR − 1 and [i] we can calculate 'gmK_i' from Gaddum's equation (1):
by modifying the relationship to show gmK_i:

$$DR - 1 = \frac{[i]^n}{gmK_i^n} \tag{9}$$

$$gmK_i = \left(\frac{[i]^n}{DR - 1}\right)^{1/n} \tag{10}$$

simplifying:

$$gmK_i = \frac{[i]}{(DR - 1)^{1/n}} \tag{11}$$

In cases where n is unknown and is assumed to be say 1 or 2 for the purpose of calculating a K_i value, the magnitude of the error introduced depends on the true value of n and on DR − 1. As DR − 1 approaches 1 this error becomes infinitesimal.

Preliminary experiments and reliability of results. Preliminary experiments were carried out in all cases to determine whether clumping took place in the absence of chloroquine, and the concentration of each drug which would inhibit CIPC by 50% at 10^{-6} and 10^{-7} M chloroquine. Then a sigmoid competitive inhibition curve was plotted from an experiment using a fixed concentration of inhibitor and varying chloroquine concentrations. The value of gmK_i determined from the 50% value at 10^{-7} M chloroquine was closely similar in all cases to that determined from the sigmoid, and the latter value is reported in the 'results', for quinidine, cinchonidine, cinchonine, isoproterenol, WR 177,602, WR 30,090, WR 165,355 and WR 33,063. For quinine, Ro 21-0960, dichloroisoproterenol, WR 93,156, WR 142,490 and WR 122,455, several sigmoid competitive inhibition curves were plotted and gmK_i values obtained as described above.

Source of drugs. Chloroquine diphosphate was given by ICI Limited. Quinine dihydrochloride was purchased from BDH Ltd. WR 93,156 HCl was given by Dr. C. D. Fitch. Dichloroisoproterenol HCl, isoproterenol HCl, quinidine HCl·H_2O, and cinchonidine base were obtained from Sigma (London) Ltd. Dr. J. Williamson supplied cinchonine base and Drs. Uskokovic and Brossi of Hoffman la Roche Inc. gave 9-epiquinine 2HCl and Ro 21-0960 base. WR 142,490, WR 177,602, WR 122,455, WR 165,355, WR 30,090 and WR 33,063 were supplied as the hydrochloride salts by the US Army Research and Development Command under contract No. DAJA37-74-C-1496 to Prof. W. Peters. The stereochemistry of the cinchona alkaloids used in this paper is taken from Lyle and Keefer [11]. Chemical names and stereochemistry of the drugs used in this study are given in Table 1. WR 177,602 was synthesized by Dr. R. E. Olsen, Cordova Chemical Co.

RESULTS

A. Pigment clumping effect of chloroquine

In five experiments, each using a range of chloroquine concentrations from 10^{-8} M to $1{\cdot}6 \times 10^{-7}$ M, between 90 and 96% of pigmented parasites were clumped by the top concentration of chloroquine (mean 93·8, standard deviation (S.D.) ± 2·3). Control values ranged from 3 to 9 (mean 5·4 ± 2·5). When, after subtracting control values, the results were plotted against chloroquine concentrations, it was found that as chloroquine concentration increased, percentage clumping increased and approached a maximum of 88%. The results were therefore recalculated taking 88% as the maximum value attainable. Plotting the

Table 1. Drugs used in this study

Name	Chemical Name	Enantiomer	Salt	Relative stereochemistry	Configuration[b]
Chloroquine	7-chloro-4-(4′-diethylamino-1′-methylbutylamino) quinoline	±	diphosphate	a	1′RS
Quinine	3-vinyl-6′-methoxyruban-9-ol	−	dihydrochloride	8,9 *erythro*	9R, 8S 4S, 3R
9-Epiquinine	3-vinyl-6′-methoxyruban-9-ol	+	dihydrochloride	8,9 *threo*	9S, 8S 4S, 3R
Quinidine	3-vinyl-6′-methoxyruban-9-ol	+	hydrochloride monohydrate	8,9 *erythro*	9S, 8R 4S, 3R
Cinchonidine	*des*methoxyquinine	−	base	8,9 *erythro*	9R, 8S 4S, 3R
Cinchonine	*des*methoxyquinidine	+	base	8,9 *erythro*	9S, 8R 4S, 3R
Ro 21-0960	2′,8′-bis(trifluoromethyl) dihydrocinchonine	±	base	8,9 *erythro*	9RS, 8SR, 4RS, 3SR
Isoproterenol	isopropylaminomethyl-(3,4-dihydroxyphenyl)-methanol	±	hydrochloride	a	1′RS
Dichloroiso-proterenol	isopropylaminomethyl-(3,4-dichlorophenyl)-methanol	±	hydrochloride	a	1′RS
WR 93, 156	7-chloro-4-(6′-ethyl-2′-octyl amino)quinoline	±	hydrochloride	a	2′RS
WR 142,490	α-(2-piperidyl)-2,8-bis (trifluoromethyl)-4-quinoline methanol	±	hydrochloride	*erythro* [13]	1′RS, 2′SR
WR 177,602	α-(2-piperidyl)-2,8-bis (trifluoromethyl)-4-quinoline methanol	±	hydrochloride	*threo* [14]	1′RS, 2′RS
WR 30,090	α-((dibutylamino) methyl)-6-8-dichloro-2-(3,4-dichloro) phenyl-4-quinoline methanol	±	hydrochloride	a	1′RS
WR 122,455	3,6-bis(trifluoromethyl)-α-(2-piperidyl)-9-phenanthrene methanol	±	hydrochloride	85: 15 *erythro*: *threo* [15, 16]	1′RS, 2′SR: 1′RS, 2′RS
WR 165,355	3,6-bis(trifluoromethyl)-α-(2-piperidyl)-9-phenanthrene methanol	±	hydrochloride	*threo* [16]	1′RS, 2′RS
WR 33,063	6-bromo-α-(diheptylamino-methyl)-9-phenanthrene methanol	±	hydrochloride	a	1′RS

Notes—a: one asymmetric carbon atom; b: according to the sequence rule [12]; R: *rectus*; S: *sinister*.

results against log chloroquine concentration gave a sigmoid dose–response curve (Fig. 1). When probit % clumped was used the curve approximated to a straight line with a slope of 3·2 probits/log, indicating that clumping responses to chloroquine were lognormally distributed in the parasite population. The 50% value, read off from the sigmoid, was 8·30 ± 0·08 logs. 50% of the parasites capable of clumping showed a response to 2×10^{-8} M chloroquine. Inhibition experiments where the chloroquine control series had a 50% point less than $1{\cdot}38 \times 10^{-8}$ M or more than $2{\cdot}88 \times 10^{-8}$ M (mean ± 2 × S.D.) were ignored.

B. Inhibitory effect of chloroquine

Chloroquine at higher concentrations inhibited pigment clumping. (As in Section A, corrected percentage of clumped pigment was expressed as a percentage of 88, but in order to express inhibition this value was then subtracted from 100) (Fig. 2). When the probit of the percentage inhibition was plotted against the log of the chloroquine concentration, a straight line was produced with a slope of 1·5 probits/log (mean of 2 experiments). This indicates that sensitivities of parasites to clumping inhibition by chloroquine were lognormally distributed. Fifty % of the parasites capable of clumping were inhibited by $3{\cdot}6 \times 10^{-5}$ M chloroquine. In experiments on the inhibition of CIPC by other drugs, chloroquine concentrations less than 1/10 of this value were always used.

C. Competitive inhibition of CIPC

(*i*) *Cinchona alkaloids.* None of the cinchona alkaloids tested caused pigment clumping when added alone. When a range of chloroquine concentrations was tested in the presence of 10^{-6} M quinine it was noted (Fig. 3) that the maximum degree of clumping attained was unaffected, but that an increased concentration of chloroquine was needed to give 50% clumping. The slope and shape of the curve were unaffected. It was noted earlier [7] that quinine concentrations producing 50% inhibition of clumping were not in simple direct proportion to chloroquine concentrations. A series of six experiments, using varying concentrations of quinine, was therefore carried out to investigate the point further. The results were

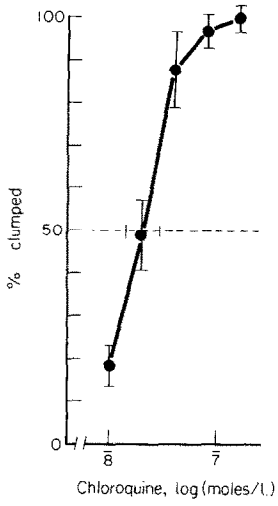

Fig. 1. Dose–response curve relating percentage of parasites with clumped pigment to log concentration of chloroquine (Dose-response sigmoid). S.D. is indicated. The interrupted line crosses the sigmoid at the 50% point and values + and − 2 S.D. are marked on this line. (From 5 experiments using duplicates.)

analysed by plotting log (DR − 1) against log quinine concentration. This gave a straight line (Fig. 4) with a slope of 2·30 (correlation coefficient 0·991, $P < 0·001$). The intercept on the abscissa was $\bar{7}·61$, corresponding to a gmK_i of $4·1 \times 10^{-7}$ M.

The 9-epimer of quinine, 9-epiquinine, has little antimalarial activity (see Table 3). When tested *in vitro* it had no effect on CIPC at 10^{-4} M.

Quinidine, the dextrorotatory natural diastereomer of quinine has similar antimalarial activity [17]. Quinidine (2×10^{-7} M) did not affect maximum CIPC but raised the 50% point to 10^{-7} M chloroquine (DR − 1 = 4). Slope and shape of the curve were unaffected. The gmK_i value, assuming $n = 2$, was 10^{-7} M.

Cinchonidine is demethoxyquinine and is antimalarially active [17]. Cinchonidine ($1·2 \times 10^{-6}$ M) did not affect maximum CIPC but raised the 50% point to $8·3 \times 10^{-8}$ M (DR − 1 = 3·2). The gmK_i value, assuming $n = 2$, was $6·7 \times 10^{-7}$ M.

Cinchonine is demethoxyquinidine and is also antimalarially active [17]. Cinchonine ($5·1 \times 10^{-7}$ M) did not affect maximum CIPC but raised the 50% point to $1·1 \times 10^{-7}$ M (DR − 1 = 4·5). The gmK_i value, assuming $n = 2$, was $2·4 \times 10^{-7}$ M. Ro 21-0960 is a highly antimalarially active derivative of cinchonine (see Table 3). In the presence of $2·1 \times 10^{-7}$ M Ro

Table 2. Comparison of 'clumping' and 'high-affinity' sites

Drug	K_i or gmK_i (clumping) nM	K_i (high affinity) nM
Quinine	410	2000*
WR 93,156	1230	4000†
WR 142,490	4	2000*
WR 30,090	25	2000*
WR 122,455	11	700*
WR 33,063	29	1000*

* Ref. 22. † Ref. 21.

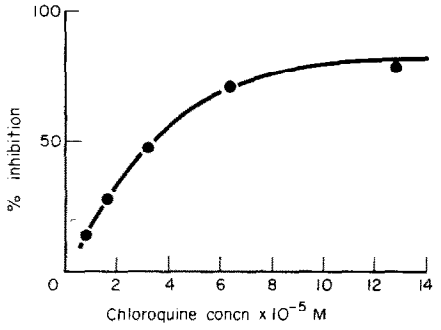

Fig. 2. Graph showing inhibition of clumping by chloroquine concentrations above 10 μM.

21-0960 maximum CIPC was unaffected but the 50% point was moved along to $1·82 \times 10^{-7}$ M. The slope and shape of the curve were unaffected (Fig. 5). The relationship between log (DR − 1) and log Ro 21-0960 concentration was plotted (Fig. 6), giving a straight line with a slope of 1·74 (correlation coefficient 0·938, $P < 0·001$). The intercept on the abscissa was $\bar{8}·72$, a gmK_i of $5·25 \times 10^{-8}$ M.

(ii) Dichloroisoproterenol and isoproterenol. The β-adrenergic inhibitor dichloroisoproterenol (DCI) was originally tested by us *in vitro* because of similarities between the side chain and that of quinine, and the lipophilic character of the molecule [18]. In the presence of 2×10^{-6} M DCI the maximum CIPC produced was unaltered. Whilst the 50% point was raised to $1·5 \times 10^{-7}$ M chloroquine (Fig. 7). A series of nine experiments was carried out to study the relationship between log (DR − 1) and log DCI (Fig. 8) and a straight line with a slope of 0·83 was produced (correlation coefficient 0·985, $P < 0·001$). The intercept on the abscissa was $\bar{7}·58$, corresponding to a gmK_i of $3·8 \times 10^{-7}$ M.

The β-adrenergic agonist isoproterenol had a very slight competitive effect in CIPC, the K_i value, assuming $n = 1$, being approximately $1·3 \times 10^{-3}$ M. (DR − 1 = 1·5). Neither DCI nor isoproterenol caused pigment clumping when added alone.

(iii) WR 93,156. WR 93,156 is an isostere of chloroquine, where the terminal nitrogen atom of the side chain is replaced by –C–H. Unlike chloroquine, this agent causes no pigment clumping alone. 4×10^{-6} M WR 93,156 did not affect maximum CIPC, but the

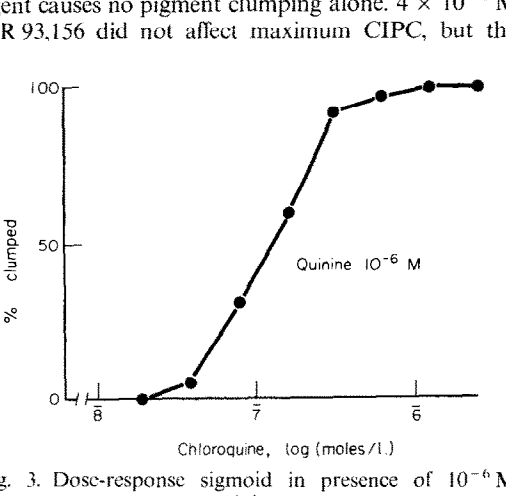

Fig. 3. Dose-response sigmoid in presence of 10^{-6} M quinine.

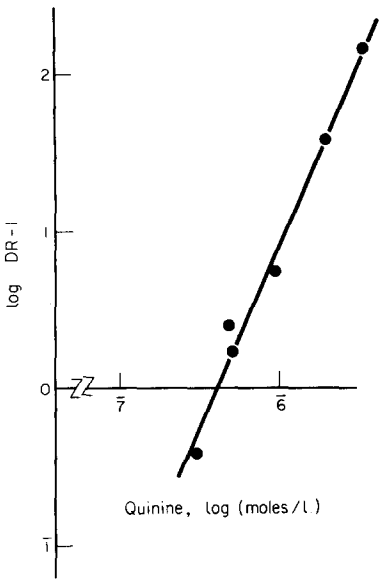

Fig. 4. Log (DR − 1) plotted against log quinine concentration.

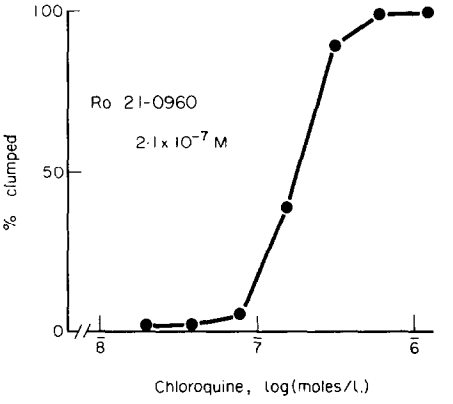

Fig. 5. Dose–response sigmoid in presence of $2{\cdot}1 \times 10^{-7}$ M Ro 21-0960.

50% point was shifted to $6{\cdot}9 \times 10^{-7}$ M chloroquine. The slope and shape of the curve were unaffected (Fig. 9). The competitive relationship was further studied in six experiments. When the log (DR − 1)/log WR 93,156 relationship was plotted it was a straight line with a slope of 2·43 (correlation coefficient 0·938, $P < 0{\cdot}01$). The intercept was 6·09 corresponding to a gmK_i of $1{\cdot}23 \times 10^{-6}$ M (Fig. 10).

(*iv*) *WR* 142,490, *its* threo *epimer WR* 177,602 *and WR* 30,090. WR 142,490 is a substituted quinoline methanol similar to Ro 21-0960. It is highly active *in vivo* against normal and chloroquine-resistant malaria (see Table 3). In the presence of 2×10^{-8} M WR 142,490 maximum CIPC was unaffected but the 50% point was shifted to $1{\cdot}9 \times 10^{-7}$ M (Fig. 11). After eight competition experiments the log (DR − 1)/log WR 142,490 relationship was plotted (Fig. 12) and was found to be a straight line with a slope of 1·02 (correlation coefficient 0·97, $P < 0{\cdot}001$). The intercept 9·6 corresponds to a K_i of 4×10^{-9} M.

WR 177,602 has marked activity against *P. berghei in vivo* [19]. In CIPC it was competitive and the K_i, assuming $n = 1$, was estimated to be 10^{-8} M. $4{\cdot}3 \times 10^{-8}$ M WR 177,602 moved the 50% point along to 10^{-7} M chloroquine (DR − 1 = 4).

WR 30,090, a substituted quinoline methanol, is active against normal and chloroquine-resistant malaria (Table 3). $9{\cdot}38 \times 10^{-8}$ M WR 30,090 did not affect maximum CIPC but moved along the 50% point (DR − 1 = 3·8). K_i, assuming $n = 1$, was estimated to be $2{\cdot}5 \times 10^{-8}$ M. WR 142,490, WR 177,602 and WR 30,090 caused no clumping when added alone.

(*v*) *WR* 122,455, *its* threo *epimer WR* 165,355 *and WR* 33,063. WR 122,455 is a substituted phenanthrene methanol, active *in vivo* against normal and chloroquine-resistant malaria (Table 3). In the presence of $4{\cdot}2 \times 10^{-8}$ M WR 122,455 maximum CIPC was unaffected, but the 50% point was raised to $1{\cdot}5 \times 10^{-7}$ M (Fig. 13). WR 122,455 alone caused no

Table 3. Comparison of gmK_i and antimalarial activity *in vivo*

Drug	gmK_i (nM)	n	Activity *in vivo* (given s.c.) against *P. berghei* [31]. ED_{90} N	I_{90} NS	I_{90} RC	Activity against chloroquine-resistant *P. falciparum* in man [32, 33].
Quinine	410	2·3	87	0·8	inactive	+ −
9-Epiquinine	10^5	ND	inactive	ND	ND	ND
Ro 21-0960	53	1·7	3·7	2·6	inactive	ND
WR 142,490	4	1·0	8·5	1·3	2·3	+
WR 30,090	25	ND	2·7	1·2	66	+
WR 122,455	11	1·3	10	1·9	37	+
WR 165,355	7	ND	2·6	1·5	ND	ND
WR 33,063	29	ND	18·5	ND	150	+

ED_{90} = daily dose of drug in mg/kg reducing parasitaemia to 10% of control in a 4-day test.

$$I_{90} = \frac{ED_{90} \text{ in resistant strain}}{ED_{90} \text{ in sensitive strain}}.$$

Strain N is chloroquine-sensitive *P. berghei*, Strain NS is chloroquine-resistant, quinine-sensitive *P. berghei* and strain RC is *P. berghei* resistant to chloroquine and quinine. The values for WR 30,090 are for oral dosing.

ND = not tested; + = active; + − = active only against lower levels of chloroquine-resistance.

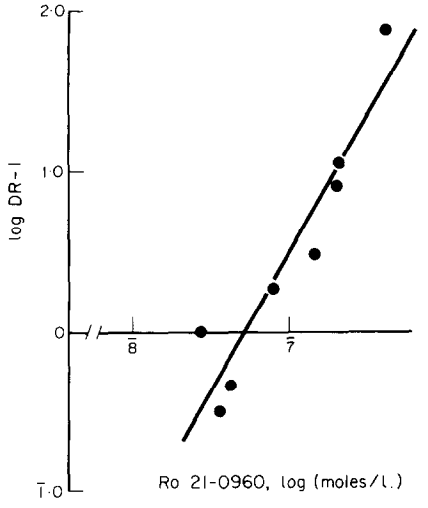

Fig. 6. Log (DR − 1) plotted against log Ro 21-0960 concentration.

clumping. The log (DR − 1)/log WR 122,455 relationship was plotted (Fig. 14) and was found to be a straight line with a slope of 1·27 (correlation coefficient 0·989, $P < 0{\cdot}01$). The intercept, $\bar{8}{\cdot}02$, corresponds to a gmK_i of $1{\cdot}05 \times 10^{-8}$ M.

WR 165,355 is antimalarially active against *P. berghei in vivo* (Table 3). It has marked clumping and inhibitory effects *in vitro* at 10^{-5} M, but at 10^{-6} M and below it is a strong competitive inhibitor of CIPC and causes no clumping alone. 4×10^{-8} M WR 165,355 moved the 50% point to $1{\cdot}3 \times 10^{-7}$ M chloroquine (DR − 1 = 5·5). The K_i value, assuming $n = 1$, was estimated to be $7{\cdot}3 \times 10^{-9}$ M.

WR 33,063, a substituted phenanthrene methanol, is active *in vivo* against normal and chloroquine-resistant malaria (Table 3). $6{\cdot}95 \times 10^{-8}$ M WR 33,063 did not affect maximum CIPC but moved the 50% point to $6{\cdot}76 \times 10^{-8}$ M chloroquine (DR − 1 = 2·4). K_i, assuming $n = 1$, was estimated to be $2{\cdot}9 \times 10^{-8}$ M. The drug caused no clumping when added alone.

DISCUSSION

*A. Clumping response to chloroquine**

The 50% clumping concentration of 2×10^{-8} M chloroquine which we observe at 37° is very close to the K_m of 10^{-8} M reported by Fitch [20] for high affinity accumulation of 14C chloroquine by *P. berghei* at 22°. Fitch *et al.* [21] have published a figure indicating that at 37° their K_m value is closer to 2×10^{-8} M. In addition to this similarity in K_m values, the time course of the early 'granulation' stage of pigment clumping [5] is similar to that of the early rapid stage of 14C chloroquine accumulation [20].

* (+) and (−) chloroquine are reported to be equivalent in antimalarial activity [23].

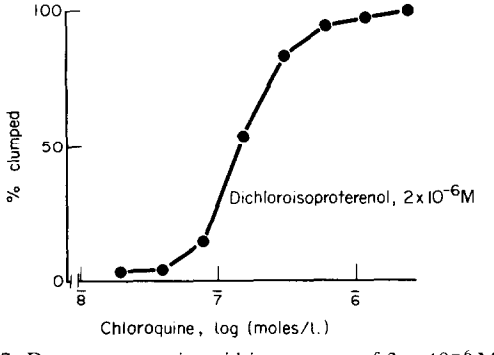

Fig. 7. Dose–response sigmoid in presence of 2×10^{-6} M DCI.

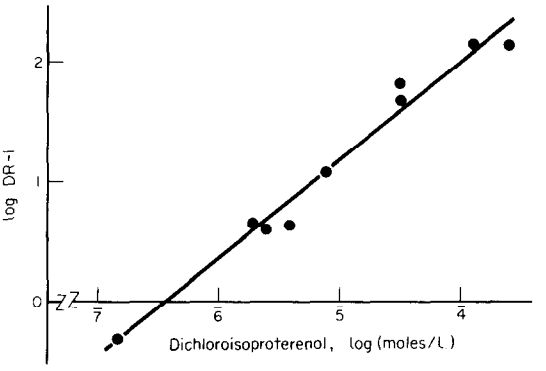

Fig. 8. Log (DR − 1) plotted against log DCI concentration.

B. Inhibitory effect of chloroquine

At the high concentrations necessary for inhibition of clumping, chloroquine is probably acting on synthetic or energy-supplying reactions responsible for the second part of the clumping process [5].

C. Competitive inhibition of CIPC

Quinine showed marked competitive inhibition of CIPC ($gmK_i = 4 \times 10^{-7}$ M). The slope of the line in Fig. 4 indicates that two molecules of the drug compete with each molecule of chloroquine. Fitch [22] found the K_i of quinine to be 2×10^{-6} M, in competition with 14C chloroquine for the high affinity site. His results gave no reason to suspect that more than one molecule of quinine competed with each molecule of chloroquine.

Epiquinine, the 9-epimer of quinine, shows little antimalarial activity, and no detectable affinity for the clumping site, whilst the active antimalarial quinidine also a diastereomer of quinine, has a similar affinity for the clumping site to that of quinine. The importance of the 8–9 *erythro* relationship (see Table 1)

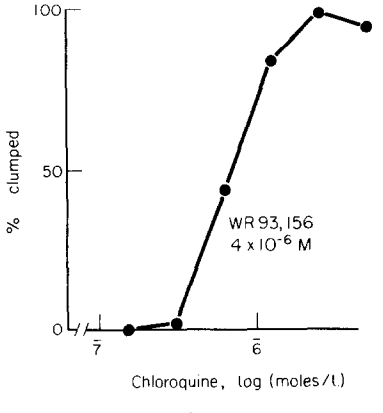

Fig. 9. Dose–response sigmoid in presence of 4×10^{-6} M WR 93,156.

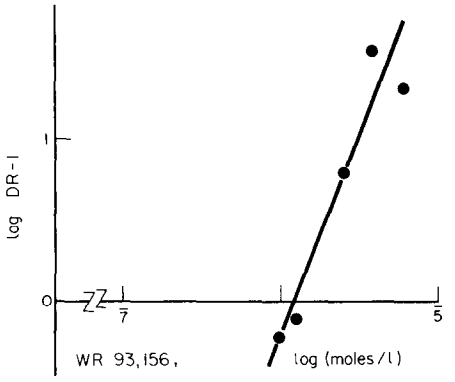

Fig. 10. Log (DR − 1) plotted against log WR 93,156 concentration.

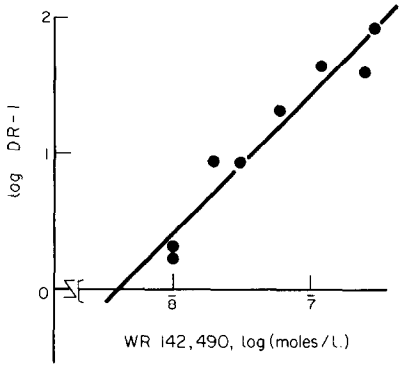

Fig. 12. Log (DR − 1) plotted against log WR 142,490 concentration.

for antimalarial activity in the cinchona alkaloids (Table 3) is reflected in requirements for inhibition of CIPC, and is probably a consequence of the bulky nature of the quinuclidine ring, which hinders rotation around the C8–C9 linkage. It has been postulated that in amino-alcohol antimalarials [24] and in all blood schizontocides [25] the presence, or potential presence, of an intramolecular hydrogen bond between the protonated side-chain nitrogen function and a nearby proton-acceptor group (—$\ddot{O}$H or ≥$\ddot{N}$H) is a prerequisite for antimalarial activity. Clearly a stereochemical alteration changing the relationship between C8 and C9 from *erythro* to *threo* will affect the likelihood of this hydrogen bond being made in cinchona alkaloids. It will also influence whether the intramolecular link is made directly or via a water molecule as has been suggested for the 9-epimers [26] (see Fig. 15 for drug structures).

It is interesting to note that the dextrorotatory quinidine and cinchonine have a higher affinity for the clumping site than do their respective diastereomers the laevorotatory quinine and cinchonidine.

Chloroquine and similar 4-aminoquinolines have strongly basic nitrogen functions on either side of a planar ring system. One of these basic groups (the terminal side-chain nitrogen) is almost certainly protonated and positively charged in the active species of the drug. These three areas of the drug, electropositive, planar ring and electronegative aromatic nitrogen probably interact with complementary areas on the receptor. In the active monocation of chloroquine, incorporating a possible intramolecular hydrogen bond [27, 25] the three areas are likely to be coplanar. The important binding determinants of the receptor (electronegative area, planar area, electropositive area) are therefore also likely to be arranged in one plane. In cinchona alkaloids the protonated, and internally hydrogen bonded, quinuclidine nitrogen cannot readily become coplanar with the aromatic ring. In addition, the aromatic amino group is not so electronegative as in 4-amino quinolines [28] (see Fig. 16).

In 4-aminoquinolines and 9-aminoacridines substitution of bulky groups adjacent to the aromatic nitrogen markedly reduces antimalarial activity [23, 29]. This is not the case for quinoline methanols. It appears that the distance between the aromatic nitrogen and the electropositive part of the receptor is critical in 4-aminoquinolines and 9-aminoacridines, suggesting a direct interaction of a sterically restricted nature (hydrogen-bonding or a coordination link?) between the aromatic nitrogen and the receptor. The quinoline- (and probably also the phenanthrene-) methanols may interact by less sterically restricted ring-ring interactions, possibly involving some degree of charge-transfer, with planar aromatic groups surrounding the electropositive area.

The lack of planarity, and/or probable weakness of interaction of the aromatic nitrogen with the receptor may be a reason why two molecules of quinine

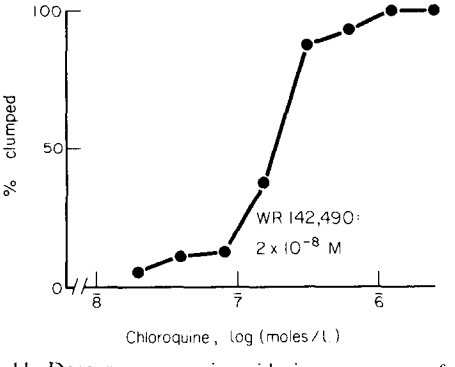

Fig. 11. Dose–response sigmoid in presence of 2×10^{-8} M, WR 142,490.

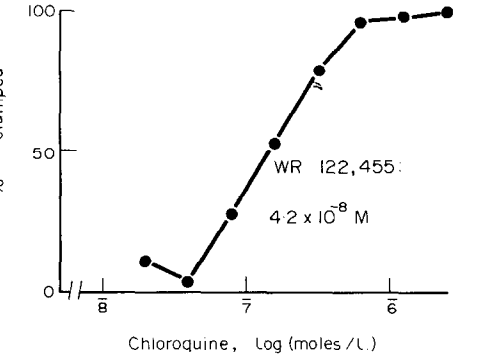

Fig. 13. Dose–response sigmoid in presence of $4{\cdot}2 \times 10^{-8}$ M WR 122,455.

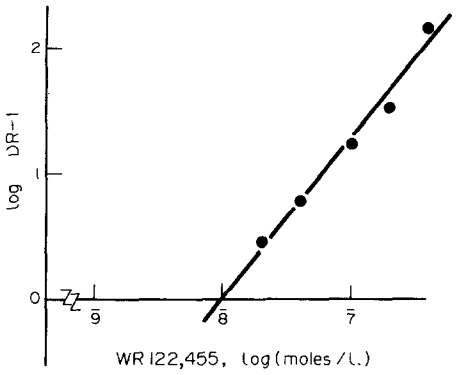

Fig. 14. Log (DR − 1) plotted against log WR 122,455 concentration.

interact with the receptor filled by one chloroquine molecule. Again, in the case of the chloroquine isostere WR 93,156, lack of the protonated side chain nitrogen leads to poor interaction with the receptor, and the involvement of two molecules where only one chloroquine molecule is needed.

Although only one aromatic ring is present, reducing ring-ring interactions with the site, dichloroisoproterenol presumably fits well since $n \simeq 1$. The agent has an easily protonatable nitrogen function capable of hydrogen bonding to the alcoholic hydroxyl group in the side chain. The positively charged group can easily be coplanar with the aromatic ring, and the electronegative function is well represented by the 3 and 4 chlorine substituents. The *N*-diethyl derivative of this agent, SN 9516, is an active antimalarial [29]. The poor interaction of isoproterenol with the site is probably related to the polar nature of the phenolic hydroxyl groups which prevent association with this highly lipophilic area. Insensitivity to isoproterenol indicates that the chloroquine receptor is not closely related to normal β-adrenergic recoptors.

The quinoline methanol WR 142,490 (the *erythro* racemate; see Table 1) has a very high affinity for the clumping site, 100 times that of quinine. WR 142,490 apparently interacts with the whole receptor, since $n = 1$. The structures of WR 142,490 and dichloroisoproterenol are directly comparable. The protonatable nitrogen of the piperidyl side chain can easily become coplanar with the aromatic ring, unlike the very similar drug Ro 21-0960 which has a quinuclidine side chain. The electronegative and lipophilic characters of the region around the aromatic nitrogen function in WR 142,490 and in Ro 21-0960 are enhanced by trifluormethyl groups. The *threo* diastereomer of WR 142,490, WR 177,602 has an affinity for the clumping site similar to that of WR 142,490. This is unlike the quinine/epiquinine relationship and reflects the freer rotation around the link between the asymmetric carbon atoms.

The last quinoline methanol in the series, WR 30,090, was originally reported [7] to be relatively inactive *in vitro* against CIPC. This was incorrect and was due apparently to inadequate methods of dissolving the drug. When an alcoholic solution was first made, and as short a dilution series as possible was used, the drug showed a high affinity for the clumping site. The comments made about the structure of WR

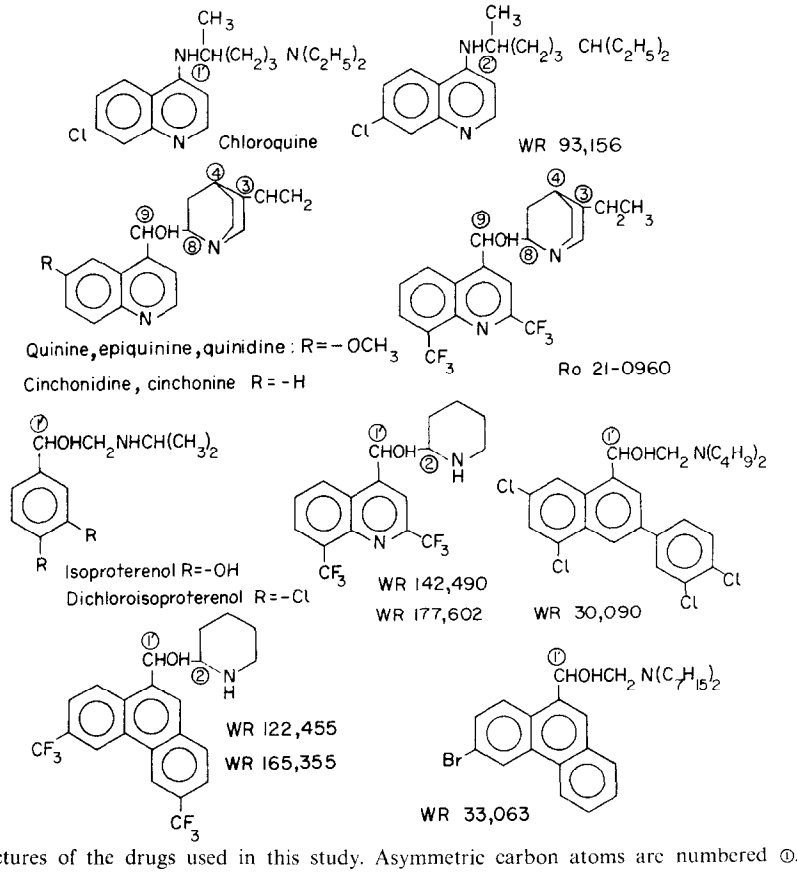

Fig. 15. Structures of the drugs used in this study. Asymmetric carbon atoms are numbered ①, ② etc.

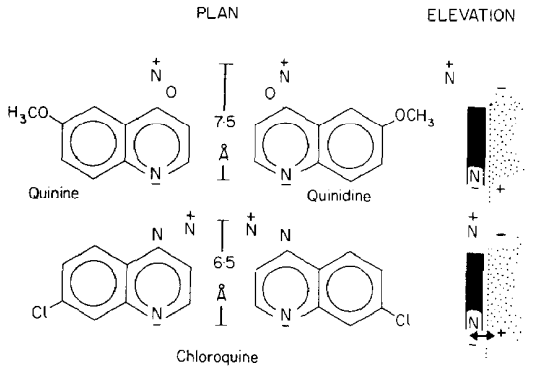

Fig. 16. (−) quinine, (+) quinidine, (+) chloroquine, and (−) chloroquine drawn to show a possible arrangement (arrived at by the use of Corey–Pauling–Koltun models) of the proposed receptor-binding determinants. The approximate distance between protonated and aromatic nitrogen atoms is indicated. Hydrogen bonding may occur between N^+ and the adjacent nitrogen or oxygen atoms. In the side elevation the possible relationship of the drugs to a relatively planar receptor site is indicated. The possibility of hydrogen bonding or a coordination link between the 4-aminoquinoline aromatic nitrogen and the receptor is indicated by a double arrow.

142,490 with regard to the ability of the side chain nitrogen to become coplanar with the ring, and the enhancement of lipophilic and electronegative characters near the aromatic nitrogen function apply equally here.

WR 122,455, a phenanthrene methanol, is a *threo-erythro* mixture of two racemates (Table 1). It has an affinity for the clumping site 40 times that of quinine, and occupies it fully ($n \simeq 1$). As in other drugs which fill the receptor the protonatable side chain nitrogen can become coplanar with the aromatic ring, and extra electronegative and lipophilic character is lent to the area opposite to the side chain by trifluoromethyl substituents. The *threo* epimer of WR 122,455, WR 166,355 has similar, possibly slightly higher, affinity for the receptor. In this drug the amino group can easily become coplanar with the aromatic ring. According to Olsen [16] strong hydrogen bonding occurs between the hydroxyl and piperidyl amino groups.

Although the phenanthrene methanol WR 33,063 was reported earlier to be relatively inactive *in vitro* on CIPC [7], work on this drug was repeated in view of the new results in WR 30,090, which has a comparable side chain. Satisfactory results were not obtained unless the alcoholic solution of the drug was diluted in saline containing 0·1% (v/v) Tween 80. Again, when dissolved satisfactorily, this drug had a high affinity for the clumping site. The protonatable side chain amino group can become coplanar with the aromatic ring, and bromine substitution increases the electronegative and lipophilic character of the area opposite the side chain.

It is informative to compare the characteristics of the 'clumping site' with the 'high affinity site' studied by Fitch and colleagues. When the K_m value, the apparent dissociation constant of chloroquine at the two sites, is compared the results are identical (at 37°). Fitch [22] showed that the K_i value, using 'cold' chloroquine as an inhibitor of [^{14}C]chloroquine accumulation, was 5×10^{-7} M, indicating that the high affinity site is heterogeneous. We are unable to carry out similar experiments using our technique. Using the same drugs (Table 2) K_i values measured at the high affinity site range from 7×10^{-7} to 4×10^{-6} M, less than 6-fold, whilst at the clumping site they range from 4×10^{-9} to $1{\cdot}2 \times 10^{-6}$ M, 300 fold. The high affinity site is not highly structure-specific [21], whilst the clumping site is. Although our comparison between the two sites is somewhat inadequate, because the high affinity site is studied at 22° or 25° whilst the clumping site is studied at 37°, it does seem that in spite of broad overall similarities the two sites are distinct. This comparison between high affinity and clumping sites recalls the observation [30] that most receptors binding [^{3}H](−) noradrenaline were not stereospecific, yet the response to (−) noradrenaline was.

Possible differences in the chloroquine-resistant receptor

It is probable that clumping receptors are not 'lost' from chloroquine resistant malarial parasites, but are present in a modified form, accessible to suitable drugs. Moderate degrees of chloroquine resistance generally involve retention of sensitivity to quinine. With higher levels of chloroquine-resistance, sensitivity to quinine is lost, but quinoline and phenanthrene methanols with piperidyl or dialkyl side chains are still relatively effective. As mentioned earlier, chloroquine's interaction with the electropositive area of the receptor is likely to differ from that of quinine, and it is therefore probable that in moderate chloroquine-resistance the electropositive area is modified —perhaps being shielded by a lipophilic group, or the distance between electronegative and electropositive areas of the receptor is slightly increased. These modifications would be unlikely to affect the interaction of quinine with the receptor. Where quinine is ineffective, at high levels of chloroquine-resistance, one can imagine that the electronegative area of the receptor is also modified in such a way as to reduce its affinity for the positively charged part of the drug. This would have a marked action on the effectiveness of cinchona alkaloids and derivatives, where the positively charged group is incapable of becoming coplanar with the aromatic ring. Drugs such as WR 122,455 and WR 142,490 where the receptor-fitting determinants are coplanar, and lipophilic electronegative groups, which can enhance charge-transfer interactions, are available on the aromatic ring, are still capable of binding to the modified receptor.

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