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THE SUSCEPTIBILITY OF THE MALARIAL PARASITE *PLASMODIUM FALCIPARUM* TO QUINOLINE-CONTAINING DRUGS IS CORRELATED TO THE LIPID COMPOSITION OF THE INFECTED ERYTHROCYTE MEMBRANES

GAVRIEL SHALMIEV and HAGAI GINSBURG*

Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

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Abstract—The anti-malarial action of quinoline-containing compounds depends on various membrane-related processes, and drug resistance could depend, among other factors, on the membrane lipid composition. To verify this hypothesis, the constitution of phospholipid classes and the content of cholesterol of various strains of *Plasmodium falciparum*-infected human erythrocytes grown in *in vitro* cultures have been assessed in conjunction with drug susceptibility. It was found that uninfected erythrocytes in the culture serve as a major source for the increased lipid content of malaria-infected cells. Alterations of the phospholipid composition of infected cells that result from parasite lipid metabolism are also reflected in the constitution of uninfected red cells, implying lipid exchange between infected and uninfected cells. An inverse relationship between the content of acidic phospholipids and cholesterol has been observed. Some strains resistant to chloroquine and quinine were sensitive to mefloquine, and *vice versa*. Resistance to chloroquine or quinine was found to be directly related to the content of acidic phospholipids, while that of mafloquine displayed an inverse correlation. Concomitantly, the resistance to chloroquine was inversely related to the content of cholesterol, while the sensitivity to mefloquine decreased with cholesterol concentration. The possible mechanisms that could account for these observations are briefly discussed.

Malaria still constitutes a major human health problem in most of the tropical areas of the world. The efficiency of classical anti-malarial drugs and insecticides is declining with increasing resistance of parasites and their vectors, respectively. Only recently has the anti-malarial mode of action of quinoline containing anti-malarial drugs (QCDs†), begun to be unraveled. QCDs translocate into the malaria-infected red blood cells (IRBC) in their free base form [1], and accumulate inside the acidic food vacuole of the malarial parasite by virtue of their weak base properties [2, 3]. Inside the food vacuole they complex with free heme [4, 5] formed by the degradation of ingested host cell hemoglobin [6–8], thus inhibiting the heme polymerase-dependent sequestration of heme into the malarial pigment hemozoin [9]. Free heme demonstrably inhibits vacuolar proteases [10] and drugs inhibit acid phospholipase A [11, 12], thereby interfering with digestion [13]. Heme is able to translocate across membranes [14], and we have recently shown that it nicks the parasite DNA when infected cells are treated with therapeutic concentrations of QCDs.‡

The global resurgence of malaria is mostly due to the advent of drug-resistant parasites. There is sufficient evidence showing that chloroquine-resistant (CQR) strains of *Plasmodium falciparum* accumulate less drug, probably due to a less active vacuolar proton pump [15]. This putative mechanism implies cross-resistance to all QCDs, but in many cases this has not been observed, and some CQR clones are resistant to mefloquine and *vice versa* [16–18], indicating that decreased accumulation *per se* is not the sole factor in determining resistance, as previously contended [19]. Since heme has to translocate from the food vacuole into the parasite's cytosol in order to exert its effect on parasite DNA, and because the transport of heme depends on the lipid composition of membranes [20, 21], it was interesting to investigate the dependence of drug sensitivity on lipid composition. We show that the parasite's resistance to chloroquine and quinine is directly related to the relative content of acid phospholipids and inversely related to that of cholesterol (C). Mefloquine resistance displays an opposite relationship.

MATERIALS AND METHODS

Materials. Phospholipid (PL) standards and RPMI-1640 were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Silica gel G TLC plates were from Analtech Inc. (Newark, NJ, U.S.A.). All

* Corresponding author. Tel. (972) 2-585539; FAX (972) 666804.

† Abbreviations: RBC, red blood cells; NRBC, normal RBC; URBC, uninfected RBC isolated from parasite culture; IRBC, infected RBC; PL, phospholipid; PC, phosphatidylcholine; LPC, lysoPC; PE, phosphatidylethanolamine; LPE, lysoPE; PS, phosphatidylserine; PI, phosphatidylinositol; SPM, sphingomyelin; PG, phosphatidylglycerol; C, cholesterol; QCD, quinoline containing anti-malarial drugs; CQR, chloroquine-resistant.

‡ Ginsburg *et al.*, submitted.

other chemicals were of best available grade. Fresh blood was kindly donated by the Shaarei Zedek Hospital (Jerusalem) and human plasma by The Hadassah Hospital (Jerusalem).

Parasite cultivation. The FCC1, FCR1, FCR3, D6, ITG, W2 and K1 strains of *P. falciparum* were cultivated *in vitro* using A^+ or O^+ human erythrocytes at 1% hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 10 mM glucose and 20 mM $NaHCO_3$, and 10% (v/v) heat-inactivated AB^+ human plasma. Erythrocytes were inoculated with the desired parasite strain at 1–2% parasitemia in culture flasks and gassed with a mixture of 90% N_2, 5% CO_2 and 5% O_2. Cultivation proceeded at 37° with daily changes of medium and gassing. The cultures were synchronized in isoosmotic sorbitol [22]. When the cultures reached the trophozoite/schizont stage and a parasitemia of 10–15%, the erythrocytes were washed twice with serum-free growth medium and fractionated by the Percoll–sorbitol method [23] using alanine instead of sorbitol and only two layers of different Percoll concentrations: 90% for the recovery of uninfected erythrocytes (<2% parasitemia) and 60% for the enrichment of mature trophozoites and schizonts (≥85% parasitemia). Normal erythrocytes maintained under culture conditions were used as control. The number of cells was determined with a cell counter (Analys, Stockholm, Sweden) and the parasitemia was assessed by microscopic inspection of Giemsa-stained thin blood smears.

Determination of parasite sensitivity to anti-malarial drugs. The method of Desjardins *et al.* [24] was used to determine the IC_{50} values of chloroquine, mefloquine and quinine. Cultures at the ring stage (1% hematocrit, 2% parasitemia) were exposed to increasing drug concentrations. After 24 hr in culture, [^{3}H]hypoxanthine was added (5 μCi/mL final), and after an additional 24 hr incubation period, cells were harvested in triplicates (Dynatech, Inc. cell harvester). Filters were dried for 1.5 hr at 100° and counted in a toluene-based scintillation fluor. The IC_{50} values of growth inhibition were calculated on the Enzfitter programme.

Lipid extraction. Packed trophozoite-infected (1×10^9 cells) or uninfected erythrocytes (4×10^9 cells) retrieved from the Percoll gradient were washed twice in plasma-free culture medium supplemented with 50 mM sucrose plus 150 mM alanine to avoid osmotic lysis, then in phosphate-buffered saline (140 mM NaCl, 20 mM Na-phosphate), lysed on ice with 2 vol. of 1% acetic acid and immediately supplemented with 10 vol. of 110 mM $MgCl_2$ plus 2 mM EGTA. EGTA was included to prevent the action of calcium-dependent endogenous phospholipase(s). The 18,000 *g* (15 min, 4°) pellet was washed again in the same medium and resuspended in turn with 3 vol. each of water, methanol and chloroform, with intermittent vigorous mixing. After centrifugation at 3500 *g* for 15 min at room temperature, the lower organic layer was collected and evaporated to dryness under a stream of nitrogen, the lipids were dissolved in a small volume chloroform:methanol (2:1 v/v) and kept at −20° until used.

Analysis of PL composition. Separation of PLs was performed by TLC on Analtech silica gel G plates (250 μm thick) by bidimensional chromatography [25]. A sample containing 100–200 nmol of lipid phosphorus were chromatographed in the first dimension with chloroform:methanol:33% methylamine in ethanol (13:6:1.5, by vol.), diethylether:glacial acetic acid (19:1, v/v), and then chloroform:acetone:methanol:acetic acid:water (10:4:2:3:1, by vol.) in the second dimension. PLs were revealed by iodine vapor and identified by comparison of the R_f values with those of authentic standard PLs.

The PL spots were scraped and extracted three times with 0.8 mL of chloroform:methanol:water (65:25:4, by vol.) mixture. PL phosphorus content was determined colorimetrically [26]. This method was also used for analysis of total PL phosphorus prior to chromatography. Although the recovery of PL from the plates varied between 35 and 80% (based on phosphorus content relative to that in samples prior to chromatography), no correlation between PL composition and % recovery could be observed. Hence, we assume that the PL composition as determined by TLC reflects the composition of the total extract.

Analysis of total C. C was determined by the Liebermann–Burchard colorimetric method [27]. Aliquots of total lipid extracts were placed in glass tubes and evaporated to dryness under a stream of nitrogen, redissolved in 1.25 mL of chloroform (on ice), and 0.25 mL of the Liebermann–Burchard colorimetric reagent added (10 vol. of acetic anhydride were chilled to a temperature lower than 10° in a glass-stoppered container, 1 vol. of concentrated sulfuric acid was added and the well shaken mixture kept cold for 9 min). The tubes were incubated with shaking for 10 min at 37°, and returned to the ice. The optical density at 670 nm was recorded in a Spectronic 3000 Array spectrophotometer in quartz cuvettes. Concentrations of cholesterol were computed from a standard curve (5–160 μg).

Statistical analysis. Data were analysed essentially by linear regression. Slopes and their standard errors and correlation coefficients (r) were calculated. The square of r reflects the proportion of the correlation that is explained by the independent parameter. Since the correlations were usually rather low, the derived values of the slope and its standard error were used to find the P range that would account for a significant difference from a slope of zero (no correlation).

RESULTS

The analysis of PL composition was performed on cultures of seven different strains of *P. falciparum*. For three strains (D6, W2 and FCR3) experiments were repeated three times using different red cells and different batches of plasma. Although some variation was observed in the values obtained, they did not change the overall trends observed, and for reasons of economy replicate experiments were not performed on all strains.

The PL composition of normal red blood cells (NRBC) was found to be somewhat different from

Table 1. PL composition of erythrocytes infected with various strains of *P. falciparum*

PL type	*P. falciparum* strain FCC1	FCR1	FCR3	D6	ITG	W2	K1
LPC	2.11	2.98	4.52 ± 0.42	2.57 ± 0.34	3.84	2.75 ± 0.54	3.58
PI	3.98	6.88	6.16 ± 0.57	5.90 ± 1.03	6.67	6.28 ± 1.18	7.55
PS	6.41	4.57	8.68 ± 0.17	5.80 ± 0.56	5.95	8.96 ± 1.30	6.29
SPM	6.61	5.91	7.36 ± 3.20	10.85 ± 3.84	10.83	8.17 ± 3.16	10.64
PC	47.07	44.80	37.78 ± 2.86	40.81 ± 5.39	44.77	41.93 ± 4.01	41.20
PE	22.87	22.57	22.60 ± 0.29	21.34 ± 1.17	17.76	19.33 ± 1.28	20.90
LPE	6.30	6.33	7.55 ± 1.00	9.45 ± 2.36	5.49	6.47 ± 2.29	6.35
PG	4.65	5.96	3.55 ± 0.30	3.48 ± 0.59	4.69	6.12 ± 2.00	3.47
Total PL	22	14.7	18.92 ± 1.34	17.12 ± 1.48	22.39	15.38 ± 2.29	22.14

Parasite cultivation, lipid extraction and determination were performed as described in Materials and Methods.

Results are presented as percentage of total PLs, and the total content is given in mg/10^{10} cells.

For three strains, FCR3, D6 and W2, experiments were repeated three times and results are shown as means ± SD.

Table 2. PL composition in normal and uninfected erythrocytes

PL type	Normal cells	Uninfected cells in parasite cultures FCC1	FCR1	FCR3	D6	ITG	W2	K1
LPC	1.58	1.43	1.48	2.77 ± 0.42	2.19 ± 0.82	2.10	1.96 ± 0.10	2.07
PI	1.9	1.47	2.67	4.17 ± 0.84	3.78 ± 1.40	3.44	3.49 ± 0.32	2.98
PS	9.1	10.32	8.95	8.24 ± 2.19	11.04 ± 0.94	9.93	9.26 ± 1.96	9.85
SPM	18.49	15.48	11.82	16.49 ± 2.70	16.74 ± 0.53	14.70	17.09 ± 3.67	14.9
PC	36.66	41.60	45.93	34.45 ± 4.57	34.62 ± 3.30	40.20	37.32 ± 4.20	43.18
PE	24.81	22.24	22.76	21.86 ± 2.55	21.99 ± 0.54	19.20	19.67 ± 2.55	18.32
LPE	7.46	7.47	6.39	12.03 ± 2.10	9.62 ± 1.79	10.40	11.21 ± 1.33	8.69
Total	5.29	4.48	5.76	3.25 ± 0.34	3.26 ± 0.20	3.4	3.79 ± 0.96	5.4
Parasitemia (%)		14.3	9	16.33 ± 0.94	19.77 ± 1.93	15	15.13 ± 2.91	7

Uninfected cells were isolated from cultures of different parasite strains by Pecoll–sorbitol fractionation. Lipids from these and NRBC were extracted and analysed as described in Materials and Methods.

Results are given as percentages of total PLs. Total PL content is given in mg/10^{10} cells. The parasitemia in the cultures used for cell fractionation is given in % infected cells.

For three strains, FCR3, D6 and W2, experiments were repeated three times and results are shown as means ± SD.

that reported previously [28], notably being richer in phosphatidylinositol (PI), lysophosphatidylethanolamine (LPE) and sphingomyelin (SPM). This divergence could have resulted from the use of outdated blood maintained under parasite culture conditions. As reported previously by several investigators (for reviews see Refs 29–31), the PL content of IRBC was also found in this work to be appreciably higher than that of NRBC (Tables 1 and 2). The 3–4-fold increase suggests that the majority of the membranes are of plasmodial origin and the total lipid composition of the membrane fraction of IRBC would closely resemble that of the parasite. The same conclusion was reached from the published compositions of separated host cell and parasite membranes [32]. The membranes of IRBC are much richer in their relative content of PI, phosphatidylcholine (PC), lysoPC (LPC), and far poorer in phosphatidylserine (PS), sphingomyelin (SPM), phosphatidylethanolamine (PE) and LPE. Phosphatidylglycerol (PG) present only in trace amounts in NRBC, appears in appreciable amounts in IRBC membranes. The relative content of individual phospholipids depends somewhat on the type of *P. falciparum* strain.

Although malarial parasites have all the necessary pathways for *de novo* synthesis of PL [30, 31], it seems that non-infected cells serve as major sources for parasite PL in culture. Cultivation at low hematocrit allows relatively high parasitemias as well as an assessment of changes in the PL composition of the uninfected cells (URBC) in the same culture (Table 2). Indeed, we observed a proportional decrease in the PL content of URBC with increasing parasitemia (Fig. 1), implying that they are the major source for parasite PL. The fact that the results obtained from the various strains fall on the same line, indicates that there is no strain-specificity with respect to preference of PL source. A closer inspection of the results depicted in Table 2, reveals that the major substrates of parasite lipids are PE and SPM, because these are the only species whose content in uninfected cells consistently decrease with increasing parasitemia irrespective of the strain. The

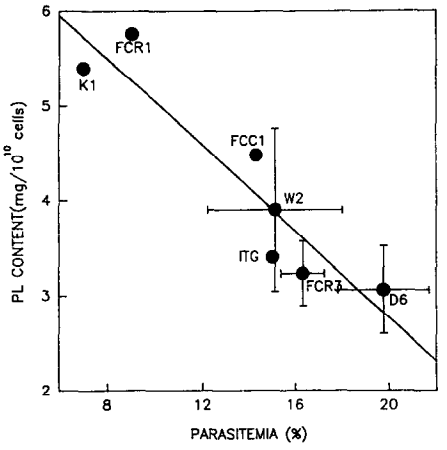

Fig. 1. The total PL content of uninfected erythrocytes in culture. Different strains were grown in culture till the trophozoite stage, and IRBC were separated from URBC by the Percoll–sorbitol method. Lipids were extracted and their total PL content as phosphorus was determined as described in Materials and Methods. Results are expressed as PL content against the parasitemia of culture prior to separation of URBC from IRBC. Slope = -0.228 ± 0.041; $r = 0.927$.

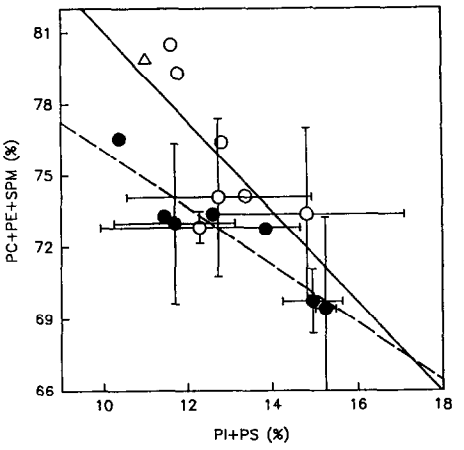

Fig. 2. Relationship between the relative content of neutral and acidic PLs in infected and uninfected erythrocytes. Separation of IRBC from URBC from cultures of different parasite strains and lipid extraction, were performed as described in the legend to Fig. 1. The different PLs were separated by TLC and their content determined as described in Materials and Methods. The percentages of PC + PE + SPM were summed and plotted against the serum of PI + PS. The different points were obtained from the different parasite strains. The lines were obtained by linear regression. URBC (○—○) $r = 0.696$, slope = -1.97 ± 0.91; IRBC (●---●) $r = 0.922$, slope = -1.22 ± 0.23. NRBC (△).

relative content of other PL species either increases, e.g. for PI, PC, and in some cases LPE, or remains unaltered, as is the case for PS and LPC. One has to conclude that there is net transport of the former from IRBC to uninfected cells.

Analysis of the PL composition of IRBC (Table 1) reveals that in the various parasite strains the relative content of the acidic PL, PS and PI is inversely correlated to that of the neutral PL, PC, PE and SPM (Fig. 2). The slope of this correlation (-1.21 ± 0.23; $r = 0.922$) suggests that there is a one-to-one replacement of one type of PL by the other. A similar correlation exists for uninfected cells (the slope is larger -1.97 ± 0.91, but the correlation coefficient is lower $r = 0.696$, and the slopes of the two lines are significantly different at $0.4 > P > 0.3$). Since in some cases the ratio of neutral to acidic PL in uninfected cells is vastly different from that of control cells (RBC which were not exposed to infected cells, NRBC), it is tempting to propose that their composition may be under control of the PL metabolism of infected cells in the same culture.

The C content of IRBC is invariably higher than that of either normal control or uninfected cells (Table 3). Here again the C/PL ratio in NRBC is smaller than previously reported values [28]. However, the C/PL ratio of IRBC is always lower than that in RBC or URBC. The C/PL ratio of uninfected cells is sometimes larger than that of normal control, suggesting that the C content of the growth medium plasma (most likely lipoproteins) constitutes the major source of C for infected cells. Interestingly, an inverse correlation (significantly different from zero slope at $0.2 > P > 0.1$) was found between the relative content of acidic PL and the C/PL ratio (Fig. 3A). Since an inverse correlation also exists for acidic and neutral PL (Fig. 2), a direct correlation between the relative content of neutral PL and the C/PL ratio was also found as expected, though at a low level of significance (Fig. 3A). No such correlations were found for uninfected cells (Fig. 3B).

The effects of the quinoline-containing antimalarial drugs quinine, chloroquine and mefloquine on the seven different strains of *P. falciparum* were tested in culture under conditions which preclude any inoculum effect [34]. Results shown in Table 4 indicate that the variance in drug sensitivity, as assessed by the ratio of maximal to minimal IC_{50} values, differed among the drugs; 2 for quinine, 3.8 for mefloquine and 23 for chloroquine. A correlation analysis revealed some degree of cross-resistance between quinine and chloroquine, and an inverse correlation between the sensitivity to these two drugs and the susceptibility to mefloquine (Fig. 4). The correlation coefficient of log(IC_{50}) mefloquine to that of quinine was 0.795. Testing for parallelism between the regression lines of IC_{50} for quinine vs IC_{50} of chloroquine and that of IC_{50} of mefloquine vs IC_{50} of chloroquine, the slopes of the two correlations were significantly different at $0.1 > P > 0.05$.

Comparison of drug sensitivity to lipid composition in the various strains revealed that the susceptibility to chloroquine and quinine decreased (increased IC_{50}) while that to mefloquine increased, with increasing content of acidic PL (PI + PS + PG) (Fig. 5). The increase in C/PL ratio caused an opposite

Table 3. C content in uninfected and infected erythrocytes

Parasite strain	Uninfected erythrocytes Total PL	C	C/PL ratio	Infected erythrocytes Total PL	C	C/PL ratio
FCC1	4.48	0.83	0.19	22	3.00	0.136
FCR1	5.76	1.17	0.20	14.7	2.07	0.141
FCR3	3.25 ± 0.34	0.59 ± 0.05	0.18 ± 0.02	18.92 ± 1.34	2.09 ± 0.04	0.111 ± 0.01
D6	3.26 ± 0.20	1.01 ± 0.37	0.31 ± 0.10	17.12 ± 1.48	2.58 ± 0.17	0.151 ± 0.01
ITG	3.4	0.74	0.22	22.39	2.19	0.098
W2	3.79 ± 0.96	0.75 ± 0.10	0.20 ± 0.02	15.38 ± 2.29	1.63 ± 0.23	0.109 ± 0.02
K1	5.4	1.42	0.263	22.14	2.309	0.104
Normal cells	5.29	1.007	0.190			

Values are presented as mg/10^{10} cells.

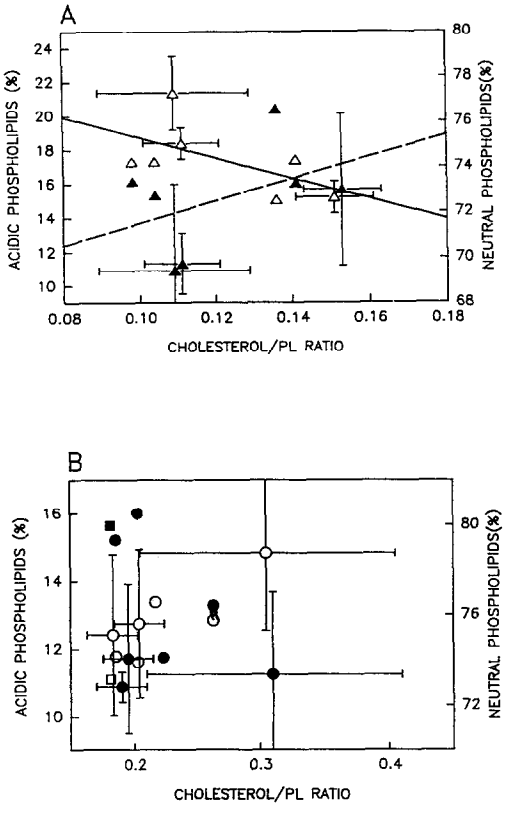

Fig. 3. Relationships between the relative content of acidic or neutral PLs and the C to total PL ratio. Determination of the various PLs in IRBC (panel A) and URBC (panel B) from cultures of different strains was performed as described in the legend to Fig. 2. The C content was determined as described in Materials and Methods. The sum of PI + PS + PG (acidic, open symbols) or of PC + PE + SPM (neutral, full symbols) were plotted against the C/PL ratio (w/w). The lines in panel A were drawn by linear regression: acidic $r = 0.587$, slope = -60.1 ± 37.1, $0.1 > P > 0.05$; neutral $r = 0.437$, slope = 50.8 ± 46.8, $0.4 > P > 0.3$. Test of parallelism between the two regression lines indicated that their slopes were different at a significance level of $0.1 > P > 0.05$. The square in panel B symbolizes NRBC.

effect for the susceptibilities to chloroquine and mefloquine, whereas no correlation could be found between the sensitivity to quinine and the C/PL ratio (Fig. 6).

DISCUSSION

The metabolism and dynamics of lipids in malaria-infected erythrocytes have been extensively reviewed recently [30, 31]. The PL composition of IRBC is substantially different from that of NRBC [29]. This altered composition is achieved through various processes, of which *de novo* synthesis could supply all the needs of the parasite lipid anabolism provided adequate concentration of substrates is supplied [30, 31]. The *in vitro* culture system is obviously different from *in vivo* conditions where the large systemic increase in plasma fatty acids, triacylglycerols and C upon infection can serve the parasite's lipid metabolism. In the present study we have clearly demonstrated that uninfected erythrocytes can serve as a source for PL of the growing parasite, as evidenced by the parasitemia-dependent decrease of PL content of URBC. Since NRBC have very limited phospholipase activity, it must be concluded that PL translocate as such from NRBC to IRBC. Indeed, we found that IRBC can accumulate PL from exogenous sources. They are first incorporated into the host cell membrane and subsequently are exchanged into the parasite membranes and undergo modifications to suit the parasite's needs [31, 35, 36]. However, the exit of PL from NRBC is very much restricted to those species present in the outer PL bilayer leaflet, namely PC and SPM, because flip-flop is limited under normal conditions [37]. To explain the observed net transport of PL from URBC to IRBC, we suggest that IRBC modify the URBC, probably through the generation of oxidative radicals (URBC display high rates of hexose-monophosphate activity and are avidly phagocytosed *in vitro*. Ginsburg *et al.* unpublished observations). Oxidative stress is known to increase the rate of flip-flop [38]. Interestingly, increased flip-flop of PL in uninfected erythrocytes obtained from *P. knowlesi*-infected rhesus monkey has been reported [39], indicating that such effects

Table 4. Inhibition of parasite growth by antimalarial drugs

Parasite strain	IC_{50} (nM) Quinine	Chloroquine	Mefloquine
FCC1	136.4 ± 13.9	121.1 ± 9.3	21.9 ± 7.2
FCR1	265.5 ± 31.7	209.9 ± 108.5	25.5 ± 5.9
FCR3	205.1 ± 37.5	155.6 ± 26.4	18.1 ± 17.0
D6	166.7 ± 11.9	25.6 ± 3.7	62.2 ± 1.4
W2	199.1 ± 193.6	582.1 ± 14.9	23.1 ± 8.1
ITG	135.5 ± 14.8	190.6 ± 146.0	43.8 ± 5.2
K1	220.8 ± 9.0	257.0 ± 58.5	16.6 ± 6.2
MAX/MIN IC_{50}	1.96	22.7	3.75

The effect of various drugs on different strains of *P. falciparum* was assessed as described in Materials and Methods.

Results are given in terms of IC_{50} ± SD, the drug concentrations that causes 50% inhibition of parasite growth in Nm.

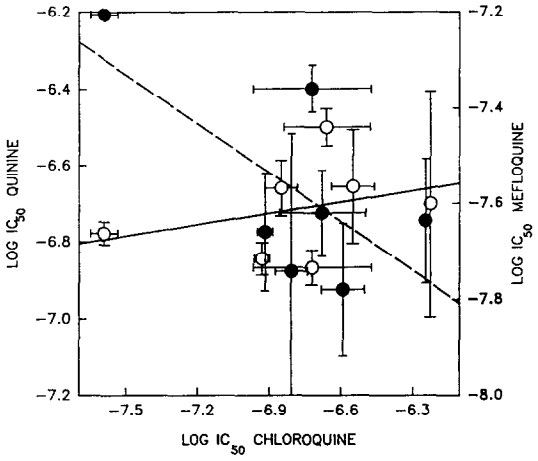

Fig. 4. Correlation between quinine or mefloquine sensitivity and chloroquine sensitivity of various strains of *P. falciparum*. Results shown in Table 4 were used to draw the relationships between the sensitivity to quinine (○—○) or mefloquine (●- - -●) and that to chloroquine. Lines were obtained by linear regression of the log(IC_{50}) values. The regression analyses yielded the following parameters: quinine vs chloroquine: $r = 0.376$ (0.284 when the IC_{50} were used as such), slope = 0.09 ± 0.11, $0.5 > P > 0.4$; mefloquine vs chloroquine: $r = 0.688$ (0.456), slope = −0.34 ± 0.17, $0.2 > P > 0.1$. Test of parallelism between the two regression lines indicated that their slopes were different at a significance level of $0.1 > P > 0.05$.

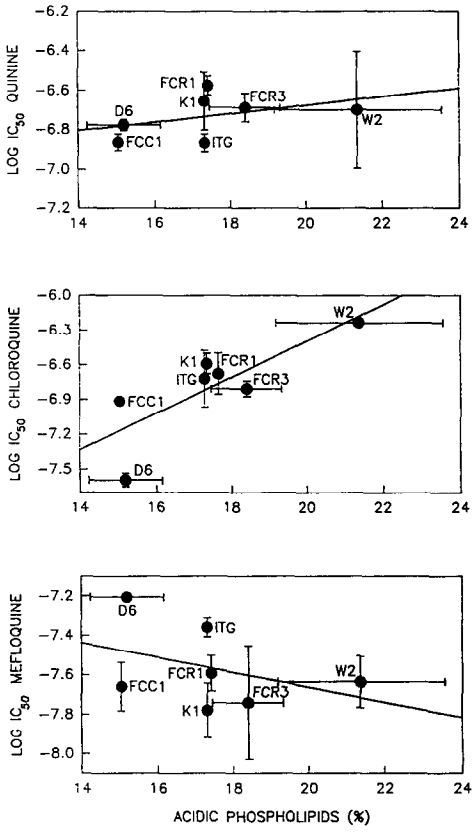

Fig. 5. Relationship between the sensitivity of various parasite strains to chloroquine, quinine or mefloquine, and the relative content of acidic PLs. Results depicted in Tables 1 and 4 were used to draw these graphs. Acidic PLs are the sum of PI, PS and PG. Lines were obtained by linear regression. The correlation coefficients were 0.906 ($P > 0.005$) for chloroquine, 0.42 ($0.4 > P > 0.3$) for quinine and 0.428 ($0.4 > P > 0.3$) for mefloquine.

may also occur *in vivo*. Other investigators could not find increased rates of flip-flop either *in vivo* or in *P. falciparum* cultures [31].

The observation that PE and SPM are the major PL species depleted from URBC is consistent with PE being a substrate for the major route of PC synthesis [33], and that the parasite is unable to synthesize SPM. The latter probably distributes into the newly formed parasite membranes. Alternatively, the parasite could have some sort of sphingomyelinase activity in order to use SPM.

The increase in relative content of some PL in URBC, namely, PI, PC, LPE and LPC, suggests the net translocation of some PL occurs also from IRBC to URBC. In cell fractionation studies, it has been demonstrated that the host cell membrane is richer in PI, PC and LPC [32], suggesting that those

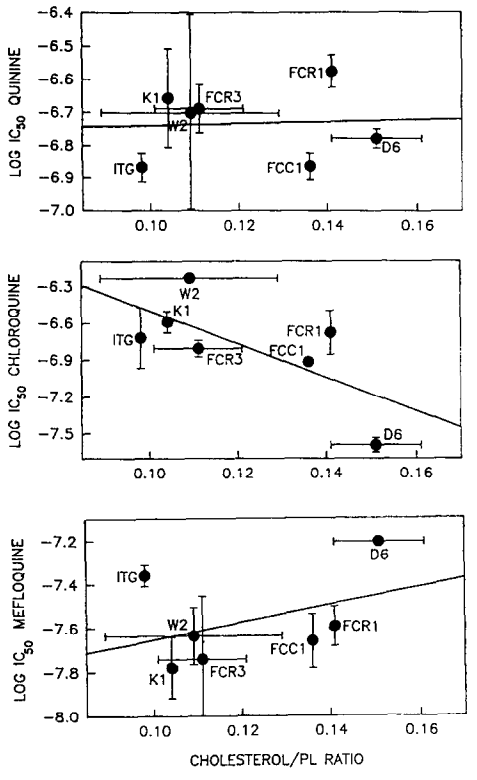

Fig. 6. Relationship between the sensitivity of various parasite strains to chloroquine, quinine or mefloquine, and the relative content of C. Results depicted in Tables 1, 3 and 4 were used to draw these graphs. Lines were obtained by linear regression. The correlation coefficients were 0.511 ($0.25 > P > 0.2$) for chloroquine, 0.078 ($0.9 > P > 0.8$) for quinine and 0.415 ($0.4 > P > 0.3$) for mefloquine.

PL species that are actively synthesized or modified by the parasite, can get into the host cell membrane, and, through the increased flip-flop in this membrane (reviewed recently in Refs 30, 31, 38), be exposed at the exofacial side. Through the mediation of non-specific PL transport proteins present in the plasma they could eventually exchange into the membranes of URBC. The yet unequal composition of IRBC and URBC suggests that the rates of flip-flop and transfer of individual PL species may be different, and that some PL species may be compartmentalized in the various parasite membranes.

A rather unexpected result of the present study was that different parasite strains have different PL compositions. This finding may suggest that enzymes involved in PL synthesis may be expressed to different levels in diverse lines, or that their regulation may be different, or that the transport of substrates/products of PL metabolism is strain-dependent. The complexity of the interrelationships between different pathways of PL synthesis [31] precludes any further interpretation of the results without direct determination of enzyme activities. However, the inverse relationship found between the relative content of acidic and neutral PL, implicates PS decarboxylase and phospholipase C activities, the latter being instrumental for the production of phosphatidic acid as a substrate for PI and PG syntheses from imported PL. Interestingly, the only diversities between parasite strains so far described were related to drug susceptibility, but none of the other biochemical or physiological parameters of parasites have thus far been explored.

The C content of IRBC is known to be higher than that of NRBC, but the C/PL ratio is smaller, as found in the present study. Although it was suggested that increased C is due to the systemic presence of low-density lipoproteins in the malaria-infected animal [30, 31], we see here that using normal plasma also results in increased C levels in IRBC. It has been suggested that all the C of IRBC is confined to the host cell membrane [31]. If this were true, the C/PL ratio of this membrane would be higher than that of NRBC. Such a situation would be incompatible with the higher fluidity observed in the membranes of IRBC. Once again, C levels and the C/PL ratios varied among the various strains. The correlation found between the content of neutral PL (mostly PC) and C, is consistent with the higher affinity of C for PC, a factor thought to govern the asymmetric distribution of C in NRBC membranes [37, 41]. Thus, it is conceivable that C, originating mostly in the plasma's lipoproteins, partitions rapidly into the membranes of IRBC. This partitioning may be governed by the acidic/neutral PL ratio of IRBC, but not exclusively, because no correlation could be found between C/PL and neutral PL in URBC. It would be interesting to study the other factor(s) involved, since C/PL is known to control a variety of membrane-mediated processes that could affect parasite metabolism and drug sensitivity, as shown below.

The physiological and biochemical details of parasite resistance to QCD are not well established. CQR strains of *P. falciparum* accumulate less drug [19], and we have recently suggested that this may be due to a weakened vacuolar H^+-pump that fails to provide the driving force for drug accumulation [15]. If this were true, we would expect CQR strains to be cross-resistant to quinine and mefloquine, since these are also weak bases anticipated to accumulate by the same pH gradient-driven mechanism. Lack of cross-resistance is widely reported for both clinical isolates and cultured *P. falciparum* [16–18]. This paradigm was also found to exist in the present study. While, chloroquine-resistant strains were to some extent cross-resistant to quinine, an inverse correlation was found between the response to chloroquine or quinine, and mefloquine. In this study we provide a possible clue for this enigma. We found that resistance to chloroquine and quinine is correlated to the relative content of acidic PL in the IRBC membranes, while that for mefloquine was inversely related. An inverse relationship is obtained when the IC_{50} values are correlated against the C/PL ratio, though, in this case the sensitivity to quinine is not correlated with C/PL. Since C/PL is inversely correlated with the relative content of acidic PL, it is impossible to conclude which of these parameters is dominant in generating drug resistance. It is worth noting that the dependence of chloroquine resistance on C/PL contradicts our observation that

high C diet results in higher chloroquine resistance of *P. berghei in vivo* [42]. This divergence may be due to parasite species, to the fact that the murine parasite develops primarily in reticulocytes, or to ancillary biochemical effects of the diet.

The low values of r (correlation coefficient) and the high levels of P (low significance) derived from the regressions do not invalidate the tendency of the correlation. Although they could be due to the restricted number of strains that have been used for the present analysis, they could also suggest that the dependence of drug resistance on the lipid composition explains only a part of the various mechanisms that could be involved in drug resistance. This conclusion is not surprising since drug action seems to be pleiotropic [12], meaning that any process that may be affected by the drug may also be differentially sensitive to drug action in each parasite strain. The possible relationships between lipid composition and drug action have been recently discussed by Zidovetzki and Sherman [40]. Drugs could interfere with the digestion of host cell cytosol which requires the action of phospholipase(s) [11, 12]. Protonated forms of cationic amphiphiles (as QCDs are) interact with PL by both hydrophobic and electrostatic forces. Binding alters the physicochemical properties of PL and reduces their susceptibility to hydrolysis by phospholipases. Both chloroquine [43] and quinacrine [44] are potent inhibitors of phospholipases at concentrations lower than those achieved in the parasite acid vacuole. NMR measurements [45, 46] indicate that drug–PL interactions require a protonated amino group in the drug and a charged moiety in the PL, but the affinity of binding is primarily dependent upon the hydrophobicity of the drug [47]. Quinine and mefloquine, but not chloroquine, produce significant disordering of the PL side chains and induce non-bilayer phases preferentially with PE [48]. PL–drug interaction reduces the electrostatic charge of membrane–aqueous interfaces and results in a reduction of phospholipase-mediated hydrolysis of PL [45]. Chloroquine and mefloquine bind preferentially to acidic PL [49, 50], but due to the relatively high electrostatic charge of chloroquine, it binds to an extent less than expected from its hydrophobicity. This is presumably the reason of its inability to intercalate into PL monolayers [51], whereas substantial intercalation is observed with quinine and mefloquine [52]. However, contrary to expectations, higher concentrations of cationic amphiphiles are necessary to inhibit the hydrolysis of acidic PL by lysosomal phospholipase A than those required for the same inhibition of neutral PL [53]. This may explain the reduction of parasite sensitivity to chloroquine and quinine with the increase of the acidic PL content of their membranes. The opposite effect of acidic PL content on mefloquine sensitivity can not be related to the induction of hexagonal phases and non-bilayer structures by this drug, since quinine has a similar effect [48].

C inhibits drug binding to PL [46] and should have increased drug resistance. While this is observed with mefloquine, an opposite effect is seen with chloroquine. No correlation could be found between the sensitivity to quinine and the C/PL ratio. From the above deliberations it seems that inhibition of the parasite vacuolar phospholipase [11] and hence, the feeding process [13], could account in part for drug effects. However, the mere impediment of feeding has a reversible effect on parasite growth [7], while that of drugs is irreversible [54]. The irrevocable effect is best explained by the drug-induced nicking of parasite DNA.* Since this would require the egress of heme from the food vacuole, one should consider the effect of lipid composition on this process. Free heme is able to diffuse across PL bilayers [14, 20, 21]. C and positively charged PLs reduce the rate of heme transport. This could explain the lipid composition-dependent inhibition of parasite growth by mefloquine. However, the demonstrable complexation of QCDs with heme [4, 5], and the chloroquine-dependent increases of heme intercalation into PL monolayers [51], require further investigations that would clarify the effect of drugs on heme translocation across membranes of different lipid composition.

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REFERENCES

1. Cutler DJ and Ferrari V, Uptake of chloroquine by human erythrocytes. *Biochem Pharmacol* **39**: 753–762, 1990.
2. Yayon A, Cabantchik ZI and Ginsburg H, Identification of the acidic compartment of *Plasmodium falciparum* infected human erythrocytes as the target of the antimalarial drug chloroquine. *EMBO J* **3**: 2695–2700, 1984.
3. Krogstad DJ, Schlesinger PH and Gluzman IY, Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J Cell Biol* **101**: 2302–2309, 1985.
4. Blauer G and Ginsburg H, Complexes of antimalarial drugs with ferriprotoporphyrin IX. *Biochem Int* **5**: 519–523, 1982.
5. Blauer G, Optical properties of complexes of antimalarial drugs with ferriprotoporphyrin IX an aqueous medium. I. The system ferriprotoporphyrin IX-quinine. *Arch Biochem Biophys* **251**: 306–314, 1986.
6. Yamada KA and Sherman IW, *Plasmodium lophurae:* composition and properties of hemozoin, the malarial pigment. *Exp Parasitol* **48**: 61–74, 1979.
7. Rosenthal PJ, McKerrow JH, Aikawa M, Nagasawa H and Leech JH, A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. *J Clin Invest* **82**: 1560–1566, 1988.
8. Goldberg DE, Slater AFG, Cerami A and Henderson GB, Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. *Proc Natl Acad Sci USA* **87**: 2931–2935, 1990.
9. Slater AFG and Cerami A, Inhibition by chloroquine of a novel haem polymerase activity in malarial trophozoites. *Nature* **355**: 167–169, 1992.

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10. Vander Jagt DL, Hunsaker LA and Campos NM, Characterization of haemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. *Mol Biochem Parasitol* **18**: 389–400, 1986.
11. Krugliak M, Waldman Z and Ginsburg H, Gentamicin and amikacin repress the growth of *Plasmodium falciparum* in culture, probably by inhibiting a parasite acid phospholipase. *Life Sci* **40**: 1253–1257, 1987.
12. Ginsburg H and Krugliak M, Quinoline-containing antimalarials—mode of action, drug resistance and its reversal. An update with unresolved puzzles. *Biochem Pharmacol* **43**: 63–70, 1992.
13. Zarchin S, Krugliak M and Ginsburg H, Digestion of the host erythrocyte by malaria parasites is the primary target for quinoline-containing antimalarials. *Biochem Pharmacol* **35**: 2435–2442, 1986.
14. Light III WR and Olson JS, Transmembrane movement of heme. *J Biol Chem* **265**: 15623–15631, 1990.
15. Ginsburg H and Stein WD, Kinetic modelling of chloroquine uptake by malaria-infected erythrocytes: assessment of the factors that may determine drug resistance. *Biochem Pharmacol* **41**: 1463–1470, 1991.
16. Geary TG and Jensen JB, Lack of cross-resistance to 4-aminoquinolines in chloroquine-resistant *Plasmodium falciparum in vitro*. *J Parasitol* **69**: 97–105, 1983.
17. Oduola AMJ, Weatherly NF, Bowdre JH and Desjardins RE, *Plasmodium falciparum*: cloning by single-erythrocyte micromanipulation and heterogeneity *in vitro*. *Exp Parasitol* **66**: 86–95, 1988.
18. Cowman AF and Foote SJ, Chemotherapy and drug resistance in malaria. *Int J Parasitol* **20**: 503–513, 1990.
19. Geary TG, Jensen JB and Ginsburg H, Uptake of [^{3}H]-chloroquine by drug-sensitive and -resistant strains of the human malarial parasite *Plasmodium falciparum*. *Biochem Pharmacol* **35**: 3805–3812, 1986.
20. Cannon JB, Kuo F-S, Pasternack RF, Wong NM and Mueller-Eberhard U, Kinetics of interaction of hemin liposomes heme binding proteins. *Biochemistry* **21**: 3715–3721, 1985.
21. Rose MY, Thompson RA, Light WR and Olson JS, Heme transfer between phospholipid membranes and uptake by apohemoglobin. *J Biol Chem* **260**: 6632–6640, 1985.
22. Lambros C and Vanderberg JP, Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**: 418–420, 1979.
23. Kutner S, Breuer WV, Ginsburg H, Aley SB and Cabantchik ZI, Characterization of permeation pathways in the plasma membrane of human erythrocytes infected with early stages of *Plasmodium falciparum*: association with parasite development. *J Cell Physiol* **125**: 521–527, 1985.
24. Desjardins RS, Canfield CJ, Haynes JD and Chulay JD, Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**: 710–718, 1979.
25. Yavin E and Zutra A, Separation and analysis of ^{32}P-labeled phospholipids by a simple and rapid thin-layer chromatographic procedure and its application to cultured neuroblastoma cells. *Anal Biochem* **80**: 430–437, 1977.
26. Svanborg A, Svenerholm L, Myren I, Soomagi MR, Claesson B and Rohman S, Plasma total lipid, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. *Acta Med Scand* **169**: 43–61, 1961.
27. Abell LL, Levy BB, Brodie BB and Kendall FE, A simplified method for the estimation of the total cholesterol in serum and demonstration of its specificity. *J Biol Chem* **195**: 1357–1366, 1952.
28. Van Deenen LLM and De Gier J, Lipids of the red cell membrane. In: *The Red Blood Cell* (Ed. Surgenor D), pp. 147–211. Academic Press, New York, 1975.
29. Holz GG, Lipids and the malarial parasite. *Bull WHO* **55**: 237–248, 1977.
30. Vial HJ, Ancelin M-L, Philippot JR and Thuet MJ, Biosynthesis and dynamics of lipids in *Plasmodium*-infected mature mammalian erythrocytes. *Blood Cells* **16**: 531–555, 1990.
31. Vial HJ and Ancelin M-L, Malarial lipids, an overview. In: *Subcellular Biochemistry Vol. 18 Intracellular Parasites* (Eds. Avila JL and Harris JR), pp. 259–306. Plenum Press, New York, 1992.
32. Hsiao LL, Howard RJ, Aikawa M and Taraschi TF, Modification of host cell membrane lipid composition by the intra-erythrocytic human malaria parasite *Plasmodium falciparum*. *Biochem J* **274**: 121–132, 1991.
33. Vial HJ, Thuet MJ and Philippot JR, Phospholipid biosynthesis in synchronous *Plasmodium falciparum* cultures. *J Protozool* **29**: 258–262, 1982.
34. Geary TG, Divo AA, Jensen JB, Zangwill M and Ginsburg H, Kinetic modelling of the response of *Plasmodium falciparum* to chloroquine and its experimental testing *in vitro*. *Biochem Pharmacol* **40**: 685–691, 1990.
35. Van der Schaft PH, Beaumelle B, Vial HJ, Roelofsen B, Op den Kamp J and Van Deenen L, Phospholipid organization in monkey erythrocytes upon *Plasmodium knowlesi* infection. *Biochim Biophys Acta* **901**: 1–14, 1987.
36. Moll GN, Vial HJ, Ancelin ML, Op den Kamp JAF, Roelofsen B and van Deenen LLM, Phospholipid uptake by *Plasmodium knowlesi* infected erythrocytes. *FEBS Lett* **232**: 341–346, 1988.
37. Op den Kamp JA, Lipid asymmetry in membranes. *Annu Rev Biochem* **48**: 47–71, 1979.
38. Arduini A, Stern A, Storto S, Belfiglio M, Mancinelli G, Scurti R and Federici G, Effect of oxidative stress on membrane phospholipid and protein organization in human erythrocytes. *Arch Biochem Biophys* **273**: 112–120, 1989.
39. Gupta CM and Mishra G, Transbilayer phospholipid asymmetry in *Plasmodium knowlesi*-infected host cell membrane. *Science* **23**: 1201–1209, 1981.
40. Zidovetzki R and Sherman IW, Lipid composition of the membranes of malaria-infected erythrocytes and the role of drug-lipid interactions in the mechanism of action of chloroquine and other antimalarials. In: *Biochemical Protozoology* (Eds. Coombs G and North M), pp. 336–348. Taylor and Francis, London, 1991.
41. Yeagle PL, Cholesterol and the cell membrane. *Biochim Biophys Acta* **822**: 267–287, 1985.
42. Ginsburg H, Landau I and Baccam D, Effect of cholesterol-rich diet on the susceptibility of rodent malarial parasites to chloroquine chemotherapy. *Life Sci* **42**: 7–10, 1987.
43. Matsuzawa Y and Hostetler KY, Inhibition of lysosomal phospholipase A and phospholipase C by chloroquine and 4,4′-bis(diethylaminoethoxy)*a*,*b*-diethylphenylethane. *J Biol Chem* **255**: 5190–5194, 1980.
44. Naor Z and Catt KJ, Mechanism of action of gonadotropin-releasing hormone. *J Biol Chem* **250**: 2226–2269, 1981.
45. Schwarting H, Seiler KU and Wasserman O, Mechanism of drug-induced phospholipidosis. *Naunyn Schmiedbergs Arch Pharmacol* **293**: R57, 1976.
46. Seydel JK and Wasserman O, NMR-studies on the molecular basis of drug-induced phospholipidosis—II. Interactions between several amphiphilic drugs and phospholipids. *Biochem Pharmacol* **25**: 2357–2364, 1976.
47. Lullmann H, Plosch H and Ziegler A, Ca replacement by cationic amphiphilic drugs from lipid monolayers. *Biochem Pharmacol* **29**: 2969–2974, 1980.
48. Zidovetzki R, Sherman IW, Maguire PA and De Boeck H, A nuclear magnetic resonance study of the

interactions of the antimalarials chloroquine, quinacrine, quinine and mefloquine with lipids extracted from normal human erythrocytes. *Mol Biochem Parasitol* **38**: 33–40, 1990.
49. Harder A, Kovatchev S and De Buch H, Interaction of chloroquine with different glycerophospholipids. *Hoppe Seylers Z Physiol Chem* **361**: 1847–1850, 1980.
50. Chevli R and Fitch CD, The antimalarial drug mefloquine binds to membrane phospholipids. *Antimicrob Agents Chemother* **21**: 581–586, 1982.
51. Ginsburg H and Demal RA, The effect of ferriprotoporphyrin IX and chloroquine on phospholipid monolayers: Possible implications to antimalarial activity. *Biochim Biophys Acta* **732**: 316–319, 1983.
52. Ginsburg H and Demel RA, Interactions of hemin, antimalarial drugs and hemin-antimalarial complexes with phospholipid monolayers. *Chem Phys Lipids* **35**: 331–347, 1984.
53. Pappu A and Hostetler KY, Effect of cationic amphiphilic drugs on the hydrolysis of acid and neutral phospholipids by liver lysosomal phospholipase A. *Biochem Pharmacol* **33**: 1639–1644, 1984.
54. Krugliak M and Ginsburg H, Studies on the antimalarial mode of action of quinoline-containing drugs: time-dependence and irreversibility of drug action, and interactions with compounds that alter the function of the parasite's food vacuole. *Life Sci* **49**: 1213–1219, 1991.