Chapter 2

Lysosomal Membrane Stabilizing Effect of Antimalarials - An In Vitro Study

Introduction

Malaria is the world's most devastating human parasitic infection causing about 2-5 million deaths per year [1]. After an initial drop in the death incidence in 1960s again an increase is observed, with a parallel increase in the production and consumption of the antimalarials [1]. The major market share of antimalarials is of chloroquine, quinine and primaquine.

Chloroquine is one of the highest consumed drugs It is not only used for treatment but also for prophylaxis and prevention of malaria. Beside, chloroquine is also used for the treatment of rheumetoid arthritis, discoid lupus erythematous and higher dosages are taken in porphyria cutanea tarda, solar urticaria and polymorphous light eruption [1]. However due to the increasing resistance to chloroquine in plasmodium, the cinchona alkaloid quinine is currently receiving more attention as a therapeutic drug [2].

Toxicity of quinine with respect to central nervous system (CNS) is well recognized [3] and when given by intravenous route, quinine is reported to result in high mortality rate [4]

Primaquine is the only tissue schizontocide and hence is the drug of choice, although its hydrogen peroxide producing property is the main reason of anemia in black population [5].

The mechanism of action of these antimalarials in general is less clearly understood, although various explanations have been proposed for their antimalarial action as well as their effects on the biomolecules [1]. Inhibition of enzymes of antioxidant defense

mechanism [6,7]and of mitochondrial respiration, cytochrome oxidase and several dehydrogenases by antimalarials have been demonstrated [8]. Antimalarial actions in terms of the protozoan enzyme inhibition and DNA integration are found true even in the mammalian system [9]. Chloroquine accumulates in very high amounts in the food vacuoles of plasmodium as well and this may show antilysosomal effect in the parasite itself. It has been reported that when given in very high dose, chloroquine accumulates in very high amounts in lysosomes and causes their rupture [10]. However, the effects of the antimalarials in the concentration ranges nearing the plasma levels after giving therapeutic doses are not known.

It would therefore be of interest to find out the effect of the different antimalarials in concentrations simulating therapeutic plasma concentrations, on the lysosomal membrane. With this objective, the effects of the three antimalarials i.e. quinine, primaquine and chloroquine on the lysosomal membrane integrity and stability were evaluated For these studies acid phosphatase was used as the marker enzyme [11]. Studies were performed with the synthetic substrates i.e PNPP and βGP as well as with the natural substrates ATP, AMP and FDP. The results of these studies are summarized below

Materials and Methods

Chemicals

ATP, AMP, FDP, PNPP and βGP were purchased from Sigma, U.S.A All other reagents used were of analytical-reagent grade and were purchased locally.

Assay Procedures

Adult male albino rats of Charles-Foster strain weighing between 200-250 g were used. The animals were killed by decapitation and the liver was excised, washed with 0.25 M sucrose, and kept in a beaker containing chilled (0-4 °C) 0.25 M sucrose solution. The tissue was minced, washed repeatedly to free from last traces of blood and then homogenized using Potter Elvehjam type glass-teflon homogenizer to obtain 10% (w/v) homogenate. The homogenates were prepared either in 0.25 M sucrose or in 10 mM NaCl solution. The homogenates were then centrifuged at 650Xg for 10 min. to remove the nuclei and cell debris. The supernatant was used as the source of the enzyme. All operations were carried out at 0-4 °C.

Measurements of the acid phosphatase activity were carried out essentially according to the procedure described earlier [12] in an assay system (total volume 0.4 ml) containing 0 2 M sodium acetate buffer pH 5.0. 0.1 ml of nuclei-free homogenate was incubated in the buffer for 2-3 minutes and the reaction was started by the addition of the substrate. The concentration of ATP, FDP, βGP and PNPP was 10mM while that of AMP was 1 mM. In separate experiments it was established that these are the optimum concentrations for the given substrates [13]. After addition of the substrate the reaction was carried out at 37°C for 10 min. With ATP, FDP, βGP and AMP as substrates the reaction was terminated with 0.1 ml of 5%(w/v) SDS and the liberated inorganic phosphate was measured by the method of Fiske and Subba Row [14]. When PNPP was the substrate, the reaction was terminated with 2.8 ml of 0.25N NaOH, and the color was read at 420 nm [15].

The assays were performed under three experimental conditions.

First, the source of the enzyme was 10 % nuclei-free homogenate prepared in 0.25 M sucrose. The results obtained with this system reported the "free" enzyme [11, 16, 17] . It has been shown previously that under this assay conditions the lysosomal membranes are stable for 10 min, and the " free" activity is minimum. Incubations beyond this time results in partial release of the enzyme activity. [11, 16, 17]

In the second set of experiments, the above enzyme system was pre-incubated at 37°C for 10 min, with or without added antimalarials prior to starting the reaction.
In the third set of experiments homogenate prepared in 10 mM NaCl was the source of the enzyme, it is expected that the hypotonic lysis of the lysosomes will result in releasing the "total" activity.

For ATP, AMP, βGP and FDP as the substrates, the activity is expressed as μmoles of Pi liberated / 10 min /mg protein while for PNPP as the substrate, the activity is expressed as μmoles of PNP formed/ 10 min./mg protein.

The three antimalarials quinine, primaquine and chloroquine were used in the concentration range of 125 nM to 125 μM (e.g. see Figs 1-5)

Results are given as mean ± SEM of six independent observations. Statistical evaluation of the data was by Students' t-test.

Protein estimation was according to the method of Lowry *et al.*[18] with bovine serum albumin used as the standard.

Results and Discussion

The activities of the lysosomal acid phosphatase with different substrates –synthetic (PNPP and βGP) and physiological (ATP, AMP and FDP)– under isotonic and hypotonic conditions are given in Table 1. The effects of pre-incubating the isotonic homogenates at 37 °C for 10 min. are also included. It is apparent that depending on the substrate used the results fall in two different patterns.

Thus it can be noted that the pattern of activity is similar for ATP and PNPP as substrates.

With ATP as the substrate the activity is comparable under isotonic and hypotonic conditions ; with pre-incubation at 37 °C also the activity remains the same. This is not really surprising, because the lysosomal ATPase is a V type ATPase [19] which has reversed orientation in the membranes as compared to the orientation of mitochondrial FoF1 ATPase [19] Thus in the case of the lysosomal enzyme the active site is facing the cytosol in which ATP is present under in situ conditions [20] and the substrate is thus always accessible even under isotonic conditions. The enzyme generates H^+ by hydrolyzing ATP and pumps the H^+ into lysosomes thus maintaining the acidic intra-lysosomal environment [21]. Since the active site of the enzyme is already facing the cytosol, it is to be expected that the hypotonic conditions or pre-incubation at 37°C for 10 min. would not result in any further exposing of the enzyme activity.

Table 1. The effect of experimental procedure on acid phosphatase activity using different substrates.

Substrate	Acid phosphatase activity (μmoles/ min/ mg protein)		
	Isotonic	Hypotonic	Isotonic with 10 min. pre-incubation
ATP	1.01 ± 0.093	1.08 ± 0.095^{NS}	1.15 ± 0.15 ^{NS}
PNPP	3.25 ± 0.258	3.05 ± 0.759^{NS}	2.01 ± 0.129****
βGP	N.D	1.31 ± 0.320 ****	0.50 ± 0.058 ****
AMP	N.D	0.39 ± 0.040 ****	0.39 ± 0.037****
FDP	0.37 ± 0.077	1.42 ± 0.180 ****	0.60 ± 0.074 **

The activities were measured in the absence of antimalarials. For ATP, AMP, βGP and FDP as the substrates, the activity is expressed as μ moles of Pi liberated/10 min/mg protein while that for PNPP as the substrate, the activity is expressed as μ moles of PNP formed/10 min/mg protein.

Results are given as mean ± SEM of 6 indipendent observations.

N.D. Not detectable. NS Not significant; * p> 0.01,** p> 0.05, *** p> 0.002, **** p> 0 001

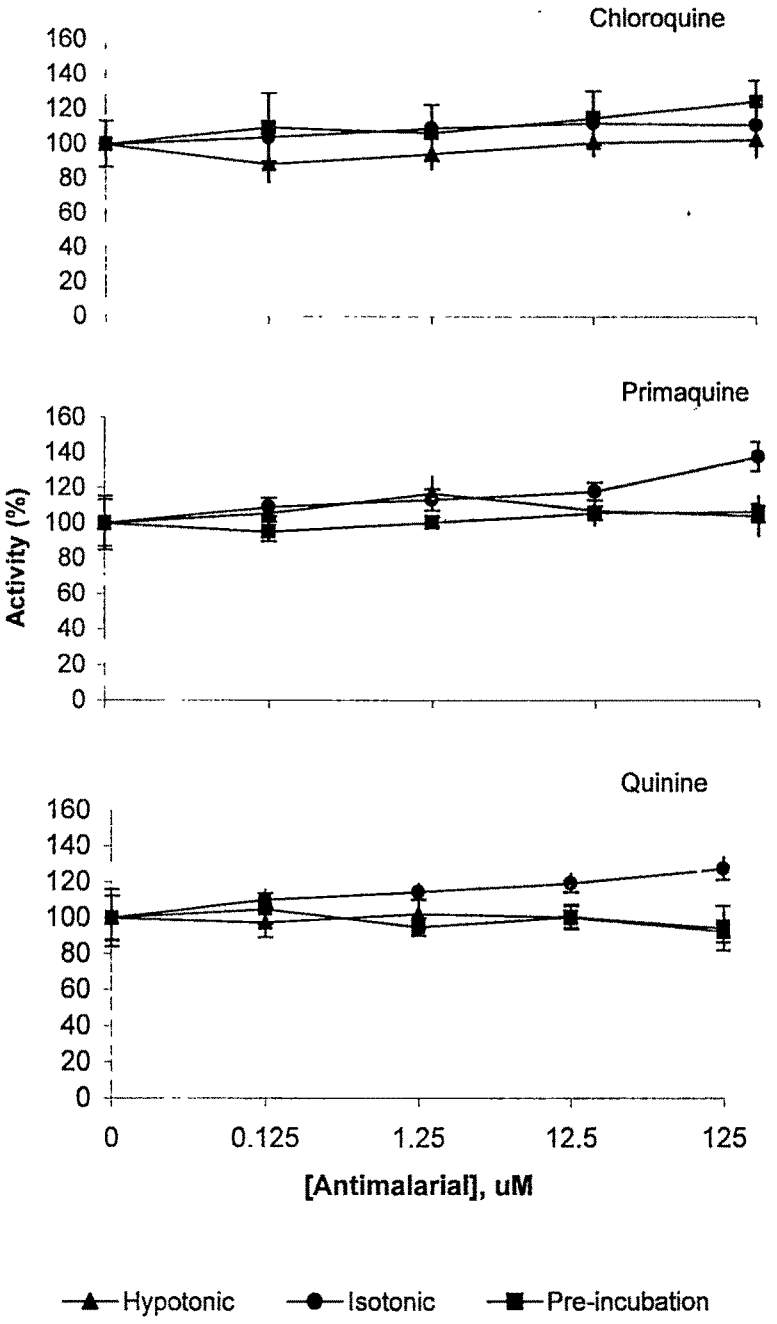
With PNPP as the substrate maximum activity is seen under hypotonic and isotonic conditions and pre-incubation at 37 °C for 10 min resulted in about 40 % loss in activity. PNPP is a synthetic, non-specific substrate and is believed to be hydrolyzed not only by acid phosphatases but also by acid phosphatases from other subcelluler origins [15]. That the enzyme activity was highest in the isotonic medium would suggest that this synthetic substrate is freely accessible under isotonic conditions. Loss of activity under pre-incubation conditions would suggest that some of the other phosphatases may be inactivated by pre-incubation; for eg. glucose-6-phosphatase is known to be inactivated by pre-incubation [15].

The pattern of activity for βGP, AMP and FDP falls in the second group. Thus for βGP and AMP as the substrates the activity was not detectable under isotonic conditions and was fully exposed under hypotonic conditions. Pre-incubation of the isotonic enzyme at 37°C resulted in partial (40%) exposure of βGP hydrolyzing activity whereas AMP hydrolyzing activity was fully exposed under pre-incubation conditions. For FDP the activity was low under isotonic condition and it increased by 4 fold and 1.7 fold respectively in hypotonic samples and upon pre-incubation. Taken together the results suggest that under isotonic conditions the lysosomal membranes were quite intact; high ratio of total / free activity is taken as the index of the lysosomal membrane integrity [11, 16, 17] It is obvious from the data presented for the three substrates βGP, AMP and FDP that this ratio ranged from 4 to infinity.

In the next set of experiments the effect of different antimalarials on hydrolysis of various substrates was evaluated. It can be noted that none of the antimalarial had any significant effect on ATP hydrolysis under isotonic, hypotonic or pre-incubation conditions. (Fig. 1).

Fig 1 Effect of antimalarials on acid phosphatase activity with ATP as the substrate. % Activity is plotted against concentration of the antimalarials: chloroquine, primaquine and quinine Activity in the absence of antimalarials for three different conditions i.e. isotonic, hypotonic and pre-incubation (Table 1) is taken as control and considered as 100%. Each data point represents mean of six independent experiments ± SEM. The error bars represent the SEM.

Fig. 1


Chloroquine
Primaquine
Quinine
Activity (%)
160
140
120
100
80
60
40
20
0
0
0.125
1.25
12.5
125
[Antimalarial], uM
Hypotonic
Isotonic
Pre-incubation

More or less similar trend was seen also for PNPP hydrolysis (Fig. 2). This is consistent with the notion that ATP and PNPP are readily accessible to the enzyme action (eg. *vide infra*).

βGlycerophosphatase activity (Fig.3) was not inhibited to any significant extent by any of the three antimalarials under hypotonic condition where the enzyme activity is fully exposed. This would suggest that the antimalarials do not inhibit the enzyme activity *per se*. When the assay tubes were pre-incubated, chloroquine caused a progressive concentration-dependent-decrease in the activity. Primaquine also decreased the activity but the effect was seen only at the highest concentration employed i.e 125 μM. Quinine had no effect. Since the antimalarials did not inhibit the enzyme activity directly (as is evident from hypotonic experiments) it may be suggested that chloroquine and primaquine may stabilize the lysosomal membrane thereby preventing the release of the enzyme from within the lysosomes to the exterior and/or preventing the access of the substrate to the enzyme and hence there is an apparent decrease rather than inhibition of the activity (Fig 3).

For AMP as the substrate chloroquine and primaquine once again did not directly inhibit the enzyme activity i e under hypotonic condition, whereas quinine at highest concentration inhibited AMP hydrolysis to an appreciable extent. Pre-incubation experiments revealed that only primaquine at all the concentrations used prevented the substrate accessibility thereby resulting in apparent decrease in the hydrolytic activity (Fig. 4).

Fig. 2. Effect of antimalarials on acid phosphatase activity with PNPP as the substrate. % Activity is plotted against concentration of the antimalarials: chloroquine, primaquine and quinine Activity in the absence of antimalarials for three different conditions i.e. isotonic, hypotonic and pre-incubation (Table 1) is taken as control and considered as 100%. Each data point represents mean of six independent experiments ± SEM. The error bars represent the SEM.

Fig. 2

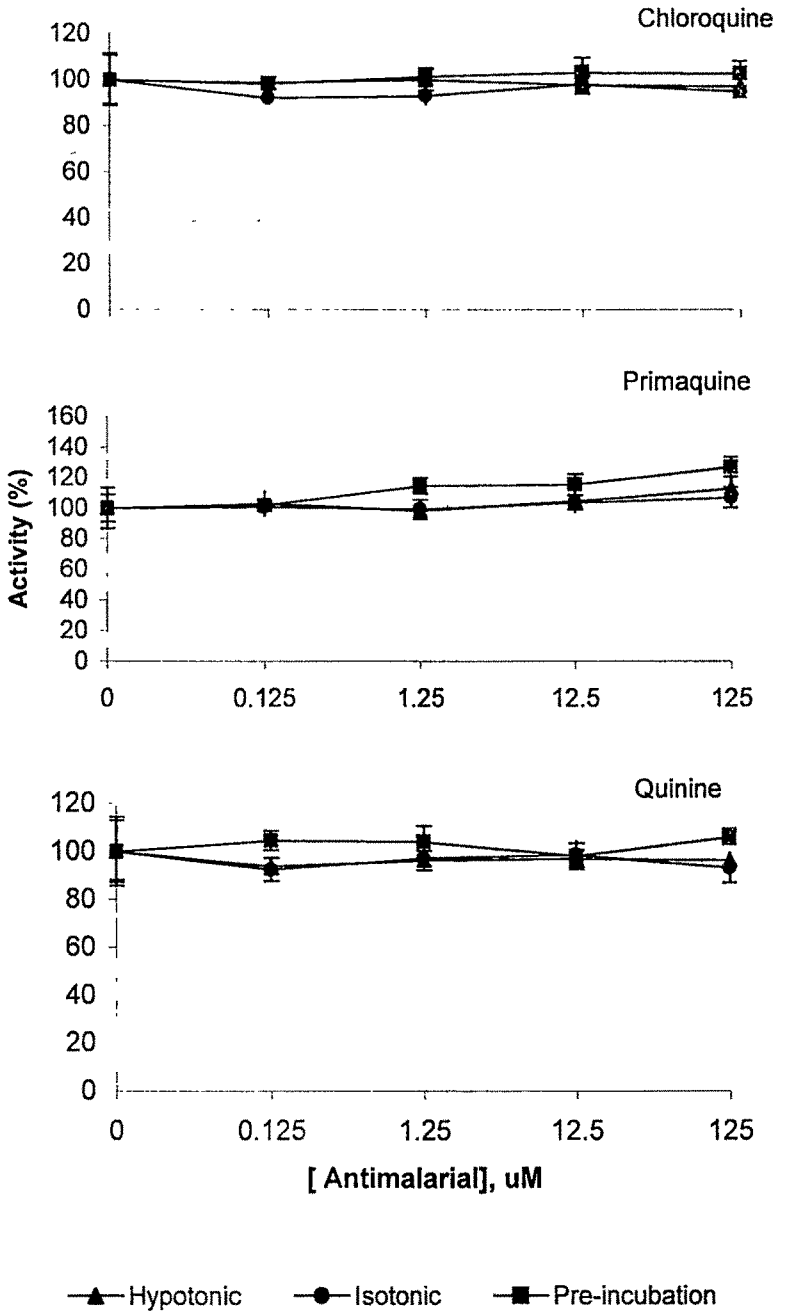

Chloroquine
120
100
80
60
40
20
0
Primaquine
160
140
120
100
80
60
40
20
0
Activity (%)
0
0.125
1.25
12.5
125
Quinine
120
100
80
60
40
20
0
0
0.125
1.25
12.5
125
[Antimalarial], uM
Hypotonic
Isotonic
Pre-incubation

Fig. 3. Effect of antimalarials on acid phosphatase activity with βGP as the substrate. % Activity is plotted against concentration of the antimalarials. chloroquine, primaquine and quinine. Activity in the absence of antimalarials for three different conditions i.e. isotonic, hypotonic and pre-incubation (Table 1) is taken as control and considered as 100%. Each data point represents mean of six independent experiments ± SEM. The error bars represent the SEM

Fig. 3

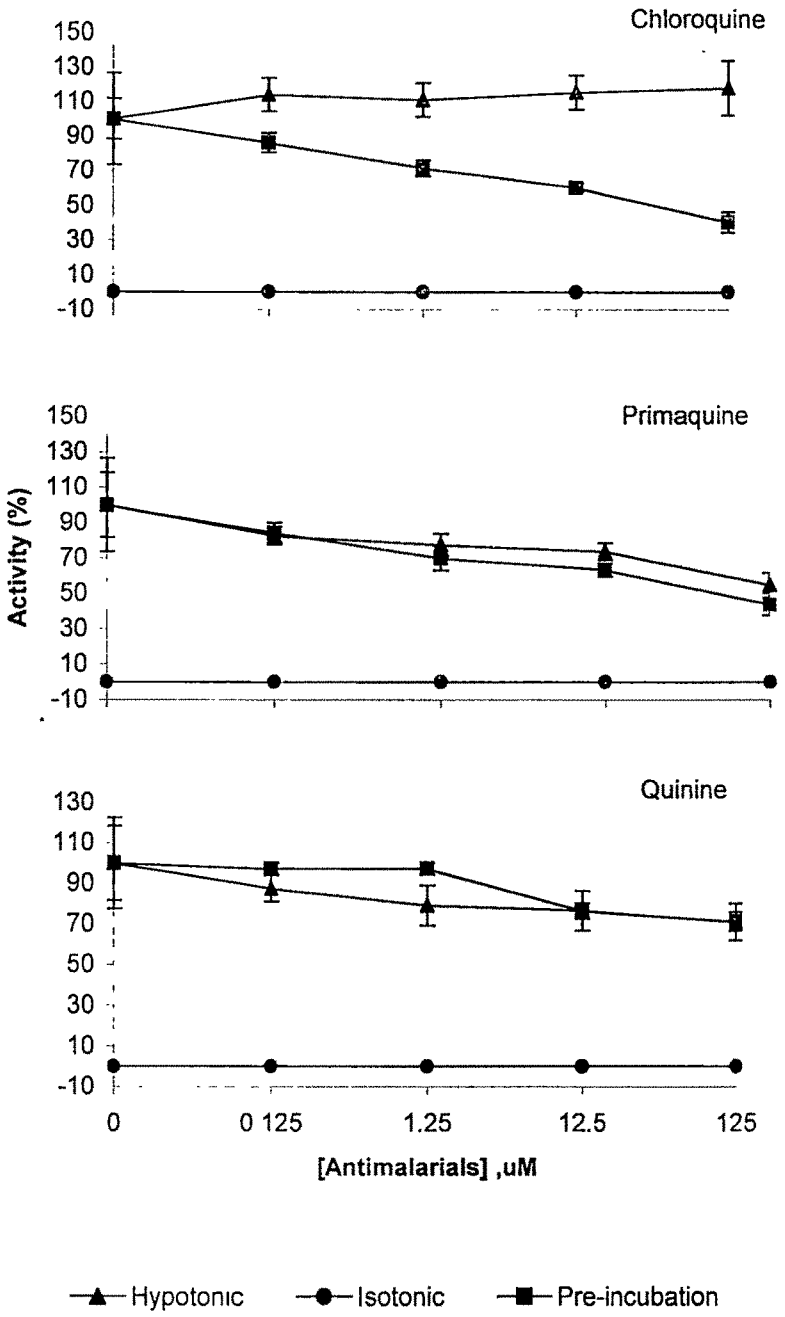
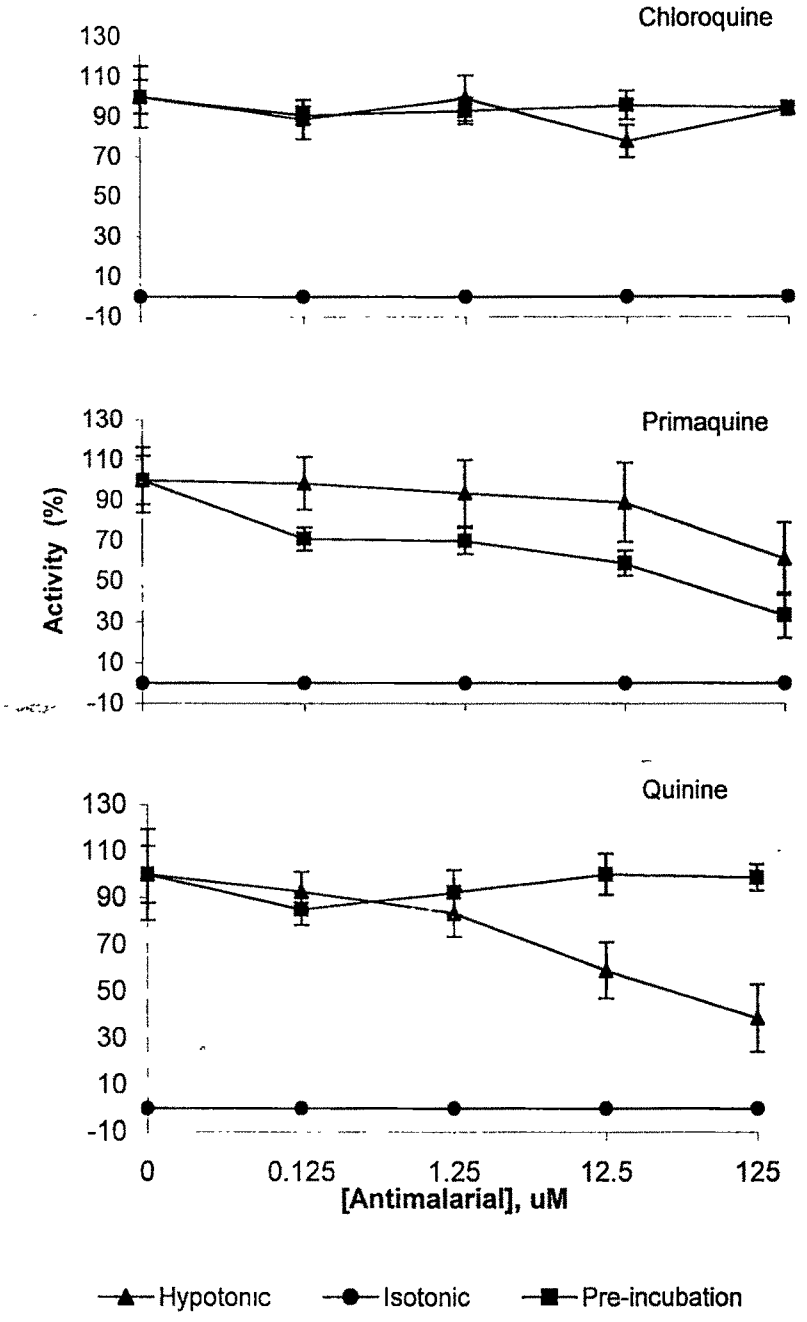

Chloroquine
Primaquine
Quinine
Activity (%)
150
130
110
90
70
50
30
10
-10
0
0 125
1.25
12.5
125
[Antimalarials] ,uM
Hypotonic
Isotonic
Pre-incubation

Fig. 4. Effect of antimalarials on acid phosphatase activity with AMP as the substrate. % Activity is plotted against concentration of the antimalarials: chloroquine, primaquine and quinine. Activity in the absence of antimalarials for three different conditions i.e. isotonic, hypotonic and pre-incubation (Table 1) is taken as control and considered as 100% Each data point represents mean of six independent experiments ± SEM. The error bars represent the SEM.

Fig. 4


Chloroquine
Primaquine
Quinine
Activity (%)
130
110
90
70
50
30
10
-10
0
0.125
1.25
12.5
125
[Antimalarial], uM
Hypotonic
Isotonic
Pre-incubation

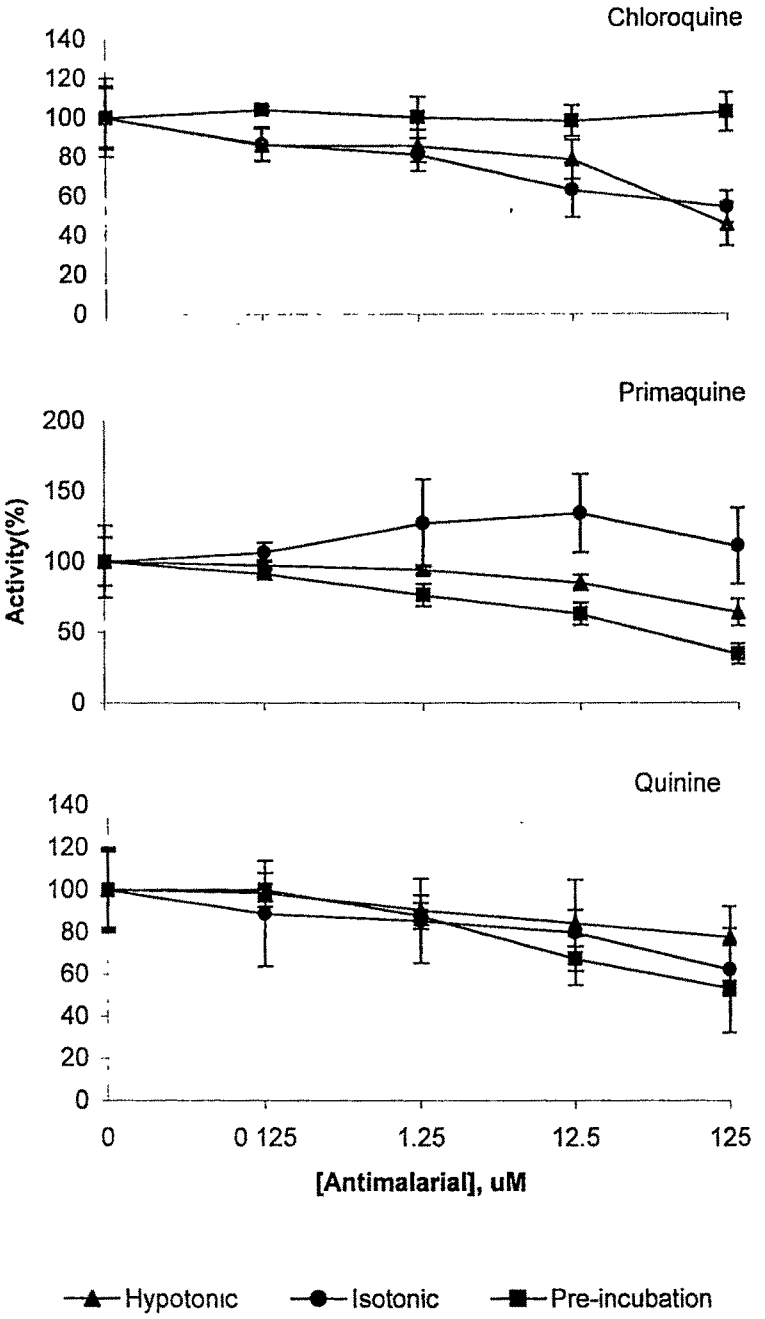
With FDP as the substrate (Fig. 5), incubation with any of the antimalarials under isotonic condition had no effect. Under hypotonic condition the enzyme activity was not inhibited by any of the antimalarials except for the highest concentration of chloroquine. Pre-incubation with antimalarials also had marginal effect except in the case of highest concentration of primaquine.

Taken together (Fig.1-5) the results would suggest that the three antimalarials stabilize the lysosomal membrane and have minimal inhibitory effect on substrate hydrolysis.

The physiological role of lysosomes in degradation of intra- as well as extracellular biological macromolecules or invading microorganisms is well recognised [20]. The lysosomal membrane has got 16-25 protein molecules which functions as the receptors for recognizing the molecules to be degraded [22]. Steps in degradation involve fusion of lysosomes with phagosomes thus forming phagolysosomes [20] Reports in the literature indicate that chloroquine is lysosomotropic and the concentration of chloroquine in the lysosomes can be 1000 times higher than that in the plasma [1]. Other antimalarials, which are also basic quinoline compound can also accumulate in the lysosomes, although to a lesser extent compared with chloroquine [1]. Chloroquine which is not only used for the treatment of malaria but also as a prophylactic agent is known to have a considerably longer half-life ($t1/2 \approx$ 9-41 days) and a considerably larger volume of distribution (120-180 L/Kg) [1], which is consistent with its accumulation within the lysosomes, referred to above [1] This would suggest that when used for treatment or for prophylaxis the drug will remain accumulated in the liver lysosomes for considerably longer periods.

Fig. 5. Effect of antimalarials on acid phosphatase activity with FDP as the substrate. % Activity is plotted against concentration of the antimalarials: chloroquine, primaquine and quinine Activity in the absence of antimalarials for three different conditions i.e. isotonic, hypotonic and pre-incubation (Table 1) is taken as control and considered as 100%. Each data point represents mean of six independent experiments ± SEM. The error bars represent the SEM.

Fig. 5


Chloroquine
140
120
100
80
60
40
20
0
Primaquine
200
150
100
50
0
Activity(%)
Quinine
140
120
100
80
60
40
20
0
0
0 125
1.25
12.5
125
[Antimalarial], uM
Hypotonic
Isotonic
Pre-incubation

Results of the present study suggest that the antimalarials in concentration range nearing those reached under therapeutic conditions stabilize the lysosomal membrane rather than causing rapture, which is noted for toxic doses [10].

Physiologically this would mean that the stabilized membrane might not be able to function efficiently towards phagolysosome formation, suggesting that long-term use of antimalarials can cause lysosomal dysfunction in liver. In this context it is of interest to note that the acid vacuoles of plasmodia also accumulate the antimalarials [1]. It is possible that lysosomal dysfunction in the plasmodia can be a possible mode of action of the antimalarials. However, such a postulate needs to be verified by carrying out more direct in vivo experiments Interesting to note in this context is the observation that the antimalarial chloroquine has been shown to stabilize the lysosomal membrane in the pancreas [23].

References

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Summary

The effect of three antimalarials chloroquine, primaquine and quinine on lysosomal enzymes and membrane integrity were examined Acid phosphatase was used as the marker enzyme and the effects were studied by employing physiological and synthetic substrates. Assays were performed using intact lysosomes, and after hypotonic lysis, in the presence of varying concentrations of the three antimalarials. The results indicate that the antimalarials do not inhibit the enzyme activity directly but they restricted the permeability and/or accessibility of the substrates The results thus suggest that antimalarials may stabilize the lysosomal membrane. Physiological significance of these finding is discussed.