SYNOPSIS

Malaria is the most devastating disease worldwide and about 300-500 million cases of malaria are reported every year. The disease is caused by the parasite *Plasmodium*. *Plasmodium* completes its life cycle in mosquitoes and humans. In humans it resides in liver (as merozoites) or in erythrocytes (as trophozoites). A variety of antimalarial drugs are being used for the treatment of malaria; chloroquine (CQ) and primaquine (PQ) are the two major drugs They have the same basic structure of quinoline but differ with respect to substitution groups. Also their site of action differs. Quinine (Q) is the oldest known drug. The use of Q has now increased due to appearance of CQ resistant strains

It has been reported that these drugs accumulate in the host tissue i.e. liver and erythrocytes. This can be quite alarming since long-term usage of antimalarials is advised for prophylaxis and treatment. This results in higher tissue concentration of the antimalarials especially in the liver in comparison to the circulating plasma concentrations. It is possible that the accumulation of antimalarial drugs in tissue such as liver and in erythrocytes can have adverse effects on the host tissue metabolism itself. The present thesis addresses this question by evaluating the effects of these three antimalarials on the function of subcellular organelles from liver The effects on lysosomal and mitochondrial functions have been examined in rat liver. In

parallel studies the effects on erythrocyte functions have been evaluated in human erythrocytes.

Chapter 1 of the thesis embodies "Introduction" which gives a general overview of the literature on malaria, types of malaria, epidemiology of malaria and the lifecycle of the *Plasmodium*. A general overview of the recent developments is also included. A large number of antimalarials used for treatment of malaria effect different developmental stages of *Plasmodium* in liver and erythrocytes. The pharmacological properties, reported mode of action, toxicity and biochemical effects etc of the three major drugs i.e. CQ, PQ and Q are described in details in the "Introduction" chapter Since the studies were carried out on lysosomes, mitochondria and erythrocytes. a brief account of these subcellular organelles and erythrocytes. and of structure-function-relationship is included. The reported effects of the three antimalarials on these cells and subcellular organelles are also described

The antimalarials are known to accumulate in very high concentrations in the lysosomes It is possible that when the antimalarials are given for treatment the circulating levels of these by themselves can effect the lysosomal functions To ascertain this possibility the effects of incubation of lysosomes in vitro with varying concentrations of CQ, PQ and Q simulating

plasma concentration ranges were examined. These results are described in **Chapter 2** of the thesis. For these studies acid phosphatase was used as the lysosomal marker enzyme. The enzyme activity was monitored with various phosphate esters such as ATP, 4-nitro phenylphosphate (PNPP), glycerol 2 phosphate (BGP), AMP and fructose 1,6-bis phosphate (FDP). ATP and PNPP were hydrolyzed under both isotonic and hypotonic conditions indicating that there is no permeability barrier for these substrates whereas BGP and AMP were hydrolyzed only under hypotonic condition. FDP was a permeable substrate. Pre-incubation with antimalarials for 10 min resulted in partial exposure of the BGP and FDP hydrolyzing activity whereas the AMP hydrolyzing activity was fully exposed. Pre-incubation with antimalarials did not affect the hydrolysis of ATP and PNPP under any of the osmotic conditions. BGPase activity was not inhibited by any of the antimalarials under hypotonic condition but pre-incubation with CQ and PQ under isotonic condition caused a progressive concentration-dependent-decrease. Similar effect of PQ was seen with AMP as substrate. None of the antimalarials affected FDP hydrolysis under isotonic condition except for a slight inhibition by CQ at highest concentration. In conclusions, the results suggest that the three antimalarials have minimum inhibitory effect on substrate hydrolysis and that CQ and PQ stabilized the lysosomal membranes

In the next set of experiments the effects of in vivo treatment with antimalarials on lysosomal functions were examined. These results are summarized in **Chapter 3** of the thesis. Treatment with CQ for 7 or 14 days resulted in a significant decrease in the body weight, liver weight and relative liver weights. PQ and Q treatment had no effect on these parameters but the total protein content of liver decreased in both the cases after 14 day treatment. Treatment with CQ showed progressive increase in free acid phosphatase activity whereas the increase in the total activity was comparatively more in the 7 day treatment group. Cathepsin D activities (both free and total) increased in the 7 day group but were comparable to the controls in the 14 day group. RNase activity also increased with time, with the extent of increase being higher in the total activity. Thus the ratio of total/ free activity (T/F ratio) also increased. Induction of free DNase activity was seen following 7 day treatment but the total activity was elevated almost to the same extent in both 7 and 14 day groups.

PQ treatment resulted in decrease in the free acid phosphatase activity; total activity decreased only after 14 day treatment. Free Cathepsin D activity decreased drastically in 7 day group. Both free and total activities increased in the 14 day group. No change in the RNase activity was seen except for a significant decrease in the PQ 14 day group. PQ also induced the DNase

activity but the extent of induction was much less compared to that seen with CQ treatment.

The free acid phosphatase activity decreased following Q treatment whereas the total activities were high especially in the 2-week group. Free Cathepsin D activity decreased in 14 day group while the total activity increased substantially in the 1 week group. The RNase activity was generally high except for the activity in 1-week group. The DNase activity was substantially elevated following Q treatment.

Taken together the results suggest that short-term exposure tends to stabilize the lysosomal membrane. However, long-term treatment, which results in accumulation of antimalarials in tissue, can cause time-dependent changes in the lysosomal hydrolases which can lead to tissue damage.

In the next series of experiment the effect of antimalarial treatment on mitochondrial functions were examined. **Chapter 4** summarizes these results. Following CQ treatment an overall 60-75% decrease in state 3 and state 4 respiration rates was noted using glutamate as the substrate. The ADP/O ratio decreases by 27 % in both the treatment groups. The ADP phosphorylation rates decreased by 83 and 72 % Similar trend was observed even with pyruvate + malate as the substrate pair, except that the extent of

inhibition was somewhat higher. The extent of inhibition of state 3 and state 4 respiration rates was in the range of 75 to 80 % when succinate was used as the substrate. However the extent of uncoupling was much higher (i.e. 65%). The overall effect was a decrease in ADP phosphorylation rate by about 95 %. With ascorbate + TMPD as the substrate the effect on state 3 and state 4 respiration was the least. The ADP/O ratios decreased to a greater extent due to which the ADP phosphorylation rates decreased by 80 - 93 %.

PQ treatment resulted in an overall 70 to 75 % inhibition of state 3 and state 4 respiration rates with glutamate as the substrate. The ADP/O ratios decreased to the extent of 20 % and ADP phosphorylation rates were lower by 80 %. With pyruvate + malate as the substrate the extent of inhibition of respiration rates was about 90 %. ADP phosphorylation rate was decreased by 95 % With succinate as the substrate, PQ treatment affected the respiration rates to a lesser extent (as was noted for CQ) and the effect was least with ascorbate + TMPD as electron donor system.

Treatment with Q showed that the state 3 and state 4 respiration rates were affected maximally with pyruvate + malate and succinate as the substrate; extent of inhibition with glutamate and ascorbate + TMPD as the substrates was lower. The pattern of uncoupling was similar to that seen for PQ. The

ADP phosphorylation rate decreased maximally with pyruvate + malate and succinate.

CQ treatment for 7 day caused a 38 % decrease in cytochrome aa_3 and cytochrome b contents. However 14 day treatment normalized the values. PQ treatment had resulted in 23 % decrease in cytochrome aa_3 content with a reciprocal 28 % increase in the cytochrome b content. Q treatment for 14 days resulted in a substantial decrease in cytochrome aa_3 content while cytochrome b content was slightly elevated (20 % increase). No appreciable changes were seen in cytochrome $c+c_1$ contents.

Since FoF1 ATPase (complex V) plays an important role in ATP synthesis, the effect of antimalarial treatment on mitochondrial ATPase were checked next and the results are summarized in **Chapter 5** of the thesis.

It was noted that the three antimalarials brought about an increase in the basal and Mg^{2+} - stimulated activities whereas the DNP stimulated activity was decreased. The total activity (i.e. DNP + Mg^{2-} activity) thus reflected the composite effect.

Substrate kinetics analysis have shown that mitochondrial ATPase can be resolved in three components. The three components have different Km and Vmax values. In view of the changes in the ATPase activities further studies were conducted to examine the effect of antimalarial treatment on substrate and temperature kinetics of the mitochondrial ATPase. Following CQ treatment it was observed that in 7 day treatment group the Vmax of the three components increased whereas Km of component I decreased and Km of component III increased. CQ treatment for 14 days resulted in loss of component III while the Vmax increased in component I and II.

PQ treatment also showed a similar effect. Component III was lost in 7 day treatment group and Vmax of components I and II increased. Though Km of component I decreased, Km of component II increased significantly. 14 day treatment with PQ resulted in change in the nature of substrate saturation curves The curves were sigmoidal. Q treatment caused an increase in Vmax of all the components in both 7 day and 14 day treatment groups. The Km was unchanged for component I and II and increase was observed for Km of component III.

Studies on temperature – dependence of ATPase activity revealed that the Arrhenius plots in the control group were biphasic with two energies of activation E_1 and E_2 and a phase transition temperature (Tt) of 14°C. These

values were in agreement with our previously reported results. Treatment with CQ for 7 days resulted in an increase in both E_1 and E_2 while Tt was unchanged. Interestingly 14 day treatment showed a chair shaped Arrhenius plots with three energies of activation and two phase transition temperatures. The corresponding energies of activation i.e. E_1 and E_3 were higher when compared to controls. Similarly Tt_1 was also higher than that in the controls. PQ treatment also showed a chair shaped Arrhenius plots and the pattern was similar to that of CQ 14 day group. 7 day treatment with Q showed a biphasic plot with increase in E2 and Tt. Interestingly, loss of phase transition was observed following 14 day treatment with Q.

The altered substrate and temperature kinetics following antimalarials treatment suggests that either the system is trying to compensate for the decreased respiratory activity by changing the kinetic properties of ATPase following antimalarial treatment or the antimalarial treatment alters the mitochondrial membrane structure-function relationship per se.

Hence further studies were carried out to study the effect of antimalarials on lipid composition of the mitochondria. These results are described in **Chapter 6** of the thesis. CQ treatment (both 7 and 14 day treatment) caused an increase in phosphotidylserine (PS), phosphotidylinositol (PI), phosphotidylethanolamine (PE) and diphosphotidylglycerol (DPG)

components while the phosphotidylcholine (PC) content was significantly decreased. The total phospholipid (TPL) was 16% higher in 7 day group while in 14 day group it was 28% higher. Cholesterol (CHL) content showed a progressive decrease resulting in an increase in the TPL/CHL molar ratio in both the treatment groups.

PQ treatment resulted in higher PS and PI contents while lysophosphatidic acid (Lyso) and sphingomyelin (SPM) increased only after prolonged treatment. PC was somewhat reduced but the change was statistically significant. TPL content increased by 38% in 7 day group but normalized in 14 day group. TPL/CHL molar ratio increased significantly in both the groups. Q treatment did not show any change in the phospholipid composition except for a slight increase in DPG contents in 7 day group.

The results taken together suggest that prolonged antimalarials treatment not only drastically impair mitochondrial energy metabolism but also results in significant alteration in cytochrome contents and phospholipid composition. The overall effect is altered membrane structure-function relationships.

As mentioned earlier, *Plasmodium* has part of its lifecycle in the erythrocytes Hence the effect of the three antimalarials on human erythrocyte functions were examined. Erythrocyte membrane

acetylcholinesterase (AChE) was used as the marker for these studies. The erythrocyte membranes were incubated with antimalarials and the effect on AChE activity was monitored. The results are summarized in **Chapter 7** of the thesis. It was observed that the three antimalarials were potent inhibitors of AChE. It was also noted that in order to obtain the required inhibition, the concentration of CQ and PQ required were much low then those of Q. Thus Q inhibited the enzyme to the extent of 33% at a concentration of about 3.2 mM whereas CQ and PQ were effective at about 22 and 40 μM concentration respectively. Similarly, 67 % inhibition could be obtained with 7.5 mM of Q whereas CQ and PQ were effective at 192 μM and 250 μM concentrations. Human erythrocyte membrane AChE activity can be resolved in two components differing in their kinetic properties. It was observed that both the components were inhibited by the three antimalarials. However, the effects were differential for the two components. The inhibition with all the antimalarials was of mixed type. The Ki for Q was in the range of 600 to 750 μM; Ki for CQ and PQ were in the range of 10-30 μM respectively; for each antimalarial the Ki was comparable for the two components

Erythrocyte AChE is a membrane-bound enzyme and the observed effects of antimalarials may result due to the effects on the enzyme per-se or may be

attributed to membrane effects. To ascertain this possibility, similar studies were performed on plasma butyrylcholinesterase (BChE). These results are summarized in **Chapter 8** of the thesis. BChE was also found to be inhibited by all antimalarials. Thus Q inhibited BChE by 33% at a concentration of 60 μM; for CQ and PQ also these concentration were comparable i.e. 40 and 50 μM respectively. Similarly, the enzyme activity was inhibited by 67 % at 500 μM concentration of Q; for CQ and PQ, the corresponding concentrations were 230 and 425 μM respectively. Kinetic analysis has shown that BChE can be resolved in three components differing in their kinetic properties. All the three components were inhibited by antimalarials and the inhibition was mixed type. The three antimalarials showed totally different values of Ki for the three components. It was found that for Q the Ki for the component I was about 13 μM and the value increased by 2 and 4 times for components II and III respectively. The pattern was similar for the other two antimalarials i.e. CQ and PQ. Compared to quinine the Ki values of CQ were about 1/3 for all the three components whereas for PQ the amount required is 1.5- 2.0 times higher than to that of Q.

Taken together, these results suggest that the antimalarials inhibit the cholinesterases per se The fact that higher concentrations of antimalarials were required for AChE inhibition suggests that the antimalarials also

accumulate in large quantities in the erythrocytes and are most possibly membrane-bound.

The results of the present studies thus suggest that antimalarials can adversely effect the functions of subcellular organelles in the liver and of erythrocytes (which the malarial parasite harbors). While these can be mechanisms contributing to the elimination of the parasite, the results also caution against prolonged use of drug for treatment or prophylaxis.

Part of the studies presented in the thesis dealt with the phosphorous estimation i.e. ATPase and phospholipid analysis. Methods for these two assays differ procedure-wise and with respect to sensitivity. Hence attempts were made to develop a method which is sensitive and at the same time applicable for both enzyme assays as well as inorganic phosphate estimation. These experiments are described in **Supplementary Chapter** of the thesis. In the present method a mixture of ascorbic acid and hydrazine sulfate was used as the reducing agent and the relative proportions of the two reducing agents were standardized. It was found that in a 4.0 ml assay system 0.4 ml of mixture containing 20mg ascorbic acid and 20mg hydrazine sulfate per ml of 0 1 N H_2SO_4 served as a good reducing agent (i.e. 8mg ascorbic acid and 8mg hydrazine sulfate per assay tube). Upon reduction of phosphomolybdate a stable color of molybdenum blue with absorption maximum at 820 nm

developed. The time course of color development was determined. Unlike as in the Bartlett procedure for color development boiling was not required in the present method. After standardizing the procedure, the applicability of the method for enzyme assays involving acid labile and acid stable substrates e.g. ATP and G6P in microsomal ATPase and G6Pase assay and for phospholipid analysis was checked. The results obtained by following with conventional Fiske and Subba Row and Bartlett methods and those obtained using our improved procedure were in excellent agreement.

In conclusion a method for phosphate estimation has been developed which is sensitive, easy, economic and applicable for both enzymatic and non-enzymatic assays. The procedure also obviates the boiling step for color development.