Effect of chloroquine phosphate and toxic concentrations of lead acetate on Ca\(^{2+}\)-ATPase activity in isolates and clones of *Plasmodium Falciparum*

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Summary: The basal activity of Ca\(^{2+}\)-ATPase in two isolates (NL56, UNC) and two clones (D6, W2) of *P. falciparum* was assessed. The effects of various concentrations of chloroquine phosphate and toxic concentrations of lead acetate were also evaluated in the clones and strains of *P. falciparum*. The Ca\(^{2+}\)-ATPase activity was measured by monitoring the rate of release of inorganic phosphate from the gamma-position of ATP on spectrophotometer at 820nm wavelength. The various concentrations of chloroquine (3, 6, 9, 12, 18µg/ml) and lead acetate (5, 10, 20, 30, 40µg/ml) on Ca\(^{2+}\)-ATPase activity were measured respectively. Chloroquine phosphate inhibited Ca\(^{2+}\)-ATPase activity in both the isolates and the cloned strains of *P. falciparum* in concentration dependent manner. Median Inhibitory concentration of chloroquine (MIC\(_{50}\)) estimated from the plot of activity against chloroquine concentration was found to be 2.6µg/ml at pH 7.4 for both the isolates and cloned strains examined. Lead acetate at concentrations 5-20µg/ml inhibited Ca\(^{2+}\)-ATPase activity in concentration dependent manner in clone W2 (Chloroquine resistant strain) while the same range of concentrations of lead acetate stimulated the activity of the enzyme in clone D6 (Chloroquine sensitive strain). The inhibitory effect of lead acetate on the enzyme in clone D6 was observed at concentrations above 20µg/ml. The result also suggests that lead ions could modulate and moderate calcium ion homeostasis in *P. falciparum* via its effect on Ca\(^{2+}\)-ATPase activity. Also sufficient influx of lead ions into *P. falciparum* may transform the biochemical or bioenergetics nature of chloroquine sensitive strain of *P. falciparum* (D6) to that similar to chloroquine resistant strain (W2). In conclusion, inhibition of Ca\(^{2+}\)-ATPase activity of *P. falciparum* may be part of the mechanism of action of chloroquine in its use as chemotherapy for malaria. The study implies that populations simultaneously exposed to lead pollution and malaria infection may experience failure in chloroquine therapy.

Keywords: *Plasmodium falciparum*, Ca\(^{2+}\)-ATPase, Chloroquine phosphate, Lead acetate, Chloroquine resistant strain, Mean Inhibitory Concentration

INTRODUCTION

The biochemical basis of chloroquine resistance by malaria parasites have been perplexing, but there have been a number of observations that suggest close similarities in the mechanism of resistance to chloroquine by *P. falciparum* and the multidrug resistant phenotype of mammalian tumor cells.(Martins *et al* 1987; krogstad *et al* 1987)

Chloroquine has been shown to be concentrated in the acid vesicle of *Plasmodium falciparum* 600 to 800 fold, and this may explain its being active against the parasite at extra cellular concentrations three orders of magnitude lower than those which affect mammalian cells (Herwaldt *et al* 1990).

Parasites (Plasmodium) resistant to chloroquine excrete chloroquine much more rapidly than susceptible parasites and accumulate significantly less drug as a result (Krogstad *et al* 1987). Studies have suggested that hydrolysis of MgATP is required for maximal chloroquine accumulation in *P. falciparum*; also vanadates did not affect chloroquine accumulation (Gluck and Awqi 1984). Ouabain, which inhibits the plasma membrane Na+/K+-ATPase, had no effect on chloroquine accumulation (Skou 1965). However, Oligomycin,
which inhibits the mitochondrial F1 F0 ATPase, reduced chloroquine accumulation by 64% (D’souza et al 1987). The possibility that chloroquine resistance may be independent of chloroquine action was first raised by the observation that chloroquine resistance could be reversed by a calcium antagonist, verapamil, and that the mechanism of chloroquine resistance may be similar to that seen in tumor cells (Martins et al 1987). This linkage was supported by the demonstration that verapamil, vinblastin and daunomycine reversed the efflux of chloroquine from chloroquine resistant \textit{P.falciparum} to varying extent, consistent with similar mechanism to that described for multidrug resistant (mdr) tumor cells (Krogstad et al 1987).

In the process of seeking an understanding into the mechanism of chemotherapy failure particularly Chloroquine treatment failure in \textit{P. falciparum} it is essential to factor into the equation the effect of environmental chemical pollution which patients may be exposed before illness, during illness and treatment thereby creating a situation that may lead to possible drug-chemical interactions.

Epidemiological and experimental evidence have shown lead to be a significant Public health problem (Debruin and Zielhuis, 1971). Automobile emission as well as lead from dust deposited on the soil are important sources of lead exposure in urban residents particularly in areas with congested traffic, (WHO, 1977). Lead in food may come from the environment or from container or from lead deposited by vehicles and machinery on and retained by agricultural crops particularly leafy vegetables (Goyer,1995). Lead inhibits enzymes, alters cellular calcium metabolism, stimulates synthesis of binding proteins in kidney and bones, and slows down nerve conduction (Goyer, 1990a). Studies have suggested similarity between the action of lead and calcium and this may be related to competition for available phosphate (Pounds, 1984; Simons, 1986). Lead alters calcium mediated cellular processes possibly by activating protein in concentrations similar to micro molar concentrations of calcium (West et al, 1994). Interactions at excessive level of lead with erythrocyte cause alteration in membrane status (Rieke, 1969; Debruin, 1971; Smith, 1976; Waldron, 1990; Goyer, 1991). It has been shown that the $K^+$ level in individuals exposed to lead may suggest alterations in membrane metabolism which could involve modulation of Na$/K^+$-ATPase and a $Ca^{2+}$-dependent ($Ca^{2+}$-ATPase) enzyme (Debruin, 1971; Goyer, 1991). In this study, we assessed the effect of chloroquine phosphate on $Ca^{2+}$-ATPase activity in the two isolates of \textit{P.falciparum} from malaria patients at University College Hospital Ibadan (NL56, UNC) and two cloned strains (W2-chloroquine resistant, D6-chloroquine sensitive) of \textit{P.falciparum} and also evaluated the modulatory effect of toxic concentrations of lead acetate on the enzyme activity in cloned strains (W2, D6) of \textit{P. falciparum}.

**MATERIALS AND METHODS**

All reagent, chemicals were of high grade and products of sigma Co .ST Louis MO USA. Reagents were filtered prior to use.

**Isolation of \textit{P.falciparum} from red-blood cells**

Two isolates (NL56, UNC) from Nigerian malaria patients and two cloned strains (W2-chloroquine resistant, D6-chloroquine sensitive) of \textit{P.falciparum} were maintained in culture according to the procedure of Oduola et al, 1992. The \textit{P.falciparum} in culture were harvested at 6-8% parasitemia and centrifuged at 250g at room temperature for 10mins. The residue of packed red cells containing the parasites (i.e. parasitised and unparasitised red cells) was washed twice with washing buffer that contained 10mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulphuric acid), 125mM KCl pH 7.4. The final washed residue was suspended in five (5) volumes of lysing buffer which contained 10mM HEPES, 125mM KCl, 0.15% saponin pH7.4 and incubated at 37°C for 30mins. Short elution column was packed with pre-equilibrated DEAE-cellulose and washed with 2-bed volumes of washing buffer. The haemolysate containing \textit{P.falciparum} and red blood cells ghost was loaded on the gel column and eluted by gravity with washing buffer using the procedure of Lanham and Godfrey (1970). Collected eluate was centrifuged at 250g for 30mins at room temperature to concentrate the isolated \textit{P.falciprnum} The harvested \textit{P.falciparum} was either immediately used or stored for a short period at -20°C for further studies ( but used within 24hrs).

**Determination of $Ca^{2+}$-ATPase activity and the effect of chloroquine phosphate**

\textit{Ca}^{2+}-ATPase activity was assessed in the isolates and clones of \textit{P.falciparum} studied by the rate of hydrolysis of ATP. Effect of chloroquine phosphate on the \textit{Ca}^{2+}-ATPase activity in the two isolates (NL56, UNC) from Nigerian malaria patients and the two cloned strains (W2, D6) of \textit{P.falciparum} was assayed by measuring the effect on the release of inorganic phosphate from the gamma-position (hydrolysis) of ATP by \textit{Ca}^{2+}-ATPase by a slight modification of the method of Ronner et al, (1977). The assay medium contained 30mM HEPES buffer pH 7.4, 120mM KCl, 2mM MgCl$_2$, concentrations of...
chloroquine phosphate (0, 3, 6, 9, 12, 15, 18 μg/ml) and 10-20 μg of prepared *P. falciparum* in a total volume of 0.8ml. The mixture was pre-incubated for 5mins at 37°C followed by adding 1mM ATP (final concentration) to start the reaction. After 30mins of incubation at 37°C with constant shaking, the reaction was terminated by addition of 0.2ml of 5% solution of sodium dodecyl sulphate (SDS) in distilled water.

The inorganic phosphate released was determined by the method of Fiske and Subbarow, (1925) ascorbic acid was used as the reducing agent and the blue colour that developed after 30mins was read at 820nm in a corning spectrophotometer model 258. Blanks were run to correct for non-enzymic hydrolysis of ATP. The Ca²⁺-ATPase activity was expressed as the activity in presence of 0.2mM CaCl₂ minus activity in presence of 0.5mM EGTA. The data represent the average of four independent experimental events performed in triplicates.

### Determination of the effect of lead acetate

The assay medium contained 120mM KCl, 2mM MgCl₂, 0.2mM CaCl₂, or 0.5mM EGTA, 30mM HEPES buffer pH 7.4 and various concentrations (0, 5, 10, 20, 30, 40μg/ml) of lead acetate respectively and 10-20μg of prepared *P. falciparum* cells. The mixture was preincubated for 5mins. Before the reaction was initiated by adding 1mM ATP, and incubated for 30mins with constant shaking. 0.2ml of 5%SDS was added to the mixture to terminate the reaction. The released inorganic phosphate was estimated as earlier described for hydrolysis of ATP. Protein concentration in each isolate and clone was determined by the method of Lowry et al (1951) using bovine serum albumin (BSA) fraction V as standard protein.

### RESULTS

Table I summarizes results of the various concentrations of chloroquine phosphate on specific activity of Ca²⁺-ATPase of the two isolates and the cloned strains of *P. falciparum* studied. The results show that chloroquine inhibited Ca²⁺-ATPase activity in the two different isolates and the cloned strains of *P. falciparum* studied. Analysis of variance through two-tailed statistical test revealed that both

<table>
<thead>
<tr>
<th>CHLOROQUINE PHOSPHATE</th>
<th>SPECIFIC ACTIVITY μmol/Pi/min/mg Protein</th>
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<tbody>
<tr>
<td>CHLOROQUINE PHOSPHATE</td>
<td>NL56</td>
</tr>
<tr>
<td>CONC. μg/ml</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.96 (0.09)</td>
</tr>
<tr>
<td>3.0</td>
<td>0.56 (0.03)</td>
</tr>
<tr>
<td>6.0</td>
<td>0.46 (0.02)</td>
</tr>
<tr>
<td>9.0</td>
<td>0.42 (0.02)</td>
</tr>
<tr>
<td>12.0</td>
<td>0.44 (0.02)</td>
</tr>
<tr>
<td>15.0</td>
<td>0.45 (0.02)</td>
</tr>
<tr>
<td>18.0</td>
<td>0.45 (0.02)</td>
</tr>
</tbody>
</table>

Data shown are Mean value and Standard deviation (SD) from four independent experiments, each performed in triplicate.

NL 56= Local isolate of unknown sensitivity profile of *P. falciparum*, UNC= Local isolate of unknown sensitivity profile of *P. falciparum*, D6=WHO standard chloroquine sensitive clone of *P. falciparum*, W2= WHO standard chloroquine resistant clone of *P. falciparum*

![](image_url)

**Fig. I:** Graph of Enzyme activity against various concentrations of chloroquine phosphate as obtained in Table I.
Studies have shown that chloroquine interact with DNA via interaction dependent on DNA secondary structure and electrostatic interaction between the positively charged diaminopentane side chain of chloroquine and the negatively charged phosphate residues in the nucleic acid polymer(Allison et al,1965;Cohen and Yielding,1965a). Chloroquine and mefloquine have been found to inhibit the ornithine decarboxylase activity present in trophozoite extracts of P.falciparum (Ginsburg and Krugliak, 1992). Chloroquine and some other quinoline containing antimalaria and have been found to inhibit hemoglobin degradation in trophozoite (Zarchin et al, 1986). Millimolar concentrations of chloroquine have also been shown to inhibit phospholipase activity in extracts of P.berghei and P.falciparum (Ginsburg and Krugliak, 1992).This study informed that chloroquine inhibits Ca^{2+}-ATPase activity of clones and isolates of P.falciparum in similar manner. The inhibition of the enzyme by lead could affect the transport of calcium ions into the cytosol and this may eventually lead to deregulation of intracellular calcium ion homeostasis in the parasites. Calcium ion deregulation may cause eventual disruption of the membrane integrity of the parasite thereby resulting into cell death as Ca^{2+}-ATPase is known to be a major regulator of calcium homeostasis in cell (Carafoli, 1988). The study also supports various other studies that have shown chloroquine to interfere with the physiology of P.falciparum (Allison et al, 1965; Cohen and Yielding, 1965a; Konig et al, 1981; Zarchin et al, 1986; Ginsburg and krogliak, 1992).

Studies have suggested that hydrolysis of MgATP is required for maximal chloroquine accumulation in a saturable dose-dependent manner with apparent km of 21μM and an apparent Vmax of 4.6 pmole/mg protein/hr and that more than one ATPase is involved in chloroquine sensitive (D6) exhibited a transformation of its Ca^{2+}-ATPase activity pattern to that of chloroquine resistant (W2) clone.

**DISCUSSION**

Chloroquine phosphate concentrations and treatment (i.e. different isolates of P.falciparum) produced statistically significant differences (P<0.05).

Inhibition of Ca^{2+}-ATPase activity in P.falciparum is concentration dependent. The inhibitory effect varies among isolates, suggesting that the level of inhibition is strain or isolate dependent. Fig. 1 is the plot of specific activity of Ca^{2+}-ATPase against various concentrations of chloroquine phosphate as given in the table. The plot revealed that the median concentration of (MIC50) chloroquine phosphate (i.e. the concentration that would inhibit 50% of the Ca^{2+}-ATPase activity) was 2.6μg/ml at pH 7.4 for the isolates and clones examined.

The effect of various concentrations of lead acetate on the specific activity of Ca^{2+}-ATPase from cloned strains (W2, D6) of P. falciparum (Table 2) showed that increasing concentration of lead acetate resulted in progressive inhibition of Ca2+-ATPase activity in a saturable manner. However, increasing concentration of lead acetate to a maximum of 20μg/ml resulted in progressive stimulation of the enzyme activity in clone D6 (chloroquine sensitive). Further increases in the lead acetate concentrations beyond 20μg/ml resulted in progressive inhibition of the enzyme activity. Two way analysis of variance revealed that there was significant difference in the effect of various concentrations of lead acetate on Ca^{2+}-ATPase activity between the two cloned strains studied (P< 0.05).Results showed that lead acetate modulated Ca^{2+}-ATPase activity in the two clones studied, however, the mechanism of the modulation differed in the two strains. Furthermore, sufficient intoxication or increased intracellular burden of lead

### Table 2.

<table>
<thead>
<tr>
<th>Concentrations of Lead acetate µg/ml</th>
<th>SPECIFIC ACTIVITY Mean (SD)</th>
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<tbody>
<tr>
<td></td>
<td>W2</td>
</tr>
<tr>
<td>0.0</td>
<td>14.32(0.56)a</td>
</tr>
<tr>
<td>5.0</td>
<td>14.00(0.59)b</td>
</tr>
<tr>
<td>10.0</td>
<td>13.16(0.65)b</td>
</tr>
<tr>
<td>20.0</td>
<td>12.79(0.82)b</td>
</tr>
<tr>
<td>30.0</td>
<td>13.90(0.28)b</td>
</tr>
<tr>
<td>40.0</td>
<td>12.70(0.65) b</td>
</tr>
</tbody>
</table>

Ca^{2+}-ATPase activity is expressed as activity in presence of 0.2 mM CaCl₂ minus the activity in the presence of EGTA. Data shown are Mean values and Standard Deviation from mean (SD) from four independent experiments, each performed in triplicate. Data with different alphabets in each column are significantly different (p<0.05) W2= WHO standard chloroquine resistant clone of P.falciparum D6= WHO standard chloroquine sensitive clone of P.falciparum.
that chloroquine is capable of reducing its own accumulation within the parasite and by implication reduce its own therapeutic activity.

Stimulation of Ca\(^{2+}\)-ATPase activity in chloroquine sensitive (D6) strain of P. falciparum at lead concentration below 20μg/ml supports previous studies (Hererman et al, 1983) which opined that lead probably mimicked calcium ion in stimulating the enzyme activity in the parasite. The ability of lead ions to modulate the enzyme activity in the parasite may signify entry of lead ions into the parasite organelles including endoplasmic reticulum and secretary granules within the parasite since lead has been shown to be taken up into isolated mitochondria by an energy dependent process and respiration is inhibited when sufficient lead has been taken up (Scott et al, 1971).

The mechanism by which lead acetate modulated Ca\(^{2+}\)-ATPase activity and by implication calcium homeostasis in cloned strains of P. falciparum may be comparable with findings in previous studies where lead intoxication have been shown to mediate cellular processes by activating synthesis of lead binding proteins at concentrations similar to micro molar concentrations of calcium (West et al,1994; Rieke 1969). The influence of lead on biological membranes have been reported as it has been shown that Ca\(^{2+}\)-ATPase (calcium pump) present in real membrane is activated by lead ion with binding constant (km) of 2pico molar compared with 0.9 micro molar for calcium ion (Pfieger and Wolf 1975). Also red blood cells loaded with lead have been shown to pump lead out provided the cells contain ATP (Simons 1984). Calcium uptake has been shown to be inhibited 50% by 0-4 micro molar lead ions in heart mitochondria and 5 micro molar lead ions in brain mitochondria (Goldstein, 1977), thus the concentrations of lead used in this report is sufficient to inhibit calcium uptake and thus interfere with normal bioenergetics of the parasites. The progressive inhibition of Ca\(^{2+}\)-ATPase (calcium pump) activity by different concentrations of lead ions observed in strain W2 (chloroquine resistant) of P. falciparum may be explained to be as a result of allosteric binding of lead ion at sites other than the normal binding sites of calcium on the enzyme in the parasite thereby altering its conformation. The conformational change could confer inhibitory effect on the functional expression of the enzyme. It is also possible that lead ions displaced calcium ions from the endogenous calmodulin to effect the observed inhibition as studies have shown that lead displaces calcium ions that are bound to calmodulin in activating bovine brain phosphodiesterase enzyme that hydrolyses 3’ 5’ cyclic AMP to 5”AMP (Hererman et al, 1983; Chou et al, 1980). The observed effect of lead ions on the Ca\(^{2+}\)-ATPase activity in clone D6 (chloroquine sensitive) of P. falciparum indicates that increasing concentrations of lead ions to a maximum of 20μg/ml enhanced the activity of the enzyme because lead seemed to compliment intracellular free calcium ions to activate the pump in accordance with the observations of Pfieger and Wolf (1925). Further increase of lead ions in the strain (D6) probably bound to calmodulin and effected inhibition of the enzyme as explained for clone W2 (chloroquine resistant). Our observations suggest that clone D6 contained less (unsaturated) intracellular free calcium ions and that lead ions complimented calcium ions to stimulate its calcium pump while strain W2 contained a full compliment (saturated) of its required intracellular free calcium ions.

In conclusion, inhibition of ca\(^{2+}\)-ATPase activity of P. falciparum may be part of mechanism of action of chloroquine in its use as chemotheraphy of malaria. Also those populations simultaneously exposed to lead pollution and malaria infection may experience failure in chloroquine therapy. However further studies are needed to know the direct mechanism of interaction of chloroquine with Calcium homeostasis as well as lead ions in relation to necrosis and apoptosis of malaria parasite.

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