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THERAPEUTIC EFFICACY OF CHLOROQUINE IN LONG CIRCULATING LIPOSOME FORMULATIONS AGAINST CHLOROQUINE-RESISTANT *PLASMODIUM BERGHEI* INFECTION IN MICE.

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ABSTRACT
Malarial parasites are developing resistance to chloroquine, the most commonly used antimalarial drug. Here, we have developed an improved long circulating liposome drug delivery vehicle for delivery of chloroquine to treat chloroquine resistant malaria in mice model. The enhancement of antiplasmodial activity was highly dependent on the size, lipid composition and presence of distearoyl phosphatidylethanolamine-methoxy-polyethylene glycol 2000 (DSPE-mPEG-2000) on liposome surface. Chloroquine in Polyethylene glycol (PEG) containing liposomal formulations exhibited spherical shape with size ranging from 80 to 100 nanometer, as measured by dynamic light scattering (DLS) and high-resolution electron microscopy (HRTEM). Chloroquine (CQ) in long circulating liposome formulations with 5mol% DSPE-mPEG 2000 resulted in enhanced killing of blood parasites compared with similar dose of free chloroquine. Compared with free drug, liposomal formulations showed enhanced efficacy assessed by clearance of parasitemia and significant delay in death of mice with *Plasmodium berghei* CQ-resistant infections. To our knowledge, this is the first report to demonstrate that chloroquine in Polyethylene glycol liposomal formulations can act as a chemotherapeutic agent against chloroquine resistant malaria.

KEYWORDS: liposomal chloroquine; PEG-liposome; erythrocytes; *Plasmodium berghei.*

INTRODUCTION
Chloroquine continues to be the mainstay of treatment for all five human malaria species. However, the efficacy of chloroquine has diminished considerably with the emergence of chloroquine-resistance in *Plasmodium falciparum* isolates in most parts of the world [1] leading to increase in mortality.[2] The intra-erythrocytic stages of the life cycle in parasite are responsible for clinical manifestations of the disease. Chloroquine acts by inhibiting free heme polymerization inside parasite food vacuole leading towards heme poisoning.[3] This detrimental effect causes irreversible growth arrest of the parasites.

The emerging resistance to antimalarial drugs has accelerated the development of new drug delivery vehicles to overcome clinical resistance and improve the efficacy of existing antimalarials.[4] It has been reported that the basis of clinical resistance towards chloroquine and other antimalarial drugs is the result of membrane associated altered polymorphic drug pumps in food vacuole of malaria parasite.[5] To obviate chloroquine resistance, liposomes as synthetic lipid vesicles were introduced to deliver chloroquine for the treatment of chloroquine-resistant malaria in mice.[6, 7] Liposomal delivery vehicles can deliver both hydrophilic and hydrophobic drugs to homing site of intracellular pathogens [8] with potential clinical applications.[9] Chloroquine targets all life stages of malaria parasites.[10-12] Targeted delivery of chloroquine in liposomes to parasite infected erythrocytes were tested using liposome surface tagged with anti-erythrocyte antibody F(ab)2 fragments against *Plasmodium berghei* infected erythrocyte membrane.[13, 14] Delivery of malarial antigen (pbCSP) in liposomes coated with oligo-mannose elicited strong protection against *P. berghei* infection in mice.[15] Notably, chitosan based nanocarriers containing chloroquine have shown superior efficacy against murine malarial infections than comparable dose of free chloroquine.[16, 17] However, the short circulatory-life of

chloroquine in conventional liposomes is a major challenge for clinical use. In the present study, we assessed the efficacy of chloroquine in long circulating liposome formulations for intracellular malarial infection and its effect on overcoming chloroquine resistance in a murine malaria model.

MATERIALS AND METHODS

2.1 Materials Soya phosphatidylcholine (SPC) was obtained as a gift from Lifecare Innovations Pvt. Ltd., Haryana, India. Cholesterol (Chol), Chloroquine di-phosphate (CQ), was purchased from Sigma-Aldrich (St. Louis, MO, USA). DSPE-mPEG-2000 (Distearoyl phosphatidyl ethanolamine-Methoxy-Polyethylene-glycol-2000), L-α-Phosphatidic acid (PA) were purchased from Avanti Polar Lipids, Inc., AL, USA. All other chemicals were analytical-grade products.

2.2 Animals All Animal experiments were performed in Inbred, female Swiss albino mice (4 to 5 weeks old, weighing 20-25 g). These mice were obtained from the Laboratory Lala Lajpat Rai University of Veterinary and Animal Sciences, India, and maintained in our animal facility at the University of Delhi South Campus, New Delhi, India. The animals were housed under standard controlled conditions at 25ºC with a 12-h light-dark cycle and access to sterilized food pellets and water. All experiments were carried out in accordance with the standard procedures approved by the Institutional Animal Ethics Committee (IAEC), University of Delhi South Campus, under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Reg.No-159/1999/CPCSEA).

2.3 Determination of Size distribution (nm) and Zeta potential (ζ) of liposomal chloroquine using DLS. The liposome size (in nanometer) and zeta potential (ζ) were measured using a Zetasizer Nano ZS (ZEN 3600, Malvern Instruments, Worcestershire, United Kingdom). For the measurements, 10µL of sample suspension was dispersed in 990µL of PBS and sonicated for 1min. The parameters set for analyses were a scattering angle of 90º and at a temperature of 25ºC. For each sample, the mean diameter and the standard deviation of 10 determinations were calculated using multimodal analysis. The zeta potential was measured by a combination of lasser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique at 25ºC.

2.4 Transmission electron microscopy (TEM) of chloroquine loaded in various PEG-liposomes `The sample preparation was performed by taking 10µL of diluted liposomal suspension placed upon 300 mesh carbon-coated copper grids under sterile conditions and air drying for analysis (Polysciences, Warrington, PA). The liposomes were visualized under a Tecnai G2 T30 U-TWIN) electron microscope, and images were obtained by using a digital imaging software at different magnifications.

2.5 Preparation of Liposomes Liposomes were prepared using soya phosphatidyl choline (SPC) and cholesterol (Chol) in a molar ratio of 7:3 by pH gradient method as reported.[18] Briefly, the lipids (total, 400 µmol) containing 10 mol% phosphatidic acid (PA) and 5 mol% of DSPE-mPEG-2000 of the lipid mixture was added during the preparation of the lipid film. The dried lipid film so obtained was desiccated overnight, and then was hydrated with 0.3M citrate buffer (pH 3.2-3.4), stored under a nitrogen atmosphere followed by freeze thawing and sonicated. The dissolved chloroquine was added to the liposomal suspension and the transmembrane pH gradient was implemented by adjusting the exterior environment (pH 7-7.5) using 1N NaOH followed by incubation at 60ºC for 15 minutes with intermittent vortex. Liposomal chloroquine was separated from unentrapped chloroquine by ultracentrifugation at 11,800×g for 2 h. The liposomal pellet was suspended in minimum volume of 20mM sterile phosphate-buffered saline (PBS) and assayed spectrophotometrically for the presence of chloroquine, according to the method.[6] The recovery of liposomes was determined colorimetrically by measuring phospholipids using Stewart's method.[19]

2.6 Development of murine model of rodent strain
For experimental infection, a murine model of malaria was developed by intraperitoneal (i.p.) injection of 10^5 to 10^7 rodent specific *Plasmodium berghei* NK65 (chloroquine resistant) infected erythrocytes to healthy Swiss albino mice weighing 20 to 25g. Parasitemia was monitored routinely by continuous weekly blood passages of parasitized erythrocytes (PE) from infected to naive mice, and survival was checked over a period of 2 weeks.

2.7 Assessment of *in vivo* antimalarial efficacy To examine the efficacy of liposomal formulations loaded with chloroquine, murine model of malaria was developed by intraperitoneal (i.p.) administration of standard inoculum of chloroquine-resistant *Plasmodium berghei* NK65 carrying 1×10^7 parasitized erythrocytes per 200µL volume to each experimental Swiss albino mouse. The *in vivo* antimalarial activity of chloroquine in liposomal formulations was carried out in accordance with a slightly modified version of the Peters 4 day suppressive test.[20] Each experimental group consists of six animals. Subsequently, after 48 h of postinfection, the parasitemia level reached 2 to 3%, and all groups of mice were treated by subcutaneous (s.c.) injection with chloroquine in free and liposomal forms at different dosages. The appropriate series of dilutions were made for free drug and liposomal suspension in buffered saline (0.2mL/day). One group kept as a control and treated with physiological saline. The efficacy of the treatment was monitored by measuring the parasitemia and survival on days 5, 8 and 15 post treatment by obtaining

thin smears of blood withdrawn from the tail vein of infected mice and staining with 10% Giemsa. The level of parasitemia was determined by counting infected and noninfected erythrocytes from 10 to 15 randomly selected optical fields at 100× magnification and expressed as the number of infected erythrocytes per 100 erythrocytes. The survival of mice was recorded and observed for external symptoms such as change in body weight, ruffled fur, lethargy, paralysis until 30 days post treatment. The reduction in the level of parasitemia was taken as the index for the curative activity of the drug. The percentage of parasitemia was calculated manually using the Cell Counting Aid software [21], using the formula.

$$\% \text{ Parasitemia} = \frac{\text{Total Number of Parasitized RBCs}}{\text{Total Number of RBCs}} \times 100$$

2.8 Statistical analysis For the *in vivo* experiments, statistical differences between multiple groups using one way analysis of variance (ANOVA), with *P* values of <0.05, by GraphPad Prism (version 5.01; GraphPad Software, Inc., CA). The survival of the mice was followed up to day 30 postinfection using the Kalpan-Meier survival analysis, and statistical differences in animal survival were analyzed by a log-rank test.

RESULTS

Determination of size (nm) and zeta potential (ζ) of chloroquine loaded in PEG liposomal formulations

The size (in nanometers) and surface charge (ζ potential) of chloroquine liposomal formulations were determined using dynamic laser light scattering (DLS). The mean diameter obtained from polyethylene glycol-phosphatidic acid, PEG-PA liposome was 98±10nm and PEG-liposome 96±11nm loaded with chloroquine. The change in surface zeta potential (ζ) values was dependent on the incorporation of charged lipid (PA). The PEG-PA liposome exhibits negative charge of -15.6±0.76 while PEG-liposome (without PA) had neutral charge of 1.98±0.48 on liposomal surface. The maximum encapsulation efficiency of chloroquine was 45-50% in PEG-PA liposome, and in PEG-liposome was 30-35%.

Transmission electron microscopy (TEM) studies of PEG-liposomal formulation containing chloroquine

The liposomal formulations predominantly consist of unilamellar vesicles in the size range of 80 to 100nm, as observed in the TEM images. The two techniques (TEM analysis and DLS measurement) showed a similar pattern of particles size and spherical morphology, as shown in Figure 1.

Effect of chloroquine in PEG-liposomal formulations on the treatment of *P. berghei* (chloroquine-resistant) infected Swiss albino mice

We studied the antimalarial efficacy of chloroquine in free and liposomal forms using a modified version of Peter's 4-day suppressive test.[22] A murine model of malaria was developed by intraperitoneal injection of 1×10^7 *P. berghei* chloroquine resistant infected erythrocytes. Chloroquine in various liposomal formulations was administered 48 h postinfection. There was progressive decrease in parasite load with increasing dose of chloroquine in PEG-liposomal formulations. In untreated group, the parasitemia was found to be 50-60% and all mice succumbed to death on day 8-10. The maximum dose of free chloroquine (80mg/kg) administered to infected mice had no effect on parasitemia (45-50%) on day15. On other hand, chloroquine in PEG-liposomes exhibited marked reduction in parasite load (11-14%) with improved survival as shown in Figure 2A. The polyethylene glycol- phosphatidic acid (PEG-PA) liposomes showed slight enhancement in parasite clearance compared to PEG-liposomes (without PA) as shown in representative blood smears in Figure 2C.

The median survival time of the treated group with PEG-PA liposomes was 23 days and PEG-liposomes were 21days. There was a marked delay in death in liposomal CQ treated group, compare with free chloroquine-treated group as shown in in Figure 2B. The animals in untreated and free chloroquine groups exhibited severe pathological damage in liver and characterized by liver enlargement and heavy deposition of hemozoin pigment, whereas the animals in treated groups showed reduced pathological liver damages as shown in Figure 2C.

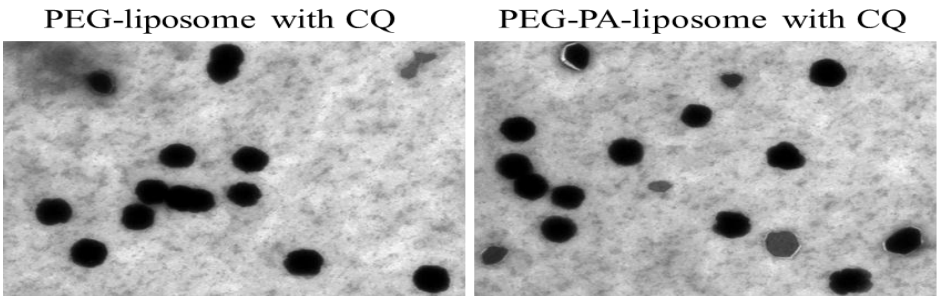

Figure 1: Characterization of liposomal formulations containing chloroquine using high resolution transmission electron microscopy (HRTEM). Representative images of various formulations of liposomes loaded with chloroquine are shown. The liposomes showed spherical morphology and size distribution, with a mean diameter of 80 to 100nm range.

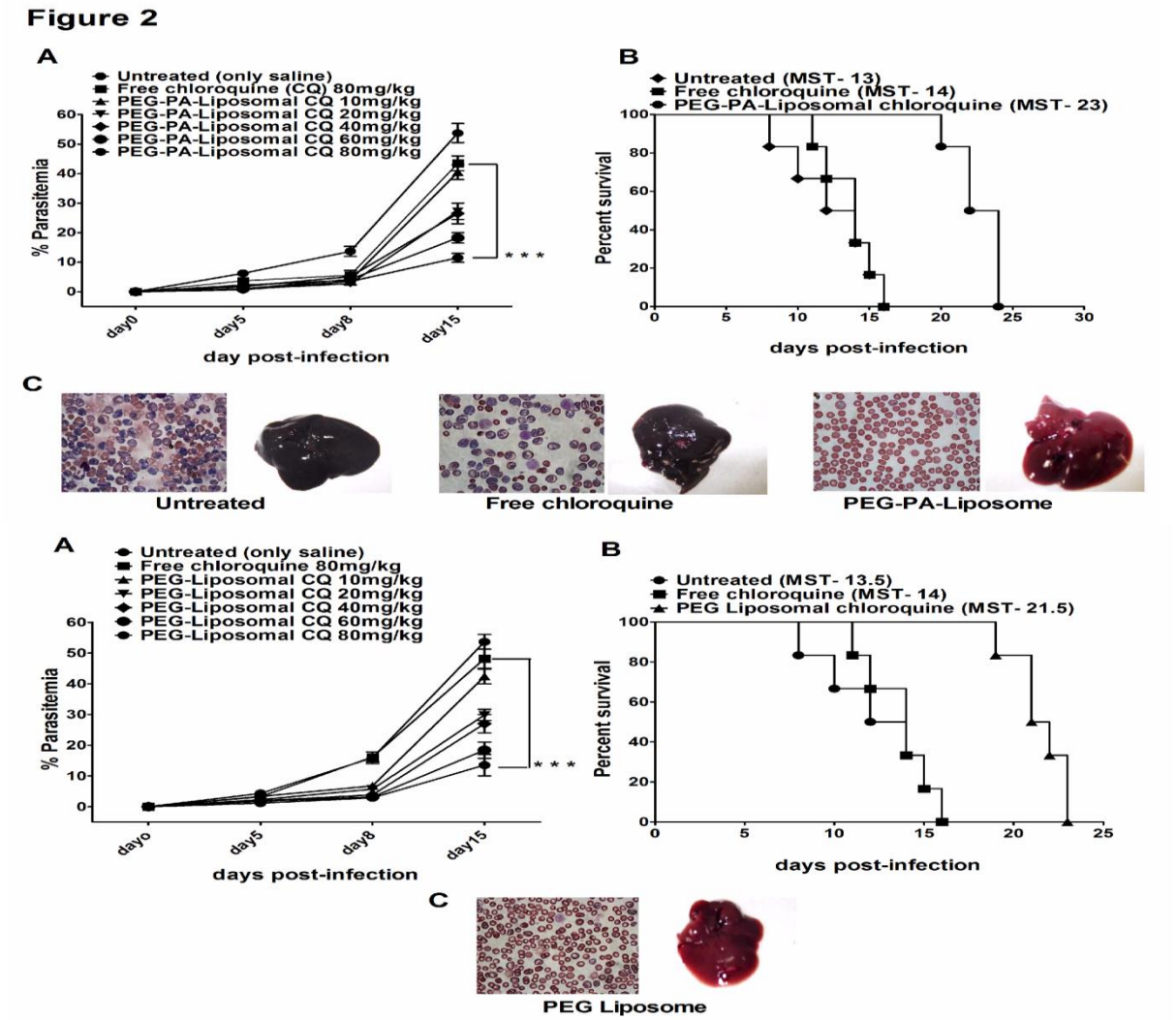

Figure 2: Effect of different doses of chloroquine in sterically stabilized liposomal formulations on parasitemia and survival of *P.berghei* NK65 (chloroquine resistant) infected Swiss albino mice. (A) Free chloroquine and PEGylated liposomal formulations containing chloroquine (with or without PA-phosphatidic acid) was administered subcutaneously. The untreated group was administered saline. The data represents mean ± SD with 6 animals in each group. (B) Survival of various liposomal formulations of chloroquine in treatment groups compared to that with free chloroquine. The median survival time (MST) (in days) of the animals in each group is shown in parentheses. (C) Photomicrograph of blood smears of untreated versus treatment groups at day 15 postinfection shown at 100× magnification with photograph of liver morphology untreated versus treated. For the determination of parasitemia in blood smears, 10 different random optical fields were counted and parasitemia calculated using the Cell Counting Aid software.

DISCUSSION

In the present study, we have demonstrated that chloroquine in PEG containing liposomal formulations are very effective in inhibiting the growth of chloroquine-resistant *Plasmodium berghei* infection in mouse model. The maximum inhibition of growth of *P.berghei* was observed when chloroquine was delivered through liposomal formulations containing 5mol% DSPE-mPEG-2000 than a comparable dose of free drug. Our previous study has reported that PEG polymer coated liposomes with surface density of 5mol% and molecular weight (MW) of PEG chain length (2000) found to be optimal for *in vivo* delivery of antimalarials.[23] Chloroquine in conventional liposomes have shown to overcome chloroquine resistance.[24] The enhancement in antiplasmodial activity through liposomal delivery of chloroquine in CQ resistant infection could be due to altered mechanism of entry of liposomal chloroquine through, membrane bilayer and membrane fusion.[25, 26] Drug loaded liposomes may overcome drug resistance by bypassing drug transporters of the parasite food vacuole and facilitate the intracellular accumulation of drug molecules to target site of action. In contrast, membrane bound drug efflux transporters (*pf*CRT) expels free chloroquine which causes CQ-resistance in parasites.[27]

Evidences suggest that delivery of chloroquine through liposomes not only increases its maximum tolerable dose but also enhances its efficacy against both chloroquine-susceptible and chloroquine-resistant *Plasmodium berghei* infections in mice.[6, 8] [28] These observations are also in agreement with previous studies that rhTNF-α delivered in sterically stabilized PEG-liposomes showed enhanced protective efficacy against *P.berghei* experimental cerebral malaria in mice.[29] Immunoliposomal based targeted delivery of chloroquine was more efficient in *P. falciparum* infected RBC in culture as the IC_{50} value was lower than comparable dose of free chloroquine.[26, 30]

It has been reported that longevity of liposomes in the circulation is significantly dependent on the lipid composition, surface charge and interaction of charged lipids with different molecular sized opsonizing proteins in bloodstream are responsible in clearance of liposomes.[31, 32] Surface modification of liposome with PEG-grafted lipids prolong the circulation time in the blood by avoiding the uptake in liver and spleen with slow release of the drug.[32, 33] The negatively charged liposomes offer better loading efficiency of positively charged hydrophilic drugs (primaquine, chloroquine) in passive and active loading of the drug.[4] Our findings illustrate that enhancement in antimalarial activity of chloroquine in liposomal formulations might be mainly due to their size 80 to 100 nm range, spherical in shape and vesicle stability which influence their preferential entry into parasitophorous vacuole membrane (PVM) of infected RBCs.[34] Liposomes may be more efficient in intracellular delivery of drug leading to enhanced cytotoxicity compared with free drug. This is supported by a report which showed that parasite infected RBCs have direct access to extracellular nanosized (80nm) latex bead particles.[35] The superior antimalarial efficacy of chloroquine loaded in PEG containing liposomal formulation may be due to their longer circulatory life in blood and preferential delivery of the drug to parasite infected erythrocytes that have enhanced membrane permeability.[26, 36] Consequently, these liposomes may enable longer exposure of parasites to chloroquine resulting in suppression of parasitemia to greater extent leading to subsequent survival of mice. Our studies suggest that liposome based delivery of chloroquine in combination therapy with potent antimalarial agents may be employed in delaying the development of clinical resistance.

CONCLUSIONS

To our knowledge, this is the first report that demonstrates chloroquine in long circulating liposomes have enhanced antimalarial activity by overcoming chloroquine resistance in murine malaria. Therefore, we propose that long circulating liposomal formulation with 5mol% DSPE-mPEG-2000 may provide potential therapeutic applications in delivering various hydrophilic antimalarials for the treatment of drug resistant plasmodial infections.

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Transparency declarations

None to declare.

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