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Improved Efficacy of Doxycycline in Liposomes Against *Plasmodium falciparum* in Culture and *P. berghei* infection in mice.

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**Running Title:** Doxycycline liposomes as antimalarial agent.
Abstract:

Drug resistance to *Plasmodium falciparum* is increasing at an alarming rate of clinically used antimalarial drugs. Therefore, there is a compelling need to develop an efficient drug delivery system to improve the efficacy of existing antimalarial agents and circumvent drug resistance. Here, we report the antibacterial drug Doxycycline (Doxy) in liposomal formulations exhibits enhanced antiplasmodial activity against blood stage forms of *P. falciparum* (3D7) in culture and established *P. berghei* NK-65 infection in murine model. Parasite killing on blood stage forms in culture was determined by a radiolabeled [$^3$H] hypoxanthine incorporation assay and infected erythrocytes stained with Giemsa were counted using microscopy under *in vivo*. The 50% inhibitory concentration (IC$_{50}$) of doxystearylamine (SA) liposome (IC$_{50}$ 0.36 µM) and doxy-SPC:Chol-liposome (IC$_{50}$ 0.85 µM) exhibited marked growth inhibition of parasites compared with free doxycycline (IC$_{50}$ 14 µM), with minimal toxicity to normal erythrocytes. Administration of polyethylene glycol Distearoyl phosphatidylethanolamine-methoxy-polyethylene glycol2000 (DSPE-mPEG-2000) coated liposomes loaded with doxycycline at 2.5mg/kg/day resulted in efficacious killing of blood parasites with improved survival in mice relative to the free drug in both chloroquine sensitive and resistant strains of *P. berghei* infection. This is the first report to demonstrate that doxycycline in liposomal system has immense chemotherapeutic potential against plasmodial infections at lower dosages.

**Keywords:** Liposomes, Plasmodium, PEG-liposomes, Doxycycline, Antimalarials
1. Introduction

Malaria is a vector-borne infectious disease, caused by protozoan parasite *Plasmodium* that is transmitted to human host through the bites of infected female *Anopheles* mosquitoes (Cox 2010). The global malarial death in 2015 was estimated to be 429,000 where young children are prone to infection majorly from African continent (World Health Organization 2016). Among all human parasites, *P. falciparum* display uncontrolled parasitization of erythrocytes and sequestration of parasitized red blood cells (PRBC) in liver, spleen and brain microvessels causing anemia and cerebral malaria (Buffet et al. 2011; Idro et al. 2010).

Emergence of clinical resistant malarial parasites to existing antimalarial chemotherapy has led to search for new class of antimalarial drugs and combination based therapy targeting multiple stages of parasite life cycle (Cui et al. 2015; Flannery et al. 2013). Importantly, *P. falciparum* and *P. vivax* show relentless development of clinical resistance against various frontline antimalarial drugs (Baird 2009; Price et al. 2014; Sinha et al. 2014; Yeung et al. 2004). Currently used artemisinin monotherapy (artemether, artemunate) to treat severe and complicated malaria, shows early stage of resistance to *P. falciparum* in patients at the risk of treatment failure (Ashley et al. 2014; Fairhurst and Dondorp 2016; Li and Weina 2010; Phyo et al. 2012). Surprisingly, evidences show that combination therapy of artesunate-mefloquine or dihydroartemisinin-piperaquine shows decreased effectiveness in falciparum infected patients of Southeast Asian countries (Amaratunga et al. 2016; Breman 2012; Wongsrichanalai and Meshnick 2008). The decreased effectiveness in conventional chemotherapy has resulted in non-specific targeting to healthy cells, recrudescence, repeated dosages thereby impair successful treatments against malaria. Thus, there is an urgent need of efficient delivery system for improving the efficacy of current antimalarial agents at reduced dosages in preventing resurgence and reinfection.

Multiple antibiotics exert profound antimalarial activity at clinically relevant dosages (Friesen et al. 2010; Gaillard et al. 2016; Pradines et al. 2001). Doxycycline (doxy) is a tetracycline based broad spectrum antibiotic widely used in treating bacterial infections by inhibiting bacterial protein synthesis of the 30S ribosomal subunit (Pioletti et al. 2001; Roberts 2003). Earlier studies have shown that doxycycline inhibits protein synthesis of bacterial origin apicoplast, mitochondria and nucleotide biosynthesis in plasmodium (Dahl and Rosenthal 2007; Dahl et al. 2006; Lin et al. 2002; Yeo et al. 1998). In addition to these effects, doxycycline has been shown to inhibit isoprenoid precursor biosynthesis pathway in
apicoplast (Bowman et al. 2014; Uddin et al. 2018; Yeh and DeRisi 2011). Currently, doxycycline is used clinically in treating malaria patients at 100mg/adult dosage alone for chemoprophylaxis and administered in conjunction with fast acting partner drugs artemisinin derivatives or quinine for falciparum malaria (Freedman 2008; Gaillard et al. 2015a; Pang et al. 1987; Sponer et al. 2002; Tan et al. 2011; Win et al. 1992). Doxycycline affects the mature stage of parasite life cycle and impairs the growth of daughter parasites leading to further inhibition (Dahl et al. 2006). Possibly, overuse of doxycycline in malaria patients may result in development of resistance to various bacterial pathogens and modifications in gut microbiota (Angelakis et al. 2014; Gaillard et al. 2016; Trzcinski et al. 2000). This may also result in development of reduced susceptibility of doxycycline in *P. falciparum* isolates (Achieng et al. 2014; Briolant et al. 2010). The advantage of using doxycycline for *P. falciparum* infection is due to its delayed killing effect against schizontocidal blood forms and well tolerated at higher dosages in patients (Gaillard et al. 2015a; Rieckmann et al. 1971). Notably, treating with doxycycline in free form causes adverse side effects in patients leading to decreased effectiveness (Gaillard et al. 2015a; Goetze et al. 2017; Tan et al. 2011). Moreover, due to its hydrophobicity and lipophilicity with shorter half-life in blood stream, hinders its therapeutic applications necessitating frequent dosages (Michel et al. 2010); (Agwuh and MacGowan 2006; Barza et al. 1975; Newton et al. 2005; Vargas-Estrada et al. 2008).

Importantly, many lipid based nanocarriers have been developed with prolonged circulation and slower release of drug targeted towards homing site of malarial pathogen (Santos-Magalhaes and Mosqueira 2010; Thakkar and S 2016). Liposomes composed of natural phospholipids are biodegradable and biocompatible by improving the bioavailability of therapeutic agents (Bookstaver et al. 2018). Therefore, delivery of doxycycline using nanosized liposomes as a carrier agent enhances the therapeutic efficacy with reduced dosing frequency, minimized toxicity and delay resistance. Delivery of chloroquine through liposomes has shown to overcome chloroquine resistance in *P. berghei* infected mice (Owais et al. 1995; Peeters et al. 1989). Besides improving the efficacy of existing antimalarial drugs, liposomal delivery of malarial antigen showed enhanced protection (Fries et al. 1992; Genito et al. 2017). The present study demonstrates improved efficacy of doxycycline in liposomal formulations against *P falciparum* (3D7) in culture and *P berghei* NK65 infection in rodent malaria.
2. Material and methods

2.1 Materials Soybean phosphatidylcholine (Lipoid S 100) was purchased from LIPOID Germany. cholesterol (Chol), stearylamine (SA), doxycycline (DOXY), coumarin-6, DAPI (4,6-diamidino-2-phenylindole), gentamycin sulphate (cell culture grade), and Histopaque-1077 were purchased from Sigma-Aldrich (St. Louis, MO, USA). DSPE-mPEG2000 (Distearoyl phosphatidylethanolamine-methoxy-polyethylene glycol2000) was purchased from Avanti Polar Lipids, (Inc., AL, USA). Powdered RPMI-1640 medium and AlbuMAX II are Gibco products from the Invitrogen Corporation. Hypoxanthine monohydrochloride, (\([^{3}H(G)]\)) was bought from American Radiolabeled Chemicals, Inc., (St. Louis, MO, 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 25 to 30 g). These mice were obtained from the Laboratory Lala Lajpat Rai University of Veterinary and Animal Sciences, India, and maintained in our animal facility (Registration No: 159/GO/Re/S/99/CPCSEA) at the University of Delhi South Campus, New Delhi, India. The animals were housed under standard humidity, temperature conditions at 25°C with a 12-h light-dark cycle and access to sterilized food pellets and water. The animals were cared for in accordance with Guide for the Care and Use of Laboratory Animals (Council 2011). Protocols for all animal 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 (Protocol Reference. No. 35/IAEC/PCG/Biochem/UDSC/03.03.2017).

2.3 Preparation of liposomes Liposomes were prepared using soya phosphatidyl choline (SPC) and cholesterol (Chol) in a molar ratio of 7:3 by hand shaken method. Briefly, the lipids (total 300 μmol) and doxycycline (10 mol% of the lipid mixture) was dissolved in a 5ml chloroform solution. To prepare other formulations, 10 mol% stearylamine (SA) and 5mol% DSPE-mPEG-2000 was added during the preparation of lipid film. The dried lipid film obtained was desiccated overnight and then was hydrated using glass beads with 20mM sterile phosphate buffered saline (PBS), stored under nitrogen atmosphere. The lipid suspension was sonicated for 45 minutes in bath sonicator and further incubated for 4h at 4°C. For fluorescent labeling of the liposomes, coumarin-6 dye was dissolved, along with the
lipid suspension. Liposomal doxycycline or liposomal dye was separated from the unentrapped doxycycline or dye, by centrifugation at 1,956 ×g for 15 min and further subjected to two successive ultracentrifugation at 11,800 × g for 1h each at 4°C. The intercalation of doxycycline in liposomes was determined by measuring the absorbance at 271nm (Satishkumar Shetty A 2014).

2.4 Determination of liposomal size distribution and zeta potential The average particle size and zeta potential of the liposomes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). About 10 μl of freshly prepared liposome suspension was redispersed in 990 μl of distilled water and placed in a clear disposable zeta cell (DTS-1060C). The parameters set for analyses were a scattering angle of 90ºC and 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 laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique at 25ºC.

2.5 In vitro culture of Plasmodium falciparum The strain of Plasmodium falciparum used in the study was CQ-susceptible (3D7) was maintained by serial passages in human erythrocytes cultured at 4-5% hematocrit in complete RPMI-1640 medium supplemented with 0.5% AlbuMAX II, and Gentamycin sulphate (50mg/litre) and incubated at 37ºC under an atmosphere of mixed gas (5% CO₂, 5% O₂ and 90% N₂). The red blood cells (RBCs) were obtained under sterile conditions by the removal of plasma and peripheral blood mononuclear cells (PBMCs) using a Histopaque gradient; the RBCs were washed 2 to 3 times using RPMI 1640 without AlbuMAX II (Incomplete media).

2.6 Evaluation of antimalarial activity of doxycycline in liposomal formulations against P. falciparum (3D7) in culture The antiplasmodial effect of doxycycline in free and liposomal formulations was determined by [³H]-hypoxanthine incorporation in the nucleic acid of parasites on blood stages (Desjardins et al. 1979). The free drug and liposomal formulations were serially diluted in hypoxanthine-free complete medium and added to each well of P. falciparum infected erythrocytes at (4% final hematocrit and 2% parasitemia) on 96-well microdilution plates. The microtitre plates were incubated in chamber under standard gas mixture condition at 37°C. After 30 h of incubation, 20μL of 0.2μCi/well [³H]-hypoxanthine (specific activity, 1.0 mCi/ml) was added to each well for an additional 18 h. At the end of incubation, the contents in each well were harvested on glass-fibre filter mats (Whatman GF/C) using a 96-well Skatron semi-automated cell harvester. The radioactive
Determined from parasite associated radioactivity by plotting the drug concentration versus the percentage of cell viability of the parasite after 48 h of a growth assay period. All data points were collected in triplicate for each experiment.

2.7 Assessment of in vivo antimalarial study To examine the in vivo efficacy of doxycycline in PEGylated liposomal formulation mice model of malaria was developed by intraperitoneal administration of standard inoculum of rodent strains (CQ susceptible and CQ resistant) of P. berghei carrying 1×10⁷ parasitized erythrocytes per 200µL volume to each experimental Swiss albino mouse. The antimalarial activity of doxycycline in liposomes in mice was carried out in accordance with a slightly modified version of the Peters 4-day suppressive test (W. Peters 1975). Each experimental group consists of six animals (n=6). Subsequently, after 48 h of post-infection, the parasitemia level reached 1 to 2% and all groups of mice were treated by subcutaneous (s.c.) injection of four consecutive doses (2.5 mg/kg/day of body weight) with free form of doxycycline suspended in 10% DMSO or doxy-liposomal formulations in PBS on the rump above the tail. One group kept as a control and treated with 10% DMSO (vehicle control). The efficacy of the treatment was monitored by measuring the parasitemia and survival on days 5, 8 and 15 post treatments by obtaining thin monolayer blood smear from the tail vein of infected mice and staining with 10% Giemsa. The level of parasitemia was determined by counting infected and non-infected erythrocytes from 10 randomly selected optical fields under oil immersion 100× objective microscope. The percent parasitemia was expressed as the number of infected erythrocytes of 1000 erythrocytes per field. The reduction in the level of parasitemia was taken as the index for the curative activities of the drugs. The percentage of parasitemia was calculated manually with the Cell Counting Aid software using the formula (Ma et al. 2010).

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

2.8 In vitro uptake of coumarin-6 tagged SA liposome on blood stages of P. falciparum 3D7 in culture: To detect the selective uptake of liposome in P. falciparum-infected erythrocytes and uninfected erythrocytes, the coumarin-6-labeled (C-6) liposome fluorescent marker was used. In brief, P. falciparum-infected erythrocytes (10% hematocrit and 10%
parasitemia) were incubated in the presence of liposomal formulation for 4h. The uptake of C-6 liposomes by parasite-infected erythrocytes, were stained with 1 µg/ml DAPI and incubated for additional 15 min at 37°C. The stained cells were visualized by fluorescence microscopy (Nikon Ellipse Ti-S) at 1000 × magnification.

2.9 Hemolytic assay The in vitro hemolytic assays of doxy loaded liposomal formulations were performed in normal human erythrocytes by measuring the lysis of RBCs in the culture medium. Different concentrations of free doxycycline and various liposomal formulations were added to normal RBCs at 4% hematocrit, as reported earlier (Rajendran et al. 2015).

2.10 Statistical analysis For in vitro studies, the IC_{50} are presented as the mean ± standard deviation of the results from at least three independent experiments. For in vivo experiments, statistical differences between two groups were determined by Student’s t test and 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 40 post infection using a Kalpan-Meier survival analysis, and statistical differences in animal survival were analyzed by a log-rank test.

3. Results

3.1 Determination of size and zeta potential of doxycycline loaded in liposomal formulations using Dynamic light scattering.

The developed doxycycline loaded in liposomal formulations and their size (in nanometers) and surface charge (zeta potential) were determined using dynamic light scattering (DLS) suspended in deionized water, as shown in Table 1. The mean diameter of liposomes showed a size range of 90 to 110 nm with a polydispersity index (PDI) of less than 0.3. The change in surface zeta potential values was dependent on the incorporation of charged lipid stearylamine (SA) and PEGylation (DSPE-mPEG-2000). The presence of SA in liposome showed positive zeta potential compared with other formulations without SA.

3.2 Effect of Doxycycline in Liposomal formulations on the growth inhibition of Plasmodium falciparum in culture. The effect of doxycycline in liposomal formulations on the growth inhibition of asynchronous P. falciparum 3D7 in culture was determined by the inhibition of the incorporation of [³H] hypoxanthine in parasites. The IC_{50} values at 48 h of incubation for the growth of the parasites for liposomes with stearylamine, SPC:Chol and free
doxycycline were found to be 0.3±0.11, 0.87±0.16 and 14±1.7 µM, respectively. There was a significant reduction in parasite growth when doxycycline was delivered through stearylamine (SA) liposome, with 38-fold enhancement and SPC:Chol liposome, with 16-fold enhancement in parasite-killing activity compared with free drug. We further extended the incubation time up to 72h the IC₅₀ values were found to be 0.15±0.85, 0.5±0.6 and 11.4 µM, respectively. However, we failed to observe any changes in the IC₅₀ value at 96h of incubation. Both the liposomal formulations exhibits superior efficacy with reduced IC₅₀ values and enhanced killing effect against parasites. However, we have previously shown that stearylamine-phosphatidylcholine liposome alone (without drug) has shown to inhibit the plasmodial growth in cultured parasites with IC₅₀ value of 5.56±0.89µM (Hasan et al. 2011) (Rajendran et al. 2015). Therefore, combined action of stearylamine and doxycycline displayed superior activity as compared to formulation without stearylamine. These results clearly suggest that inhibitory effect of P. falciparum is dependent on the size of vesicle and lipid composition on the liposomal surface by inducing enhanced cell death.

3.3 Effect of Doxycycline in long circulating PEGylated liposomal formulations on the blood stage activity against P. berghei infected Swiss albino mice. Delivery of antimalarial drugs using conventional liposomes (without PEGylation) displays modest efficacy under in vivo condition as shown in our previous findings. However, incorporation of PEG moiety on the liposomal surface can prolong the circulatory life of drugs with stealth property(Sadzuka et al. 2003). Thus, PEG 2000 chain length with 5mol% on liposomal surface was found to be optimal for efficient delivery of antimalarials (Rajendran et al. 2015). We observed a significant decrease in parasite load with 2.5 mg/kg of body weight dose of doxycycline in PEG liposomal formulation as compared to free drug showed negligible clearance in both strains. However, in chloroquine sensitive strain, on day 15 postinfection, in the untreated group, the parasitemia level was found to be 40-45%. Parasitemia in mice treated with 2.5 mg/kg and 10mg/kg of free doxycycline was 41% and 15%, free chloroquine was 8.5%, whereas it was 0.2% for SA bearing PEG-liposome, 0.5% for SPC:Chol PEG-liposome (without SA) (P<0.0001) .On the other hand, on day 11-15 post-infection, the %parasitemia in chloroquine resistant strain, in untreated group was found to be 50-55%, whereas, free drug 49% and doxy loaded liposomal formulations showed complete clearance (P<0.0001). However, free chloroquine at 5mg/kg administered to chloroquine resistant infected mice showed 30% parasitemia, free doxycycline at higher dosage 10mg/kg showed 17% parasitemia. Therefore, doxycycline loaded PEG-liposomes have shown equal effectiveness.
on clearing the blood parasites of both the strains of *P. berghei* infected mice. However, the placebo formulations without stearylamine and doxycycline had no observable effect on the killing of parasites. Therefore, the antimalarial properties of doxycycline in liposome elicits enhanced killing of parasites relative to free drug. The reduction in parasite burden in blood was reflected in the survival rates of treated animals. The median survival time of the treated group with doxy loaded liposome was more than 40 days, exhibiting a significant improved survival and showed no signs of sickness (*P*<0.0001). However, free doxycycline treated group and untreated group (control) exhibited marked death.

3.4 *Intra-erythrocytic uptake of fluorescently tagged SA-liposomes on the blood stages of* *P. falciparum* *in culture*. To determine the preferential uptake of coumarin-6 labeled SA-liposome in infected erythrocytes of *P. falciparum* in culture was studied in asynchronous of blood stage forms. The internalization of C-6 liposomes was examined after 4h of incubation. Fluorescence microscopy showed that C-6 labeled nanosized liposome were preferentially co-localized inside infected erythrocytes compared with uninfected ones as shown in Figure 3. Thus, it enables the delivery of drug contents to the homing site of the parasite infected erythrocytes more specifically. In contrast, normal cells showed negligible uptake on liposomes due to the rigidity of erythrocyte membrane.

4. **Discussion**: In the present study, we demonstrate that clinically used doxycycline was successfully loaded in liposomal formulations and the effect was determined on the growth inhibition of *Plasmodium falciparum* in culture and *P. berghei* infection model. Our results clearly indicate for the first time that antimalarial activity of doxycycline is significantly modulated following incorporation into liposomes with superior efficacy compared to free form. Among the liposomal formulations tested for the delivery of doxycycline in cultured parasites, maximum antimalarial activity was observed when delivered through SA liposome followed by SPC:Chol liposome with reduced IC$_{50}$ values. We have previously reported that stearylamine in liposome alone inhibits the growth of malaria parasite under *in vitro* and *in vivo* condition (Hasan et al. 2011; Rajendran et al. 2015). Therefore, combination of stearylamine and doxycycline display enhanced killing effect on parasites due to synergistic-additive action. Similarly, delivery of doxycycline in pheroid vesicles and other antibiotics showed enhanced efficacy with reduced IC$_{50}$ values compared to the drugs alone (Du Plessis et al. 2012). Recently development of tetracycline based derivatives display improved therapeutic effect as compared to doxycycline against malaria parasites (Draper et al. 2013). One of the most important feature of using tetracycline based antibiotics, till now no clinical
resistance been reported in human malarial species and doxycycline confer decreased susceptibility to *P. falciparum* clinical isolates (Gaillard et al. 2015 b,c). Apparently, a study has shown that doxycycline displayed slower resistance after several passages in *P. berghei* infected mice as compared to standard antimalarials such as chloroquine, quinine or pyrimethamine (Jacobs and Koontz 1976). Thus, delivery of antimalarials through nanosized vehicles would further enhance the efficacy and delay the onset of resistance for longer period in clinical settings (Bakshi et al. 2018; Urban and Fernandez-Busquets 2014).

During malarial infection, the erythrocyte membrane undergo numerous alterations with loss of rigidity leading to formation of new permeability pathways (NPP) thereby allowing the nanocarriers to reach internal site of parasites (Ginsburg and Stein 1987; Staines et al. 2005). The enhancement in antimalarial action of doxycycline in liposomes is due to their size (90-110nm) and intracellular delivery of drug molecules towards parasitophorous vacuole. Our findings suggest that preferential entry of lipid vesicles is more prominent during the mature stage (trophozoites, schizonts) of the parasites due to numerous changes and remodeling of cell permeability (Figure 3). Evidently both mitochondria and apicoplast are well developed and functionally active in mature stages of *P. falciparum* (van Dooren et al. 2005). Thus, targeting both the prokaryotic organelle of plasmodium causes detrimental effect on parasite growth. We also observed there is significant reduction in parasite load in *P. berghei* infected mice when doxycycline was delivered through long circulating PEGylated liposomes with 5mol% DSPE-mPEG-2000 on the liposomal surface. This is in agreement with our previous study and other reports that circulatory life of therapeutic agents can be significantly modulated using polyethylene glycol-coated liposomes having optimal PEG density on surface against *P. berghei* infection in mice (Mosqueira et al. 2001; Postma et al. 1999; Rajendran et al. 2015). A recent study has shown that administration of doxycycline and new synthetic tetracyclines at higher dosages (10mg/kg/day) for four days offers differential efficacy in *P. berghei* infected mice (Draper et al. 2013). Our study indicates that administration of doxycycline in PEG-liposomes at lower dosages (2.5mg/kg/day) for four days completely reduced the blood parasite load and extended survival as compared to free drug as shown in Figure 1 and 2. Moreover, doxycycline in PEG-liposomes showed equal potency in curing chloroquine sensitive and resistant strains in mice at lower dose not reported earlier. Interestingly, doxy-liposomes treated mice (*n*=5) did not show onset of activity and reappearance of parasites in the blood circulation upto day 40 post-infection. Also, the longer exposure of doxy in PEG-liposomes in blood circulation facilitates enhanced
killing of the blood stage parasites. Reduced drug dosages through liposomal delivery are favorable to host cells by overcoming undesirable toxic effects and thereby increase the clinical life of doxycycline and other drugs.

5. Conclusions

We successfully intercalated doxycycline into liposomes and evaluated its efficacy in the treatment of human falciparum malaria in culture and *P. berghei* based mouse models. The developed liposomal formulation exhibited no cytotoxic effect on normal erythrocytes. Therefore, our findings suggest that doxycycline in liposomal formulations may offer extended protection against falciparum malaria at clinically achievable dosages.

Conflicts of Interests

The authors declare that they have no conflicts of interest.

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References:


Figure legends:

Figure 1: Effect of doxycycline in long circulating PEG-liposomes on parasitemia of *P. berghei* NK65 (chloroquine sensitive and resistant) strains infected Swiss Albino mice. (A) and (B) The infected mice were treated with either free doxycycline (doxy) or doxy in liposomes daily for four consecutive days after day 2 post-infection (*n*=6/group). The mean parasitemia of the treatment and control groups were determined on day 5, day 8 and day 15 post infections with error bar representing SD.

Figure 2: Survival pattern of *P. berghei* infected mice treated with doxycycline in PEG-liposomes. (A) and (B) Survival response of *P. berghei* infected mice (CQ sensitive and resistant) to doxy in liposomal formulations versus free drug up to day 40 post-infection. Median survival time (MST) of animals in each group (*n*=6) is shown in parantheses.

Figure 3: Uptake of fluorescently labeled SA liposomal formulation by *Plasmodium falciparum* infected erythrocytes in culture. Coumarin-6 (C-6) labeled liposomes were added to culture of *P. falciparum*. The internalization of liposomes was determined after 4h of incubation by fluorescence microscopy at 1000x magnification. DAPI stains the Plasmodium nuclei (Blue), Coumarin-6 (Green).
Table 1: Determination of size and zeta potential of doxycycline in liposomal formulations.

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<th>Zeta potential (ζ) (mV)</th>
<th>PDI</th>
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<tr>
<td>SPC+Chol+DOXY</td>
<td>91.38 ± 11</td>
<td>-8.16 ± 0.54</td>
<td>0.228 ± 0.03</td>
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<tr>
<td>SPC+Chol+SA+DOXY</td>
<td>105.1 ± 5</td>
<td>16.46 ± 0.86</td>
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<tr>
<td>SPC+Chol+DSPEmPEG-2000+DOXY</td>
<td>88.18 ± 10</td>
<td>-14.13 ± 0.71</td>
<td>0.201 ± 0.01</td>
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<tr>
<td>SPC+Chol+SA+DSPEmPEG-2000+DOXY</td>
<td>101.88 ± 6</td>
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Dynamic light scattering (DLS) was used to measure the size in diameter and zeta potential of doxycycline (DOXY) loaded in liposomal formulations. The determined values are represented as the means ± standard deviations (n=5). PDI, polydispersity index.
Figure 1

*P. berghei* NK-65 (CQ-sensitive)

A

- Control (Untreated)
- Liposome formulation (placebo)
- Free Doxycycline (DOXY) 2.5mg/kg
- Free Doxycycline 10mg/kg
- Free Chloroquine 5mg/kg
- PEG-liposomal DOXY 2.5mg/kg
- PEG-SA liposomal DOXY 2.5mg/kg

% Parasitemia

Day 0, Day 3, Day 6, Day 9, Day 13

day post infection

*P. berghei* NK-65 (CQ-resistant)

B

- Control (Untreated)
- Liposome formulation (placebo)
- Free Doxycycline (DOXY) 2.5mg/kg
- Free Doxycycline 10mg/kg
- Free Chloroquine 5mg/kg
- PEG-liposomal DOXY 2.5mg/kg
- PEG-SA liposomal DOXY 2.5mg/kg

% Parasitemia

Day 0, Day 3, Day 6, Day 9, Day 13

day post infection

Figure 1

144x274mm (300 x 300 DPI)
Figure 2  

\textbf{P. berghei} NK-65 (CQ- sensitive) 

A  
- Control (Untreated) (MST -15.5) 
- Liposome formulation (placebo) (MST -14.5) 
- Free Doxycycline 2.5mg/kg (MST -16) 
- Free Doxycycline 10mg/kg (MST -24) 
- Free Chloroquine 5mg/kg (MST -32.5) 
- PEG-liposomal DOXY (MST >40) 
- PEG-SA liposomal DOXY (MST >40) 

\textbf{P. berghei} NK-65 (CQ- resistant) 

B  
- Control (Untreated) (MST -11.5) 
- Liposome formulation (placebo) (MST -11) 
- Free Doxycycline 2.5mg/kg (MST -12.5) 
- Free Chloroquine 5mg/kg (MST -15.5) 
- Free Doxycycline 10mg/kg (MST -21.5) 
- PEG-liposomal DOXY 2.5mg/kg (MST >40) 
- PEG-SA-liposomal DOXY 2.5mg/kg (MST >40) 

Figure 2 

147x277mm (300 x 300 DPI)
Figure 3

_P. falciparum_ infected erythrocytes in culture

Bright Field | DAPI | Coumarin-6
---|---|---

101x66mm (300 x 300 DPI)
PEGylated DOXY in Liposomes

Human falciparum malaria in culture

Murine model of malaria

Improved efficacy of DOXY in Liposomes in treating malaria at reduced dosages