

Co-delivery of chloroquine phosphate and azithromycin nanoparticles to overcome drug resistance in malaria through intracellular targeting.

Natarajan Jawahar^{a*}, Uday Krishna Baruah^a, Vineeta Singh^b

^a Department of Pharmaceutics, JSS College of Pharmacy, Udhagamandalam.

Faculty of pharmacy, JSS Academy of Higher education and research.

^b Scientist 'C', National Institute of Malaria Research, New Delhi.

Abstract

Malaria remains a leading cause of mortality worldwide, killing over 3,000 children every day. The resistance to all the major antimalarials drugs have been developed in majority parts of the world by the malaria parasite. To overcome the problem of drug resistance combination of drugs and nanotechnology based drug delivery systems are likely to be the new strategies in combating drug resistance. Chloroquine phosphate is the most widely used antimalarial drug with lot more advantages compared to the other antimalarial drugs. But due to the development of chloroquine resistance by the plasmodium strains in most parts of the world, it is no more effective in the treatment against malaria. In this work, we developed a polymeric nanospheres containing combination of chloroquine and azithromycin, an azalide, a subclass of macrolide antibiotic which has an antimalarial activity with the help of double emulsion technique. The least particle size obtained was 89.6 nm, polydispersity index (PDI) of 0.236, and zeta potential of -13.2mV. Combination of chloroquine and azithromycin showed synergistic effects against parasite growth in-vitro with EC₅₀ of 1.953µg/ml and IC₅₀ of 1.11µg/ml. The in-vitro data suggest that combination of chloroquine and azithromycin into PLGA-nanoparticles will have clinical utility in overcoming the problem of drug resistance through intracellular targeting as well as useful for oral administration.

Key Words: Malaria, Chloroquine phosphate, resistance, drug delivery, polymeric nanospheres, intracellular targeting

INTRODUCTION

Malaria remains a leading cause of mortality worldwide, killing over 3,000 children every day.¹ There were an estimated 247 million malaria episodes in 2006. Around 86% of these cases occurred in Africa, causing over 1.25 million deaths.² Resistance against the many existing antimalarials is well documented,³ and combining drugs can limit the emergence of resistance. The design of nanotechnology-based delivery systems applied to drugs and antigens are likely to be the main strategies in combating this disease.⁹

Polymeric nanospheres (NS) constitute the most thoroughly investigated nanocarriers for drug delivery, after liposomes.^{4,5,6} The surface properties of the NS can be modulated using a wide variety of polymers in order to obtain passive or active targeting in the body. Due to their polymeric nature, NS generally possess high stability in biological fluids and under stress conditions of preparation and storage, which qualifies them as promising drug delivery systems for antimalarials. However, when compared with other drugs, fewer reports have been published on the delivery of antimalarial drugs using polymeric systems such as NS.

The only report found on antimalarial drugs discusses the entrapment of PQ in albumin-NS prepared by a combined emulsification and congealing procedure.⁷ However, these formulations were not tested in vivo. One initial attempt has been made to demonstrate the susceptibility of *P. falciparum* to antisense oligonucleotide nanoparticles (ODN-NS).⁸ This interesting approach used antisense ODN chitosan NS about 50 nm in size to improve antisense ODN internalization by *P. falciparum*-infected erythrocytes through erythrocyte permeation pathways. The target was the malarial topoisomerase II gene.⁸ Both negatively and positively charged antisense ODN chitosan NS, as well as free antisense ODNs in a concentration of 0.5 µM, showed a sequence-specific inhibition compared with sense sequence controls. However, ODN-chitosan NS were much more sequence specific in their antisense effect than free ODNs. ODN-chitosan NS with a negatively-charged surface exhibited a significantly stronger inhibitory effect (87%) on the *P. berghei* growth in comparison with the positive ones (74%) or free ODNs

(68%). To our knowledge, this is the first study demonstrating the susceptibility of *P. falciparum* to antisense ODN-NS.⁹

To prevent drug-resistance mechanisms in malaria therapy, new strategies for intracellular antimalarial delivery are urgently needed.¹⁰ Both Chloroquine transporters could be probably bypassed by drugs encapsulated in nanosystems able to pass through the digestive vacuole membrane by different mechanisms.^{11,12} These mechanisms could be membrane fusion and membrane-induced instability triggered by pH differences in the cell compartments. These approaches can be exploited in order to reverse CQ-resistance using nanocarriers loaded with CQ. One of these suggested strategies has already been reported with CQ entrapped in pH-sensitive liposomes, even though in vivo investigations were not carried out.¹³

Combination therapy is one method of overcoming the global challenge of drug-resistance *Plasmodium falciparum* malaria. Antibiotics are used in combination with antimalarial drug against multidrug-resistant *P. falciparum*. Combination of the tetracycline group of antibiotics with quinine and mefloquine has been used successfully in Southeast Asia to treat multidrug-resistant *P. falciparum* cases.^{14, 15} The artesunate-tetracycline combination has been shown to be a useful alternative.^{16, 17} The drugs of the tetracycline group should not be used in pregnant women and young children..

Azithromycin is a semisynthetic derivative of erythromycin that differs from erythromycin by possessing methyl-substituted nitrogen in the macrolide ring. This alteration allows for greater bioavailability, increased tissue penetration and longer elimination half-life compared with those of erythromycin.¹⁸ Azithromycin has antimalarial activity against *P. falciparum* in vitro, *P. berghei* and *Plasmodium yoelii* in vivo.^{19,20,21} The clinical trial of daily oral azithromycin in adults is effective for prevention of *Plasmodium vivax* malaria.²² Azithromycin is approved for the use in children (Zithromax product information, 1999; Pfizer Inc., New York), and is safe for pregnant women.^{23,24} The objective of this in vitro study is to determine and formulate a nano drug combination between azithromycin and standard antimalarial drugs against multidrug-resistant *P. falciparum*.

MATERIALS AND METHODS

Materials

Chloroquine Phosphate was a gift from Ipca Laboratories Limited(M.P.), Azithromycin was a gift sample from Medopharm, Malur., Poly (D, L-lactide-co-glycolide) (PLGA) purchased from Sigma Chemicals, (Mumbai, India). Poloxamer 188 (Pluronic F 68) was purchased from Sigma Aldrich (USA). Dialysis bag for *in-vitro* release studies was procured from Hi Media, (Mumbai, India). All other reagents used were analytical and Pharmaceutical grade.

PREPARATION OF POLYMERIC NANOPARTICLES (PNPs)

Polymeric nanoparticles were prepared by double emulsion technique.²⁵ First, w/o microemulsions were prepared. The oil phase was DCM. Pluronic F-68 was used as a hydrophilic surfactant solution was used as continuous phase. The chosen polymer and the drug was added under stirring in DCM. Then the other drug Chloroquine Phosphate dissolved in distilled water was added drop wise into the (polymer+drug) oil phase. 40 mL of aqueous surfactant solution was prepared and the w/o phase solution prepared previously was added dropwise under stirring. After completion of stirring, the PNPs solution was subjected to ultrasonication for 1 minutes. Out of all the batches the batches with lesser particle size was selected and was freeze dried.

EVALUATION POLYMERIC NANOPARTICLES

Particle size and zeta potential

Particle size and zeta potential of the solid lipid nanoparticles were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK), which works on the Mie theory. All size and zeta potential measurements were carried out at 25°C using disposable polystyrene cells and disposable plain folded capillary zeta cells, respectively, after appropriate dilution with original dispersion preparation medium.²⁴

Polydispersity Index:

Polydispersity was determined according to the equation,

$$\text{Polydispersity} = \frac{D(0.9) - D(0.1)}{D(0.5)}$$

Where,

D (0.9) corresponds to particle size immediately above 90% of the sample.

D (0.5) corresponds to particle size immediately above 50% of the sample.

D (0.1) corresponds to particle size immediately above 10% of the sample.²⁵

Encapsulation Efficiency and Drug Loading:

Entrapment efficiency and drug loading of freeze dried Polymeric nanoparticles (PNP's) was determined according to the procedure described previously.²⁶ Weighed quantities of PNP's (10mg) were dissolved in ACN +distilled water and kept in sonicator for 1 hour Drug content in the supernatant after centrifugation (4000 rpm for 15 min) was determined by UV visible spectroscopy at 210 AND 343 nm using ACN + distilled water as blank.

Drug entrapment efficiency (%)

$$= \frac{\text{Analyzed weight of drug in SLNs}}{\text{Theoretical weight of drug loaded in SLNs}} \times 100$$

Drug loading (%)

$$= \frac{\text{Analyzed weight of drug in SLNs}}{\text{Analyzed weight of SLNs}} \times 100$$

Entrapment efficiency = 1 - Free drug/ Theoretical drug loaded × 100

External Morphological Study SEM (Scanning electron microscopy):

External morphology of nanoparticles was determined using Scanning Electron Microscopy (SEM). Samples were diluted with ultra purified water to obtain a suitable concentration. Then the

samples were spread on a sample holder and dried using vacuum. They were subsequently coated with gold (JFC 1200 fine coater, Japan) and examined by a Scanning Electron Microscopy (SEM).

Differential scanning calorimetry (DSC)

DSC analysis was performed using DSC Q200 (TA Instruments, USA). A heating rate of 10°C/min was employed at a range of 20-310°C. Analysis was performed under nitrogen purge (50ml/min). A standard aluminium sample pans were used. About 5mg sample was taken for analysis. An empty pan was used as reference. DSC thermograms were recorded for Chloroquine Phosphate (CP), Azithromycin (AZI), PLGA and (CP+AZI) loaded nanoparticles.²⁶

Drug content determination

100 mg of PNP's was taken and dissolved in ACN & distilled water (1:1) and sonicated for 1 hr. The resulted solution was filtered with 0.22µ filter to obtain clear solution. The standard plot of drugs was prepared in ACN and distilled water. Then the drug content was measured using UV at detection wavelength of 210 nm and 342nm using the formula: Conc of sample/conc of standard X 100

In-vitro release studies

The dialysis bag diffusion technique was used to study the *in-vitro* drug release of Olanzapine PLGA-nanoparticles.²⁷ The release of Chloroquine Phosphate and Azithromycin from the PNPs was studied under sink conditions. Batches which showed higher drug content and entrapment efficiency were evaluated for *in vitro* release. PNPs equivalent to 1.24mg Chloroquine phosphate and 2.5mg Azithromycin were suspended in 10ml of dissolution media (Phosphate buffer PH 7.4) and Dialysis membrane having pore size 2.4nm and molecular weight cut off 12000-14000 (Dialysis membrane-150, HiMedia, Mumbai, India) was used. The dialysis bag retains nanoparticles and allows the free drug into the dissolution media.²⁸ The dialysis bags were placed in 50mL of dissolution medium and stirred using magnetic stirring at 37±1.0 °C. Aliquots of the dissolution medium were withdrawn at each time interval and the same volume of fresh dissolution medium was added to maintain a constant volume. Samples withdrawn from pH 7.4 phosphate buffer were analyzed for Chloroquine Phosphate and Azithromycin content spectrophotometrically at 343 nm and 210nm against solvent blank.

Release kinetics

In vitro dissolution has been recognized as an important element in drug development. Under certain conditions it can be used as a surrogate for the assessment of bioequivalence. Several theories/kinetic models describe drug dissolution from immediate and modified release dosage forms. There are several models to represent the drug dissolution profiles where f_t is the function of t (time) related to the amount of drug dissolved from the pharmaceutical dosage system. To compare dissolution profiles between two drug products model dependent (curve fitting), statistic analysis and model independent methods can be used.²⁹ In order to elucidate mode and mechanism of drug release, the *in-vitro* data was transformed and interpreted at graphical interface constructed using various kinetic models.

Mechanism of Drug release

Korsmeyer et al. developed a simple, semi empirical model, relating exponentially the drug release to the elapsed time (t)

$$f_t = K t^n$$

K is a constant incorporating structural and geometrical characteristic of the drug dosage form, n is the release exponent, f_t is M_t / M_∞ (fractional release of drug).

Depending on the relative magnitude of the rate of polymer swelling to the rate of drug diffusion, various release profiles may be possible. The situation where the polymer structural rearrangement takes place rapidly in response to the swelling solvent as compared to the rate of drug diffusion generally leads

to Fickian diffusion, or the so-called first order release, characterized by square root of time dependence in both the amount released and the penetrating diffusion front position in slab geometry.

In case of sorption process is completely governed by the rate of polymer relaxation, the so-called Case II transport, characterized by linear time dependence in both the amount diffused and the penetrating swelling front position, results. In most systems, the intermediate solution, which is often termed non-Fickian or anomalous diffusion, will prevail whenever the rates of diffusion and polymer relaxation are comparable.

Kinetic constant incorporates structural and geometrical characters of the drug/polymer system. For non-Fickian release, the n value falls between 0.5 and 1.0 ($0.5 < n < 1.0$), whereas in the case of Fickian diffusion, $n = 0.5$; for zero-order release (case transport), $n = 1$, and for Supercase II transport, $n > 1$. The values of n as estimated by linear regression of $\log(M_t/M_\infty)$ vs $\log(t)$ of different formulations were calculated.

Interpretations of Diffusion Mechanisms from Dosage Forms

Release exponent (n)	Drug transport mechanism	Rate as a function of time
0.5	Fickian diffusion	$t^{-0.5}$
$0.5 < n < 1.0$	Anomalous transport	t^{n-1}
1.0	Case-II transport	Zero order release
Higher than 1.0	Super case-II transport	t^{n-1}

In-Vitro: Growth inhibition assay

In-vitro analysis was performed at National Institute of Malaria Research, New Delhi. Strain of *P.falciparum* was cultured continuously according to the candle jar method of Trager and Jensen *in vitro* in human red blood cells (blood type B) with 5% hematocrit in LIQUID RPMI-1640 medium (HIMEDIA) supplemented with 25 mM HEPES (Sigma), 0.2% sodium bicarbonate (Sigma) and 10% human B⁺ serum. Slides of culture were observed after 3, 6 and 24 h for regular development of parasite stages.

Chloroquine sensitive *P.falciparum*, 3D7 strain was used in the experiment. The culture was synchronized using sorbitol and parasitaemia was adjusted to 1-1.5% by diluting with fresh human erythrocytes. The cells were diluted with complete media to make 8% haematocrit. Again the slides of culture were prepared and observed for the calculation of parasitemia, particularly for young trophozoites or ring stages. One mg of each compound/extract was dissolved in 100 μ L DMSO and 900 μ L RPMI-1640 to obtain a stock of 1mg mL⁻¹ (stock solution). A series of eight concentrations were prepared from the stock solutions by two fold dilutions. After 24 h, thin films of the contents of each well were prepared and examined under the microscope.

Parasite count for each blood film was made using a compound microscope under the oil immersion with X100 objectives after staining the film with eosin yellow and methylene blue. Each film was observed at three different visual fields. The number of schizonts per 200 parasites were noted and compared between control and test wells for the determination of the % inhibition. All doses were studied in cultures and the mean was observed for purposes of inferences. The inhibition of parasite growth in the drug treated groups was calculated as follows: Parasitaemia in the control (non-treated) group, expressed as percentage.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistical significances in cumulative release rate. The analysis of all data was performed in the Graph Pad Prism v.5.0 software (Trial version, Graph Pad Software, CA, USA).

RESULTS AND DISCUSSION

Particle size and zeta potential

Particle size, PDI and zeta potential of the prepared formulations detected by Zetasizer are shown in Table 1 and Figure 1. The mean particle size of each formulation was approximately 99nm. The PDI of each formulation was lower than 0.4, indicating the homogeneous nature of the formulation. No multi-scattering phenomenon was observed.

As shown in Table 1, all formulations exhibit negative charge of > -10 mV. The most likely origin of this negative charge is the presence of the nonionic-type surfactant, Pluronic F-68. Compared with blank NPs, we observed a slow increase in zeta potential for CP-AZI nanoparticles, which might be caused by a partial absorption of CP-AZI on NPs surface and the masking of negative charges in surfactant.

Table 1. Characteristics of blank and drug-loaded NPs

Formulation	Size (nm)	PDI	Zeta potential (mV)
Blank NPs	101.7	0.287	-12.7
CP-AZI NPs	89.7	0.236	-13.2

Abbreviations: CP-AZI NPs, Chloroquine Phosphate-Azithromycin loaded nanoparticles

Polydispersity: The polydispersity index (PI) is the measure of size distribution of the nanoparticle formulation. PI was measured using Malvern zetasizer. PI values range from 0.416 to 0.236 i.e. monodisperse to very broad particle size distribution. PI values of all the formulations indicate that particle size distribution was unimodal. The optimized batch having least particle size (89.7nm) had a PDI of 0.236 as shown in figure 1 and figure 2.

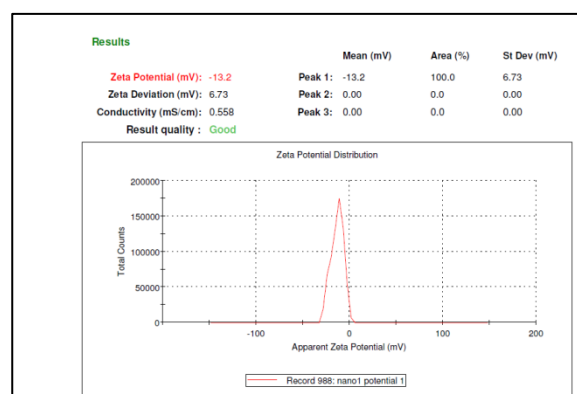


Figure 1. Zeta potential report for the nano formulation

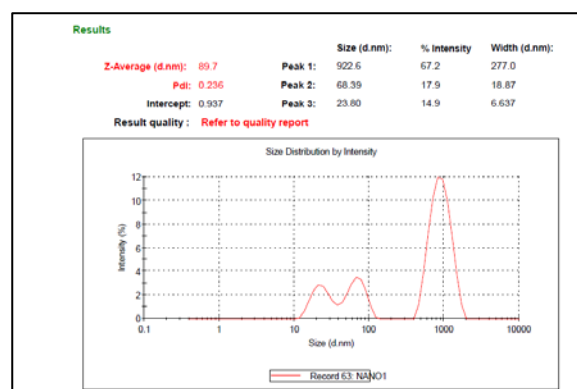


Figure 2. Particle size distribution and polydispersity Index for nano formulation

Entrapment efficiency and Drug loading:

The polymeric core was found to affect the extent of entrapment efficiency. As observed with PNP with 1:2:5 and 1:2:3.5 ratios of drug: polymer the maximum entrapment efficiency was 59.75%(CP),75.38%(AZI) and 56.85%(CP),76.83%(AZI) respectively as shown in table 2. There was not much significant difference between the entrapment efficiencies incase of 1:2:3.5 and 1:2:5. The entrapment efficiency in case of 1:2:5 and 1:2:3.5 was much higher than 1:2:2.5 and 1:2:1.25 because of presence of higher amount of polymer in the former.

Polymer to surfactant ratio can affect the loading efficiency of the formulations. The amount of pluronic F-68 was optimized in range of 2-5% w/w against a constant amount of polymer. It was observed that 5% w/w pluronic F-68 could entrap maximum drug. The possible reason might be that at this concentration surfactant provided sufficient covering to the lipid core so as to minimize possible leaching of the drug.

Table 2. Entrapment efficiency and drug loading of drug loaded batches with varying concentrations of drug: Polymer ratio

Drug: Polymer ratio	Encapsulation efficiency (%)		Drug loading (%)	
	CP	AZI	CP	AZI
1:1.25	45.52	61.4	6.78	7.12
1:2	56.85	76.83	8.4	7.84
1:3.5	52.95	71.55	8.18	9.89
1:5	59.75	75.38	7.89	9.67

Abbreviations: CP,Chloroquine Phosphate ; AZI,Azithromycin

Scanning electron microscopy (SEM): The External morphological studies (SEM) revealed that maximum nanoparticles were nearly spherical in shape (Fig. 3). The nanoparticle size observed by SEM correlated well with the particle size measured by zeta sizer (Malvern instrument).

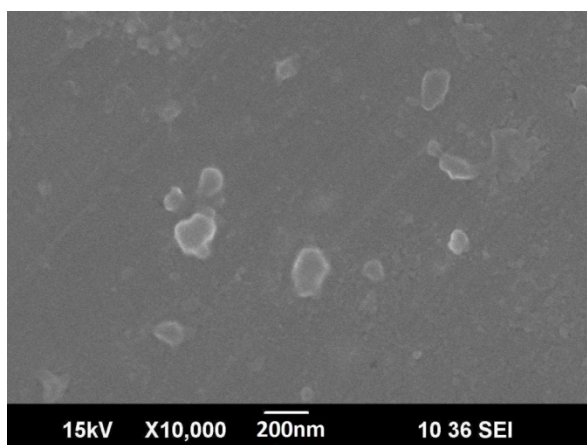


Fig 3. Scanning electron micrograph (SEM) of polymeric nanoparticles.

Differential scanning calorimetry (DSC)

DSC was employed to investigate the thermal behavior of crystalline chloroquine phosphate, azithromycin, PLGA. DSC thermograms were recorded for chloroquine phosphate, azithromycin, PLGA, nano formulation. Fig.4 shows DSC curves of chloroquine phosphate, azithromycin, PLGA and nanoparticles. The DSC curve of chloroquine phosphate, azithromycin showed a melting endotherm at 200.73°C and 126.19 °C. The peak intensity corresponding to the melting of chloroquine phosphate, azithromycin decreased in thermograms of (chloroquine phosphate+azithromycin) loaded nanoparticles (172.24 °C and 52.18 °C). These results indicate that only a small fraction of the

drug substance existed in the crystalline state. Reduction in the melting point and the enthalpy of the melting endotherm was observed. Incorporation of (chloroquine phosphate + azithromycin) inside the Polymer matrix results in an increase in number of defects in the crystal lattice, and hence causes a decrease in the melting point of the final formulations. Small particle size of nanoparticles leads to high surface energy, which creates an energetically suboptimal state causing a decrease in the melting point. This melting point depression might be due to small particle size (nanometer range), the high specific area, and the presence of surfactant. This melting point depression can be attributed to Kelvin effect.

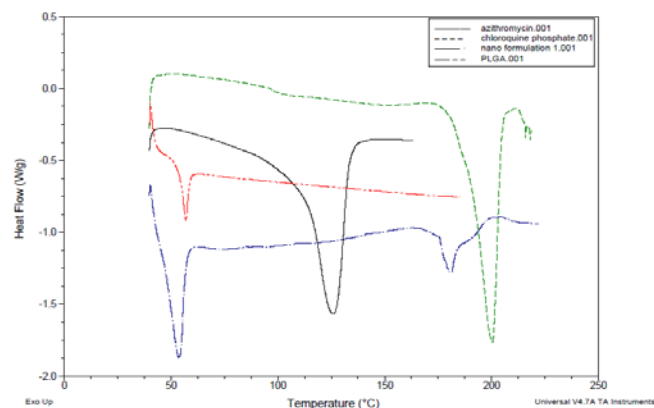


Fig 4 : DSC spectra chloroquine phosphate, azithromycin, PLGA and nanoparticles

Drug Content Determination:

The drug content was determined in 115mg nanoparticles. The drug content was found to be 28.45% and 40.9% AZI and CP when the samples were determined under UV at 210nm & 343nm.

% Drug Content = $\frac{\text{Drug analyzed in N.P}}{\text{theoretical drug Loaded}} \times 100$

AZI = $\frac{11.38}{40} \times 100 = 28.45\%$

CP = $\frac{9.41}{20} \times 100 = 40.9\%$

In vitro release studies:

In vitro dissolution studies were carried out in Phosphate buffer pH 7.4 for the PNP_s batches showing the least particle size. The release profiles indicate that PNP_s formulations showed a retarded release of the drug from the polymer matrix. Hence it proves that that the formulated PNP_s shows sustained release of the drug as shown in table 3.

Table 3. Comparative in vitro cumulative percent release data for PNP_s formulations in 7.4 pH phosphate buffer for Chloroquine phosphate(CP) and Azithromycin(AZI)

TIME (Hours)	CUMULATIVE PERCENT RELEASE (%)			
	(F1)		(F2)	
	CP	AZI	CP	AZI
0	0	0	0	0
1	20.78	5.11	20.78	5.95
2	24.36	10.14	25.52	10.15
3	30.38	17.70	29.13	19.37
4	33.92	33.63	33.92	34.48
6	41.04	47.98	39.89	45.50
12	50.52	54.91	52.83	56.58
24	65.84	68.55	64.69	72.73
36	76.61	83.09	77.77	84.79
48	86.28	88.55	89.76	91.09

Abbreviations: CP,Chloroquine Phosphate ; AZI,Azithromycin

Table 4. Regression value for various kinetic models Chloroquine phosphate(CP) and Azithromycin(AZI)

Formulation	R ²							
	Zero order		First order		Higuchi's		Peppas	
	CP	AZI	CP	AZI	CP	AZI	CP	AZI
F1	0.866	0.826	0.980	0.969	0.983	0.943	0.976	0.884
F2	0.88	0.838	0.981	0.982	0.986	0.954	0.979	0.898

Abbreviations: CP,Chloroquine Phosphate ; AZI,Azithromycin

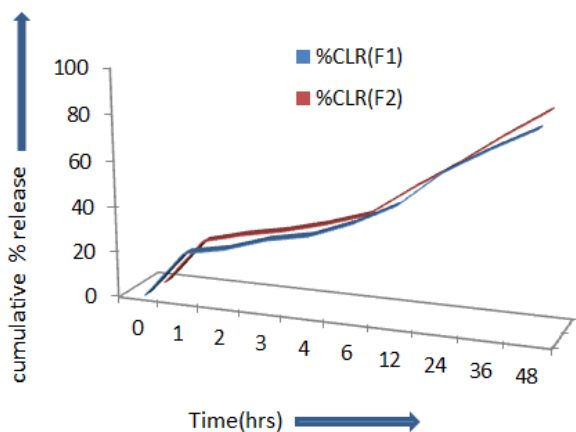


Fig 4. Comparative *in vitro* cumulative percent release graph for PNP formulations in 7.4 pH phosphate buffer for Chloroquine phosphate

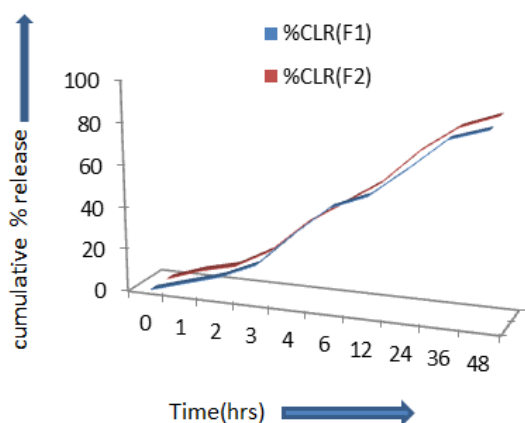


Fig 5. Comparative *in vitro* cumulative percent release (%) graph for PNP formulations in 7.4 pH phosphate buffer for Azithromycin

Release kinetics

In order to elucidate mode and mechanism of drug release, the *in vitro* data was transformed and interpreted at graphical interface constructed using various kinetic models including zero order, first order, Higuchi's and Ritger-peppas . The *in vitro* release data obtained for polymeric nanoparticle formulations, in phosphate buffer pH 7.4, was fitted into various kinetic models. The results are shown in Table.

The best linearity was obtained in Higuchi's plot for PNP formulations indicating the release from matrix as a square root of time dependent process. The Higuchi's plot for batches F1 and F2 for the drugs Chloroquine phosphate and Azithromycin are shown in Fig. 20, 21, 22 and 23.

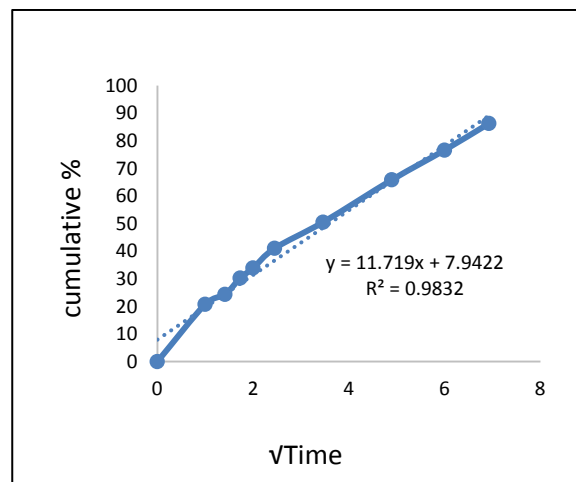


Fig 6. Higuchi's plot for Chloroquine phosphate (F1)

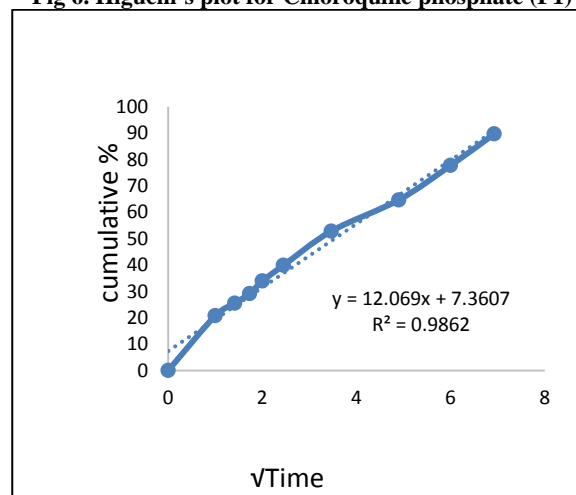


Fig 7. Higuchi's plot for Chloroquine phosphate (F2)

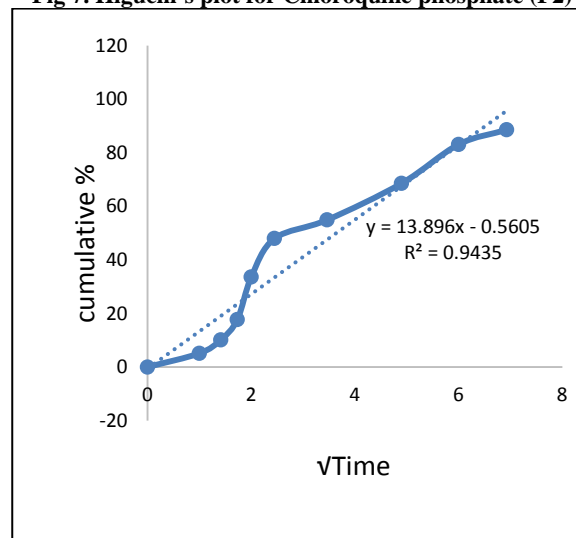


Fig 8. Higuchi's plot for Azithromycin (F1)

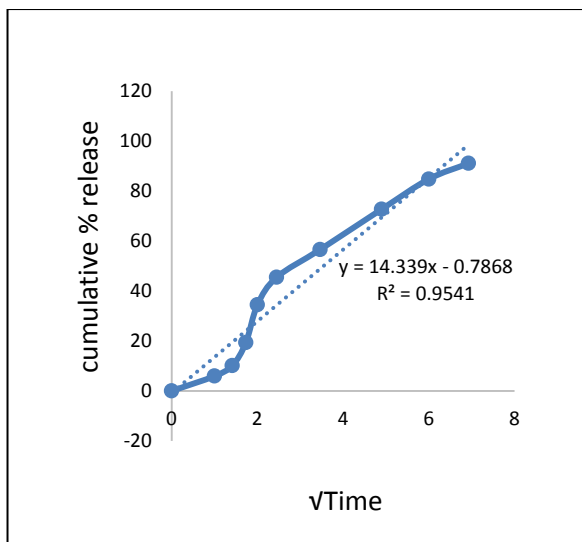


Fig 9. Higuchi's plot for Azithromycin (F2)

Release mechanism: By incorporating the release data in Korsmeyer-Peppas's equation, the mechanism of the drug release can be indicated according to the value of release exponent 'n'. Peppas's plot for PNPs are given in Fig. 24, 25, 26 and 27

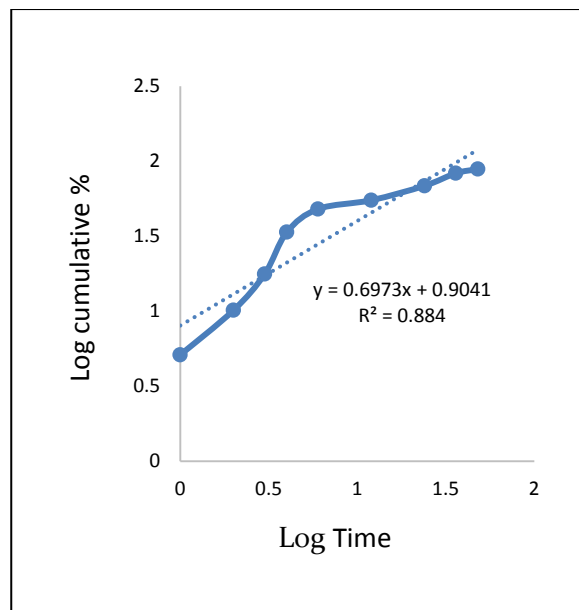


Fig 12. Korsmeyer Peppas's plot for Azithromycin (F1)

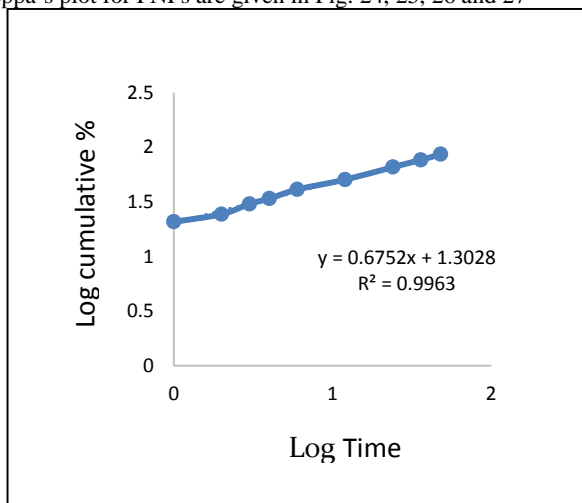


Fig. 10. Korsmeyer Peppas's plot for Chloroquine phosphate (F1)

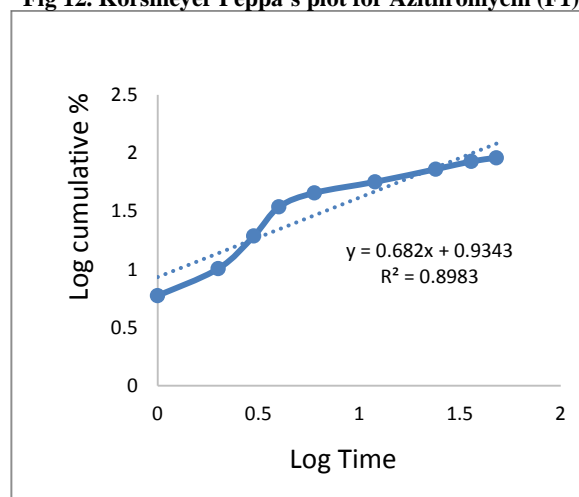


Fig 13. Korsmeyer Peppas's plot for Azithromycin (F2)

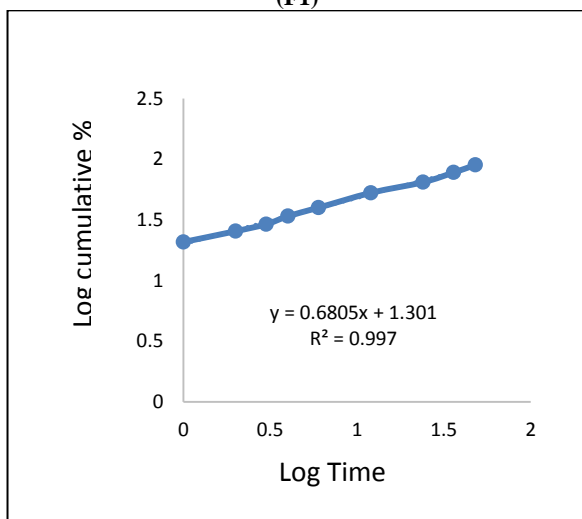


Fig 11. Korsmeyer Peppas's plot for Chloroquine phosphate (F2)

The release exponent values 'n' for F1 and F2 was found to be 0.675 and 0.680 for Chloroquine phosphate and 0.697 and 0.682 respectively for Azithromycin. Since, the release exponent 'n' values were between 0.5-1, it indicates that the PNPs formulations F1 and F2 undergo anomalous diffusion.

In-vitro: Growth inhibition assays:

The *in-vitro* growth inhibition assays were carried out for the two formulated PNPs batches having the least particle size against the Strain of *P.falciparum* cultured continuously according to the candle jar method of Trager and Jensen *in vitro* in human red blood cells (blood type B). The inhibition of parasite growth for the two formulated PNPs batches was calculated for the EC₅₀ and IC₅₀ value. The EC₅₀ and IC₅₀ value for the two formulated PNPs batches were shown in the table 18.

Table 5. EC₅₀ and IC₅₀ values for the batches F1 and F2

Formulation	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)
F1	1.953	1.11
F2	1.961	1.2
Chloroquine Phosphate	0.0768	0.0436
Azithromycin	15.86	8.4

From the obtained EC_{50} and IC_{50} value for the PNP's batches we can say the drug combination of Chloroquine phosphate and Azithromycin showed synergistic effect against the Chloroquine resistant *P.falciparum*.

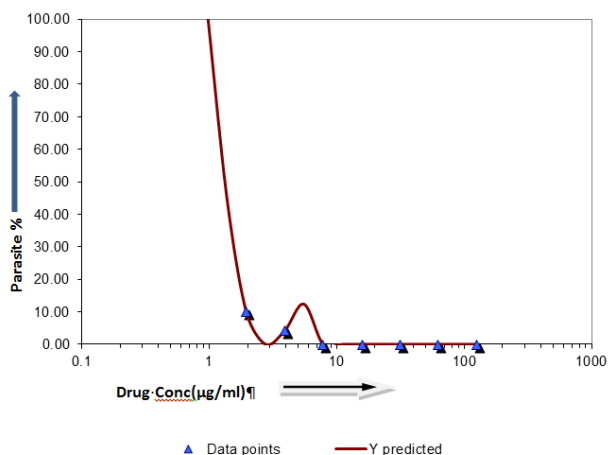


Fig 14. Graph showing growth inhibition of parasite.

DISCUSSIONS:

The study on various process/ formulation variables revealed that all the variables are important in formulation of Polymeric nanoparticles. Batch prepared with 250mg Polymer concentration, 8 hours stirring time, 4000 rpm stirring speed and 5.0% w/v surfactant concentration showed minimum particle size and was identified as an ideal batch.

The entrapment efficiency and drug loading was found to be higher in PNP prepared by 1:2:5 drug₁:drug₂: Polymer concentrations compared to 1:2:1.25, 1:2:3.5 and 1:2:2.5. Hence, 1:2:5 batch was chosen for the further studies that is *in vitro* diffusion studies.

Batches prepared with pluronic F-68 gave ideal zeta potential value.

In-vitro studies indicated that the PNP_s showed good inhibitory effect on the Strain of *P.falciparum*.

CONCLUSION:

In the present study, double emulsion technique can be used as a method for the lab-scale production of PNP's. hydrophilic drug like Chloroquine phosphate and hydrophobic drug like Azithromycin can be successfully incorporated into the polymer. The formulated polymeric nanoparticles showed inhibitory effect on the malarial strain and provided sustained release of the drugs. Thus the formulated PNP_s containing Chloroquine phosphate and Azithromycin can be used to overcome the problem of drug resistance, which was confirmed by the IC 50 value of *in vitro* growth inhibition assay. This might be due to the PNP's reaching to the intracellular level.

Acknowledgments

This work was supported by Tamilnadu Pharmaceutical Sciences Welfare Trust, Chennai and National Institute Of Malaria Research (ICMR), New Delhi.

Disclosure

The authors report no conflicts of interest in this work.

REFERENCES

1. WHO, World Malaria Report (2008), <http://www.who.int/malaria/wmr2008/malaria2008.pdf>, accessed on 15th April 2009.
2. F. Gardella, S. Assi, F. Simon, H. Bogreau, T. Eggelte, F. Ba, V. Foumane, M.C. Henry, P.T. Kientega, L. Basco, J.F. Trape, R. Lalou,

- M. Martelloni, M. Desbordes, M. Baragatti, S. Briolant, L. Almeras, B. Pradines, T. Fusai, C. Rogier, Antimalarial drug use in general populations of tropical Africa, *Malaria J.* 7 (2008).
3. Dondorp, A. M.; Yeung, S.; White, L.; Nguon, C.; Day, N. P.; Socheat, D.; von Seidlein, L. *Nat. Rev. Microbiol.* 2010, 8, 272.
4. C. Vauthier, P. Couvreur, Nanomedicines: a new approach for the treatment of serious diseases, *J. Biomed. Nanotechnol.* 3 (2007) 223–234.
5. G. Barratt, Colloidal drug carriers: Achievements and perspectives, *Cell. Mol. Life Sci.* 60 (2003) 21–37.
6. T. Xu, N. Zhang, H.L. Nichols, D. Shi, X. Wen, Modification of nanostructured materials for biomedical applications, *Mater. Sci. Eng. C* 27 (2007) 579–594.
7. V.D. Labhasetwar, A.K. Dorle, Nanoparticles — A colloidal drug delivery system for primaquine and metronidazole, *J. Control. Release* 12 (1990) 113–119.
8. F. Föger, W. Noonpakdee, B. Loretz, S. Joojuntr, W. Salvenmoser, M. Thaler, A. Bernkop-Schnürch, Inhibition of malarial topoisomerase II in *Plasmodium falciparum* by antisense nanoparticles, *Int. J. Pharm.* 319 (2006) 139–146.
9. N Stela Santos-Magalhães, V Carla Furtado Mosqueira Nanotechnology applied to the treatment of malaria, *Advanced Drug Delivery Reviews* 62 (2010) 560–575
10. D.A. Fidock, R.T. Eastman, S.A. Ward, S.R. Meshnick, Recent highlights in antimalarial drug resistance and chemotherapy research, *Trends Parasitol.* 24(2008) 537–544.
11. L.M. Bareford, P.W. Swaan, Endocytic mechanisms for targeted drug delivery, *Adv. Drug Deliv. Rev.* 59 (2007) 748–758.
12. L.W. Ho, R. Bendayan, A.M. Rauth, Y.X. Hui, K. Babakhanian, Y.W. Xiao, A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer-lipid hybrid nanoparticle system, *J. Pharmacol. Exp. Ther.* 317 (2006) 1372–1381.
13. L. Qiu, N. Jing, Y. Jin, Preparation and *in vitro* evaluation of liposomal chloroquine diphosphate loaded by a transmembrane pH-gradient method, *Int. J. Pharm.* 361(2008) 56–63.
14. Watt, G., Loesuttivibool, L., Shanks, G.D., Boudreau, E.F., Brown, A.E., Pavanand, K., Webster, H.K., Wechgritaya, S., 1992. Quinine with tetracycline for the treatment of drug-resistant falciparum malaria in Thailand. *Am. J. Trop. Med. Hyg.* 47, 108–111.
15. Looareesuwan, S., Vanijanonta, S., Viravan, C., Wilairatana, P., Charoenlarp, P., Lasserre, R., Canfield, C., Kyle, D.E., Webster, H.K., 1994a. Randomised trial of mefloquine–tetracycline and quinine–tetracycline for acute uncomplicated falciparum malaria. *Acta Trop.* 57, 47–53.
16. Duarte, E.C., Fontes, C.J., Gyorkos, T.W., Abrahamowicz, A., 1996. Randomized controlled trial of artesunate plus tetracycline versus standard treatment (quinine plus tetracycline) for uncomplicated *Plasmodium falciparum* malaria in Brazil. *Am. J. Trop. Med. Hyg.* 54, 197–202.
17. Looareesuwan, S., Viravan, C., Vanijanonta, S., Wilairatana, P., Charoenlarp, P., Canfield, C.J., Kyle, D.E., 1994b. Randomized trial of mefloquine–doxycycline and artesunate–doxycycline for the treatment of acute uncomplicated falciparum malaria. *Am. J. Trop. Med. Hyg.* 50, 784–789.
18. Dunn, C.J., Barradell, L.B., 1996. Azithromycin: a review of its Pharmacological properties and use as a 3-day therapy in respiratory tract infection. *Drugs* 51, 483–505.
19. Gingras, B.A., Jensen, J.B., 1992. Activity of azithromycin (CP-62,993) and erythromycin against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum in vitro*. *Am. J. Trop. Med. Hyg.* 47, 378–382.
20. Gingras, B.A., Jensen, J.B., 1993. Antimalarial activity of azithromycin and erythromycin against *Plasmodium berghei*. *Am. J. Trop. Med. Hyg.* 49, 101–105.
21. Puri, S.K., Singh, N., 2000. Azithromycin: antimalarial profile against blood and sporozoite-induced infections in mice and monkey. *Exp. Parasitol.* 94, 8–14.
22. Heppner Jr., D.G., Walsh, D.S., Uthaimongkol, N., Tang, D.B., Tulyayon, S., Permpanich, B., Wimonwattawatee, T., Chuanak, N., Laoboonchai, A., et al., 2005. Randomized controlled doubleblind trial of daily oral azithromycin in adults for prophylaxis of *Plasmodium vivax* malaria in Western Thailand. *Am. J. Trop. Med. Hyg.* 73, 842–849.

23. Gray, R.H., Wabwire-Mangen, F., Kigozi, G., Sewankambo, N.K., Serwadda, D., Moulton, L.H., Quinn, T.C., O'Brien, K.L., Meecham, M., et al., 2001. Randomized trial of presumptive sexually transmitted disease therapy during pregnancy in Rakai, Uganda. *Am. J. Obstet. Gynecol.* 185, 1209–1217.
24. Wawer, M.J., Sewankambo, N.K., Serwadda, D., Quinn, T.C., Paxton, L.A., Kiwanuka, N., Wabwire-Mangen, F., Li, C., Lutalo, T., et al., 1999. Control of sexually transmitted diseases of AIDS prevention in Uganda: a randomised community trial. *Lancet* 353, 525–535.
25. M.F. Zambauxa, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M.J. Alonso, P. Labrude, C. Vigneron., **1998**. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method, *Journal of Controlled Release* **50**: 31–40.
26. Jennings V, Thunemann AF, Gohla SH. Characterization of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. *Int. J. Pharm.* 2000; 199: 167-177.
27. Sanjay Sing, Madaswamy S Muthu. Preparation and characterization of nanoparticles containing atypical antipsychotic agent. *Nanomedicine.* 2007; 2: 223-240.
28. Gershon Golomb. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. *J. Control. Release.* 2002; 83: 389–400.
29. Costa P, Lobo JMS. Modeling and comparison of dissolution profiles, *Eur J Pharm Sci.* 2001; 13: 123-33.