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Research paper

Artemisinin and artemisinin plus curcumin liposomal formulations: Enhanced antimalarial efficacy against *Plasmodium berghei*-infected mice

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ABSTRACT

The therapeutic efficacies of novel liposomal delivery systems based on artemisinin or artemisinin-based combination therapy with curcumin have been investigated and reported in this study. The developed liposomal formulations had proper characteristics as drug carriers for parental administration in terms of particle size, polydispersity, encapsulation efficacy and ζ-potential. Their physical and chemical stabilities were also evaluated. Furthermore, the *in vivo* antimalarial activity of artemisinin-based liposomal formulations was tested in *Plasmodium berghei* NK-65 infected mice, a suitable model for studying malaria because the infection presents structural, physiological and life cycle analogies with the human disease. Artemisinin, alone or in combination with curcumin, was encapsulated in conventional and PEGylated liposomes and its *in vivo* performance was assessed by comparison with the free drug. Mice were treated with artemisinin at the dosage of 50 mg/kg/days alone or plus curcumin as partner drug, administered at the dosage of 100 mg/kg/days. Artemisinin alone began to decrease parasitaemia levels only 7 days after the start of the treatment and it appeared to have a fluctuant trend in blood concentration which is reflected in the antimalarial effectiveness. By contrast, treatments with artemisinin-loaded conventional liposomes (A-CL), artemisinin–curcumin-loaded conventional liposomes (AC-CL), artemisinin-loaded PEGylated liposomes (A-PL), artemisinin–curcumin-loaded PEGylated liposomes (AC-PL) appeared to have an immediate antimalarial effect. Both nanoencapsulated artemisinin and artemisinin plus curcumin formulations cured all malaria-infected mice within the same post-inoculation period of time. Additionally, all formulations showed less variability in artemisinin plasma concentrations which suggested that A-CL, AC-CL, A-PL and AC-PL give a modified release of drug(s) and, as a consequence, a constant antimalarial effect during time. In particular, A-PL seems to give the most pronounced and statistically significant therapeutic effect in this murine model of malaria. The enhanced permanency in blood of A-PL suggests the use of these nanosystems as suitable passive targeted carriers for parasitic infections; this strong effect of formulation is added up to the mechanism of action of artemisinin which acts in the erythrocyte cycle stage of human host as a blood schizonticide.

1. Introduction

The elimination of malaria is now considered a realistic goal because of good surveillance and high intervention coverage between 2000 and 2007 which have resulted in the reduction of malaria cases and deaths by 50% or more in some countries and regions of African countries [1]. Beside this background, however, it is reported that each year 300–500 million people suffer from acute malaria, and 0.5–2.5 million die of the disease of which 90% are in sub-Saharan Africa [1]. Early treatment with effective antimalarial drugs is the main life-saving intervention but, treatment is threatened by increasing of the growing resistance of *P. falciparum* to drugs that were once effective posing tremendous challenges to malaria control.

The most important antimalarial drugs (i.e. quinine and artemisinin derivatives) currently available to treat severe *falciparum* malaria are from natural origin. For instance, artemisinin and its derivatives are considered the keystones of the treatment for *P. falciparum* malaria due to their high potency and rapid action [2]; they have gametocytocidal properties by inhibiting parasite transmission which probably reduce the development of antimalarial resistance [3]. After the introduction of artemisinin-based combination therapy (ACT) interventions, morbidity and mortality

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associated with malaria were decreased in several parts of the world [4]. However, major limitations of ACTs have been ascribed to the imbalance between demand and supply, comparatively high cost, dosing complexity and the lack of clinical experience [5]. Besides the promising clinical results, artemisinin suffers of technological and biopharmaceutical issues, principally a poor bioavailability and very low solubility both in aqueous media and oils. Artemisinin has an initial burst effect and high peak plasma concentration but it metabolizes quickly *in vivo* [6]. In addition, it is not very stable and it easily decomposes, most probably by the opening of the lactone ring [7]. Recently, it has been reported that its encapsulation into conventional and PEGylated liposomes is a reasonable method to prolong the circulating time of artemisinin in blood plasma and to enhance the half-life of artemisinin [8]. Furthermore, curcumin has been proposed as an attractive partner drug for artemisinin, due to its short half-life (1–2 h), closely matching that of artemisinin [9,10]; according to a resistance prevention perspective, the combination partners should have similar pharmacokinetic properties to provide optimum mutual protection [3]. In addition, curcumin has a relative abundance and cost-effectiveness [9,10], but a similar drawback to artemisinin: a poor bioavailability [11,12].

Both *in vitro* (*Plasmodium falciparum*) and *in vivo* (*Plasmodium berghei*) studies have indicated that curcumin possesses antimalarial activity [9]. In addition, curcumin was tested in association with artemisinin using a selected clone for artemisinin resistance of the rodent malaria parasite *P. chabaudi* selected for artemisinin resistance, resulting in a statistically significant reduction of parasitaemia [10].

Delivery systems such as liposomes and nanoparticles have been studied for intracellular infections because they are able to deliver the drug to the specific target in the human body, where the parasite is located, such as tissues (spleen and liver) and cells (macrophages and Kupffer cells) [13–15].

In particular, liposomes are lipid vesicles used extensively for controlled delivery drug formulations. They inhibit rapid clearance by controlling the size, charge and surface hydration of the drug.

To the best of our knowledge, studies concerning vesicular delivery systems of artemisinin and its derivatives are very limited. It is reported that conventional liposomal formulation of artesunate, a water soluble artemisinin derivative, can increase stability of the molecule [16] and artemisinin-loaded liposomes strongly increases its bioavailability in healthy mice [8]. Moreover, liposomes containing beta-artemether (an oil soluble artemisinin derivative) are able to prevent malaria recrudescence in *Plasmodium chabaudi* malaria infected mice [17].

In this study, the therapeutic efficacies of novel liposomal delivery systems based on artemisinin or ACT containing curcumin as a partner drug are investigated. All the liposomal formulations were characterized in terms of size, polydispersity and encapsulation efficacy. In addition, their physical and chemical stabilities were evaluated. The *in vivo* antimalarial activity of the developed formulations was tested using *P. berghei*-infected mice, a suitable model for studying malaria because the infection presents structural, physiological and life cycle analogies with the human disease [18].

2. Materials and methods

2.1. Chemicals and standards

All the solvents used were HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA). NaCl 0.9%, solution was from Fresenius Kabi, Italy. Artemisinin was purchased from Sigma (Sigma–Aldrich srl, Milan, Italy). Curcumin was provided by Extrasynthese (Genay Cedex, France). Poly(ethylene glycol)-2000-distearoylphosphatidylethanolamine (PEG2000-DSPE) was purchased from Spectra2000 srl (Rome, Italy). Soy phosphatidylcholine (Phospholipon90G, P90G) was gently provided from Natterman Phospholipids, GmbH, Hermersberg, Rhineland-Palatinate, Germany. Cholesterol was analytical grade from Aldrich (Milan, Italy). Artemisinin reference standard (ST3105, batch No. C005641) was kindly provided by Sigma-Tau (Pomezia, Rome, Italy). Curcumin reference standard (10 mg) was provided by Extrasynthese (Genay Cedex, France).

2.2. Preparation and characterization of conventional and PEGylated liposomes

Conventional and PEGylated liposomes were prepared according to the film hydration method [19], according to the optimized molar ratios summarized in Table 1. Conventional and PEGylated artemisinin-loaded liposomes were prepared as described before [8]. Briefly, in the case of artemisinin-loaded conventional liposomes (A-CL) P90G (33 mg/mL), cholesterol (2 mg/mL) and artemisinin (5 mg/mL) were dissolved in dichloromethane. The organic solvent was vacuum evaporated and the dry lipid film was hydrated by addition of physiological solution (NaCl 0.9% w/v). The dispersion was stirred with a mechanical stirrer for 30 min with a water bath at the constant temperature of 38 °C. In order to reduce the dimensions of the vesicles from MLV to LUV, an high pressure homogenizer Emulsiflex C3® was used at the applied pressure of 150,000 kPa for 60 s. In order to obtain artemisinin-loaded PEGylated liposomes (A-PL), PEG2000-DSPE (6.2 mg/mL) was weighted together with P90G, cholesterol and artemisinin and then the vesicles were prepared as describe above. In the case of artemisinin–curcumin-loaded conventional liposomes (AC-CL), curcumin was added to the lipidic film mixture; in the case of artemisinin–curcumin-loaded PEGylated liposomes (AC-PL), PEG2000-DSPE (2.6 mg/mL) was weighted together with P90G (13.86 mg/mL), cholesterol (0.84 mg/mL), artemisinin (2.1 mg/mL) and curcumin (4.2 mg/mL) and then the vesicles were prepared as describe above.

Liposome size, polydispersity and ζ-potentials were measured by photon correlation spectroscopy. For these measurements, 20 μL of each liposomal dispersion was diluted 100-folds with NaCl 0.9% w/v. Measurements were carried out at the set temperature of +25 °C using a Zetasizer® Nano ZS3600 (Malvern Instruments, Malvern, UK). The encapsulation efficiency (EE) is defined as the percentual amount of drug entrapped in the vesicles in relation to the total amount of drug present during the vesicle formation and entrapment procedure. Free artemisinin or curcumin were removed by means of dialysis. Liposomal formulation was transferred in a dialysis bag. This dialysis bag was stirred in 800 mL of physiological solution (NaCl 0.9% v/v) at room temperature for 2 h. The aqueous medium was refreshed once. The content of artemisinin or curcumin into the liposomes were quantified by HPLC-ESI-MS and HPLC-DAD analysis, respectively, using artemisinin or curcumin as external standards.

Table 1
Molar ratios between the constituents of artemisinin-loaded conventional liposomes (A-CL), artemisinin-curcumin-loaded conventional liposomes (AC-CL), artemisinin-loaded PEGylated liposomes (A-PL), artemisinin-curcumin-loaded PEGylated liposomes (AC-PL). Data are shown as mean ± S.D. (n = 3).

Constituent	A-CL	AC-CL	A-PL	AC-PL
P90G	5	1.6	5	1.6
Cholesterol	0.6	0.2	0.6	0.2
PEG2000-DSPE	–	–	0.25	0.1
Artemisinin	2	0.6	2	0.6
Curcumin	–	1	–	1

Curcumin-loaded concentration was assayed by HPLC-DAD analysis performed using a HP 1100 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP 1040 Diode Array Detector (DAD), an automatic injector, an auto sampler and a column oven and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The UV–Vis spectra were recorded between 220–500 nm and the chromatographic profiles were registered at 240, 330, 420 and 430 nm. The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). Artemisinin-loaded concentration was assayed by HPLC-ESI-MS analysis. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values: negative and positive ionization mode, scan spectra from 100 to 800 m/Z, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure 30 psi, Quadrupole temperature 30 °C, Capillary voltage 3500 V. The applied fragmentors were in the range 80–180 V. Separations were performed on a reversed phase column Purosphere® Star RP-18, namely Hibar® Prepacked column RT (250 × 4.6 mm) with particle size 5 mm (Merck, Darmstadt, Germany). The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B). The multi-step linear gradient applied was described before [8]. The formulations were stored at +4 °C and physicochemical characterization was carried out according to the techniques described above. All the measurements were performed in triplicate.

2.3. Stability studies

Stability of artemisinin-loaded and artemisinin–curcumin-loaded vesicle dispersions were studied over 1 month. Vesicle dispersions were kept at 4 ± 1 °C, and at fixed time intervals, they were assayed for their physical and chemical stability. Physical stability was checked by monitoring of size and polydispersity during time, by DLS measurements and TEM visualizations. Chemical stability was checked by quantification of drug content after disruption of purified vesicles by HPLC-DAD-ESI-MS analysis.

2.4. Animals

Female Balb/C mice (18 ± 2 g) obtained from Harlan Laboratories were used for the study. The animals, kept at with a 12 h light/dark cycle and held at a temperature of 22 ± 1 °C and 60% relative humidity, were fed a standard mouse diet and provided with clean drinking water *ad libitum* throughout the experiments. Animal experiments were carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimize the number of animals used and their suffering.

2.5. Parasite and inoculation

P. berghei NK-65 strain, originally from the Istituto Superiore di Sanità (Rome, Italy) was used for evaluation of antimalarial activity. The strain provides a good model to estimate survival and antimalarial efficacy in reducing parasitaemia. It is sensitive to all currently used antimalarial drugs. The parasites kept in liquid nitrogen were thawed (at 37 °C) and maintained by serial passage of blood from mouse to mouse. The blood infected was collected in heparinizated tubes by the ocular venous sinus and diluted with isotonic saline. Each animal was inoculated by intraperitoneal injection with 1×10^7 infected erythrocytes. The parasitaemia was evaluated using peripheral blood taken from tail vein. The samples were stained with Giemsa 10% solution in phosphate buffer (pH 7.2) and examined under a light microscope with 100× magnification immersion [17]. The determination of the parasitaemia for each subject was obtained by counting the number of red blood cells until 1000. Infected mice with parasitaemia of 20–30% were allocated to several groups of three mice each.

2.6. Drug treatment protocol and antiplasmodial activity

P. berghei NK-65 infected mice were randomly divided into seven groups of 3–4 mice each and treated intraperitoneally for 12 consecutive days. The different treatments were administered from 24 h after the infection. Negative control group mice were administered 0.2 ml of saline solution. Positive control group mice were treated with artesunate at a dosage of 50 mg/kg/day. Artesunate was dissolved in 5% $NaHCO_3$ in sterile distilled water. Then, mice were treated with artemisinin dissolved in DMSO 10% v/v, A-CL, AC-CL, A-PL and with AC-PL once daily for 12 days. Artemisinin and curcumin dosages were 50 mg/kg/day and 100 mg/kg/day, respectively. The test for the determination of antimalarial activity in mice was that proposed by Gumbede and coworkers [20] and Knight and Peters [21] extending the duration of animal treatment from 4 to 12 days. At the days 3, 5, 7, 9 and 12, these mice were carefully observed with blood thin smears to determine the parasitaemia. Parasitaemia was measured by microscopic quantitation from Giemsa-stained thin blood film smears made from tail vein bleeds and observed under an immersion optical microscope.

In addition, the average parasitaemia of each group of mice was used to calculate the percentage reduction of parasitaemia using the following formula:

$$(A - B/A) \times 100$$

where A is the mean of parasitaemia in the negative control group (untreated) and B is the parasitaemia of each treated group [22]. The treatment was considered effective if the parasitaemia was reduced by 30% or more [23]. Throughout the test, the general condition of the animals in terms of behavior/clinical signs were also evaluated and the survival of the recovered mice was observed until day 30.

2.7. Statistical analysis

The significance of treatment effect was evaluated using the Wilcoxon–Mann–Whitney non-parametric test (Stat Graph Software package), with significance for $p < 0.05$. All treated groups were compared with negative control group (untreated) until day 12, before animals died.

3. Results and discussion

3.1. Characterization of conventional and PEGylated liposomes

All liposomal dispersions were analysed in terms of size (nm), polydispersity (Pd) and ζ-potentials were measured by photon correlation spectroscopy, as summarized in Table 2. The correlation coefficient is superior to 0.9 that means that the signal to noise ratio is good (noise is 10%). The mean diameters of all the artemisinin-based vesicles was ⩽200 nm and resulted suitable for the intraperitoneal administration. In particular, Pd is a dimensionless measure of the broadness of size distribution calculated from Distribution analysis and values were calculated for each peak as peak width/mean diameter.

The measurements performed three-times for each formulation were nicely reproducible. ζ-Potential is around −20 mV for all the prepared formulations, except for AC-CL which had a value of ζ-potential around −30 mV; this latter is a clue for a bigger dispersion electrostatic stabilization in physiological solution. The artemisinin loading efficiencies were ca. 78% and ca. 68% for conventional liposomes and PEGylated liposomes, respectively, evaluated by

Table 2
Size, polydispersity (Pd), ζ-potential and encapsulation efficacy (EE) of A-CL, AC-CL, A-PL and AC-PL. Data are shown as mean ± S.D. (n = 3).

	Size (nm)	Pd	ζ-Potential (mV)	EE (%)	
				Artemisinin	Curcumin
A-CL	185.33 ± 8.69	0.28 ± 0.01	−20.80 ± 0.28	78.46 ± 5.19	–
AC-CL	202.31 ± 60.59	0.15 ± 0.01	−32.30 ± 0.78	78.00 ± 0.28	62.85 ± 1.48
A-PL	92.30 ± 10.60	0.35 ± 0.01	−19.75 ± 1.77	69.93 ± 6.30	–
AC-PL	137.74 ± 31.17	0.09 ± 0.01	−21.20 ± 2.95	68.25 ± 0.64	50.10 ± 1.41

HPLC-ESI-MS analysis (Table 2). The curcumin loading efficiency for AC-PL was smaller than the in the case of AC-CL, evaluated by HPLC-DAD analysis. This fact could be related to the smaller size of PEGylated liposomes which decreases the bilayer capacity of solubilizing lipophilic drugs.

3.2. Stability studies

Stability of artemisinin-loaded and artemisinin plus curcumin-loaded vesicle dispersion was studied over 1 month according to our previous paper [24]. Physical stability was checked by monitoring of size and polydispersity during time, by DLS measurements. Size is maintained in this period of time, as reported in Fig. 1 for A-CL and AC-CL and in Fig. 2 for A-PL and AC-PL. In particular, the vesicles size variations were up to 6.24%, 6.92%, 11.28% and 14.52% of the original values for the A-CL, AC-CL, A-PL and AC-PL, respectively. Furthermore, polydispersity (Pd) of all the liposomal formulations were stable during time, as reported in Fig. 3. Indeed, the Pd variations were up to 11.78%, 8.47%, 10.44% and 10.04% of the original values for the A-CL, AC-CL, A-PL and AC-PL, respectively. According to these results, no vesicle size alterations occurred over the whole tested period. Moreover, chemical stability was checked by quantification of drug content after disruption of purified vesicles. As described in the experimental section, the content of artemisinin and curcumin into the liposomes were quantified by HPLC-ESI-MS and HPLC-DAD analyses, respectively. We found that artemisinin maintained a residual percentage of 85.1% after 1 week in the A-CL, while 90.0% of the initial artemisinin content was found after 11 days when the drug was delivered by A-PL (Fig. 4). Conversely, in the case of the combined liposomes, artemisinin maintained a residual percentage of 88.2% after 10 days and 97.2% after 15 days when vehiculated by AC-CL and AC-PL, respectively (Fig. 5). Over again regarding combined liposomes, curcumin maintained a residual percentage of 92.0% after 5 days and 89.5% after 15 days when vehiculated by AC-CL and AC-PL, respectively (Fig. 6). Both physical and chemical stability

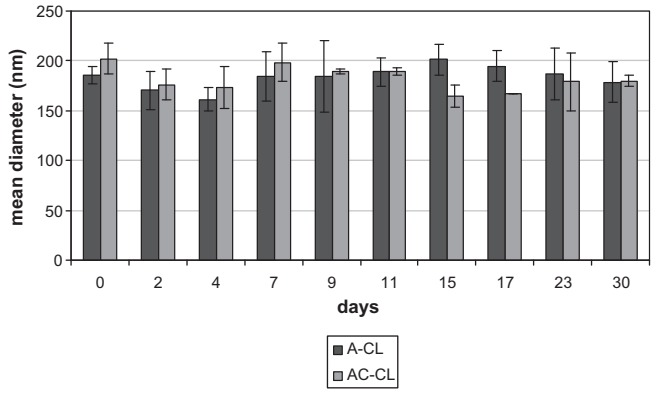

Fig. 1. Physical stability of conventional liposomes (A-CL and AC-CL) in terms of mean diameter by Dynamic Light Scattering analysis. Data are shown as mean ± S.D. (n = 3).

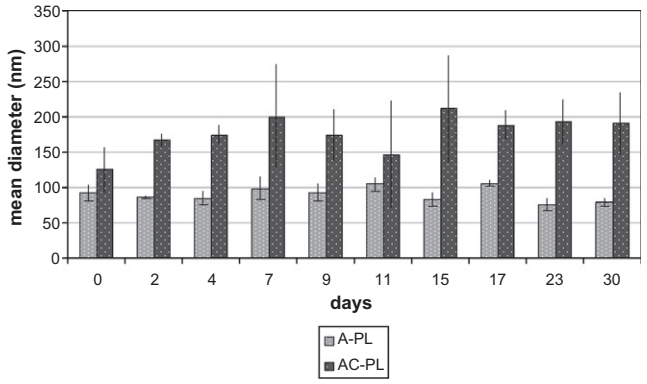

Fig. 2. Physical stability of PEGylated liposomes (A-PL and AC-PL) in terms of mean diameter by Dynamic Light Scattering analysis. Data are shown as mean ± S.D. (n = 3).

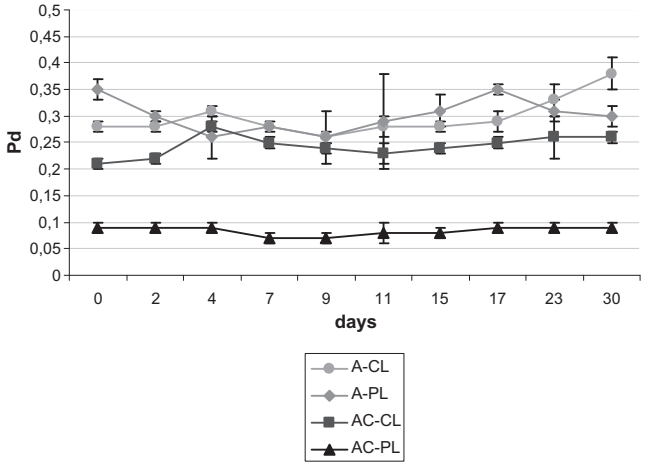

Fig. 3. Physical stability of conventional and PEGylated liposomes in terms of polydispersity by Dynamic Light Scattering analysis. Data are shown as mean ± S.D. (n = 3).

(in terms of residual percentage of drugs) were acceptable during ca. 1 week. Nevertheless, fresh solutions of all the formulations were prepared every 3-days for *in vivo* testing.

3.3. Drug treatment protocol and antiplasmodial activity

The antiplasmodial activity of all the developed artemisinin-based liposomal formulations was evaluated in *P. berghei* NK-65-infected mice and compared with the performance of free drug. Mice were treated with artemisinin at the dosage of 50 mg/kg/days alone or together with curcumin as partner drug administered at the dosage of 100 mg/kg/days. These doses were obtained from studies already present in literature, reporting the ratio 1:2 between artemisinin and curcumin after oral administration [25]. Fig. 7 shows the parasitaemia progression during time for all

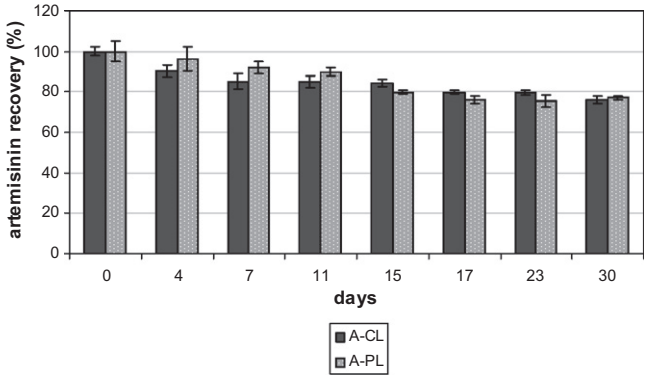

Fig. 4. Chemical stability of artemisinin-loaded liposomes (A-CL and A-PL) in terms of artemisinin content by HPLC-DAD-ESI-MS analysis. Data are shown as mean ± S.D. (n = 3).

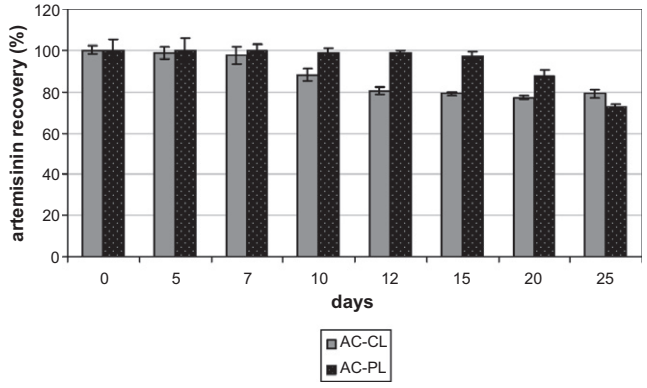

Fig. 5. Chemical stability of artemisinin-curcumin-loaded liposomes (AC-CL and AC-PL) in terms of artemisinin content by HPLC-DAD-ESI-MS analysis. Data are shown as mean ± S.D. (n = 3).

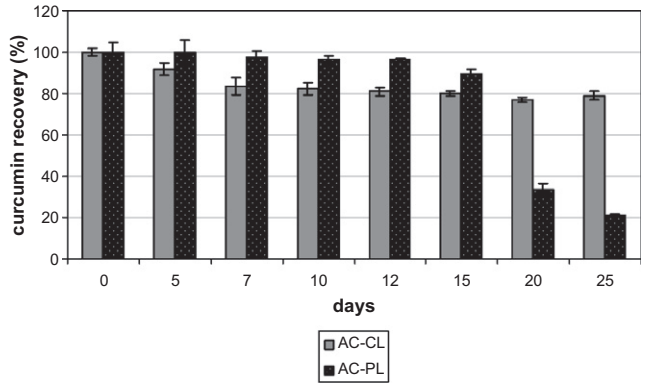

Fig. 6. Chemical stability of artemisinin-curcumin-loaded liposomes (AC-CL and AC-PL) in terms of curcumin content by HPLC-DAD analysis. Data are shown as mean ± S.D. (n = 3).

groups investigated. In parallel, a non-treated group was evaluated. As expected, negative control group registered an increasing level of parasitaemia in the blood. At the same time, positive control group maintained the level of parasitaemia under 5%. Artemisinin given as a DMSO solution at the dosage of 50 mg/kg/day for 12 days began to decrease the parasitaemia levels only 7 days after the start of the treatment and then it appeared to have a fluctuant blood concentration trend which is reflected in the antimalarial effectiveness. This variable plasma concentration of artemisinin has been already described [26]. Parasitaemia peaks reach values

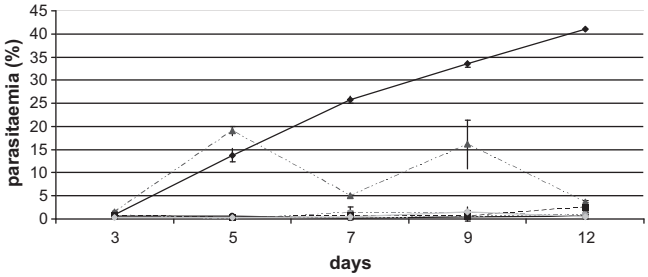

Fig. 7. Parasitaemia progression for mice groups infected with *P. berghei*. They were treated as follow: negative control; positive control; artemisinin; A-CL; AC-CL; A-PL; AC-PL. Data are shown as mean ± S.D.

>15% at days 5 and 9, although the animals were survived; alternatively, the level of parasitaemia is maintained under 5% at days 7 and 12. By contrast, the treatment of the mice with A-CL, AC-CL, A-PL and AC-PL has an immediate antimalarial effect, similar to the positive control group. All artemisinin and artemisinin plus curcumin nanoencapsulated formulations cured all malaria-infected mice within the same post-inoculation period of time. Additionally, these formulations showed less variability in artemisinin plasma concentrations, compared with the DMSO solution of artemisinin. Both conventional and PEGylated vesicles offer a modified release of drug(s) and, as a consequence, a constant antimalarial effect during time.

Going into details, Fig. 8 reported a statistical comparison of positive control group, A-CL, AC-CL, A-PL and AC-PL treated groups with the negative control group.

No remarkable differences were observed between the four liposomal treatments until day 9. However, A-PL seems to give the most pronounced and statistically significant therapeutic effect in this murine model of malaria at the day 12. All the treatments resulted to be statistically significant (p-value <0.005), but above all the A-PL treated group which had a p-value <0.001 similar to that of positive control group at day 12, as also reported in Table 3.

This result fits perfectly with the pharmacokinetic data in healthy mice reported in our previous paper suggesting artemisinin-loaded liposomes as a reasonable delivery strategy to prolong its circulating time in blood due to the passive targeting [8]. In particular, artemisinin-loaded PEGylated liposomes were able to profoundly improve both $AUC_{0-24\ h}$ and half-life of artemisinin values [8].

In the present study, the antiplasmodial effect of artemisinin is enhanced by all the vesicle formulations. Considering that artemisinin is reported to act in the erythrocyte cycle stage of human host

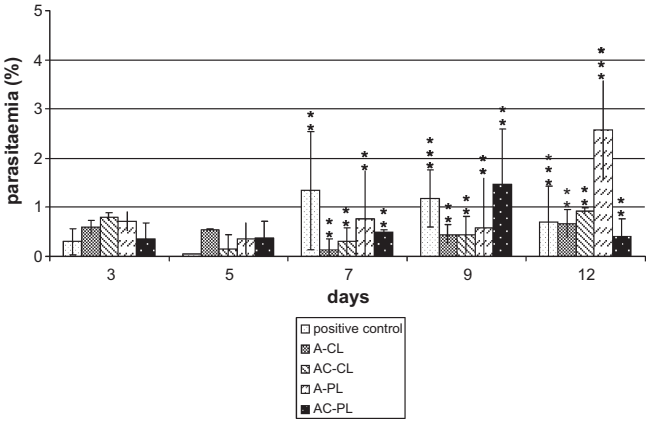

Fig. 8. Statistical comparison of positive control group, A-CL, AC-CL, A-PL and AC-PL treated groups with the negative control group. Data are shown as mean ± S.D. Significance achieved for $p < 0.05$.

Table 3
Parasitaemia of positive control group, A-CL, AC-CL, A-PL and AC-PL treated groups at the days 7, 9 and 12 were compared with the negative control group. Significance achieved for $p < 0.05$.

	p-Value		
	Day 7	Day 9	Day 12
Positive control	0.0091	0.0010	0.0004
A-CL	0.0075	0.0040	0.0020
AC-CL	0.0076	0.0038	0.0017
A-PL	0.0083	0.0038	0.0005
AC-PL	0.0078	0.0042	0.0017

Table 4
Percentage reduction of parasitaemia, considering the average parasitaemia of each group of mice, for artemisinin (A), A-CL, AC-CL, A-PL and AC-PL. Data are shown as mean ($n = 4$).

Day	Positive control	A	A-CL	AC-CL	A-PL	AC-PL
3	67.39	60.86	35.86	11.95	22.82	60.86
5	100.00	37.68	96.01	98.84	97.39	97.24
7	94.79	80.59	99.49	98.79	97.04	98.05
9	96.47	51.94	100.00	98.68	98.26	95.61
12	99.02	90.92	98.36	98.95	93.73	99.00

as a blood schizonticide [27,28], the effect of artemisinin-loaded PEGylated liposomes is the strongest in antiplasmodial activity probably due to the enhanced permanency in blood circle of this type of vesicles. Furthermore, it seems that, in the case of A-PL, the passive targeting produced by PEGylated formulation is more effective of the combination of both antimalarial drugs artemisinin and curcumin. Hence, a synergistic or additive effect of curcumin was not found, may be due to the dosage of artemisinin in the combination therapy, too high to appreciate these potential effects.

Moreover, throughout the test, the general condition of the animals in terms of behavior/clinical signs were also evaluated and the survival of the recovered mice was observed until day 30. All of the animals of the negative control group died 12 days post-inoculation, showing parasitaemia levels more than 40% of the initial value. All the liposomal treatments extended the period of survival of the mice until 30 days post-inoculation.

Finally, the average parasitaemia of each group of mice was used to calculate the percentage reduction of parasitaemia [22,23]. During the course of treatment, reduction in parasitaemia was faster in mice infected with *P. berghei* and treated with AC-PL, which reduce the parasitaemia to about 60% of the initial value after only 3 days, as shown in Table 4. In all liposomal treatments, parasitaemia was reduced more than 93% of the initial value starting from day 5. Interestingly, the infection was almost totally reverted in mice treated with A-CL after 7 days and with AC-CL, A-PL and AC-PL after 5 days.

4. Conclusions

The work presented in this paper represents a successful attempt to formulate artemisinin alone, or artemisinin combined with curcumin in liposomes. Vesicles have proper physical characteristics as drug carriers for parental administration in terms of particle size, polydispersity, encapsulation efficacy and ζ-potential. In addition, they provide simultaneously an appropriate solvent for the drugs, a stabilizing system for its/their maintenance/storage during a substantial time after production and a drug targeting to *P. berghei*-infected organs.

Indeed, the antimalarial activity of artemisinin and artemisinin plus curcumin liposomal formulations was evaluated in *P. berghei* NK-65-infected mice, a suitable *in vivo* model. The pharmacological results pointed out that free artemisinin began to decrease the parasitaemia levels only 7 days after the start of the treatment; by contrast, the treatment of mice with both conventional and PEGylated liposomes have an immediate antimalarial effect similar to the positive control group. Furthermore, artemisinin administered as DMSO solution appeared to have a fluctuant trend in blood concentration which is reflected in the low antimalarial effectiveness. By contrast, all the liposomal formulations showed less variability in artemisinin plasma concentrations and, as a consequence, a constant antimalarial effect during time. In particular, the best performance was obtained with PEGylated liposomes in terms of chemical and physical stabilities and antiplasmodial efficacy.

These results pointed out the optimization of existing drugs' efficacy by applying innovative formulation strategies. Further researches based on drug delivery systems could offer novel therapeutic approaches for malaria chemotherapy.

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