

Heparin-Coated Liposomes Improve Antiplasmodial Activity and Reduce the Toxicity of Poupartone B



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ABSTRACT

Poupartone B is an alkyl cyclohexenone derivative isolated from *Poupartia borbonica*. This compound demonstrated promising antimalarial activity ($IC_{50} < 1 \mu\text{g/mL}$), however, it was not devoid of toxicity. Thus, to reduce the adverse side effects of this natural bioactive molecule, a delivery strategy involving a nanostructure was formulated. Additionally, poupartone B-loaded liposomes were coated with heparin, a glycosaminoglycan that is known to target proteins on the surface of *Plasmodium falciparum*-infected red blood cells. The quantification of the compound in the formulation was performed by HPLC-DAD, while heparin was quantitated by ^1H NMR spectroscopy. The liposomes' antiplasmodial activity was tested on artemisinin-resistant *P. falciparum* isolate, and toxicity was evaluated on human HeLa cells and zebrafish embryos. Throughout this research, the formulation demonstrated higher antiplasmodial activities against both *P. falciparum* strains and a significant decrease of *in vitro* toxicity. The formulation improved the selectivity index 2 times *in vitro* and proved to be 3 times less toxic than the compound alone in the zebrafish embryo acute toxicity test. Hence, the use of this strategy to deliver natural products in *Plasmodium*-infected cells, particularly those with a narrow therapeutic margin, is proposed.

Introduction

Malaria is caused by a parasitic protozoan transmitted by *Anopheles* sp. mosquitoes. The pathology's symptoms in the human host are caused by a particular life stage of the parasite, the erythrocytic stage, that involves the invasion and destruction of infected red

blood cells (iRBCs). This process causes fever, chills, and anemia and can lead to severe symptoms, as in the case of cerebral malaria, such as coma and seizures, leading to the death of the patient. This pathology still urgently needs scientific investigation due to the resistance of parasites to available medicines. Indeed, the emer-

gence of artemisinin-resistant strains is a threat to eradication policies and reminds the community of the development of chloroquine resistance that happened in the 1960s in Asia [1], and which has since then spread to Africa, making the use of the compound more and more difficult.

According to the last World Malaria Report published by the World Health Organization (WHO), an estimated 216 million cases of malaria occurred in 2016, while 211 million cases were reported in 2015. This pathology is evidence of a high burden, having killed 438 000 people in 2015 and 445 000 in 2016, mostly children under 5 years. For the first time in the last 10 years, a substantial increase in incidence rates occurred worldwide, of which around 99% of the cases can be attributed to *Plasmodium falciparum* [2]. According to the WHO, the emergence of parasite resistance to antimalarial medicines and the lack of a sustainable and predictable international and domestic funding are involved in the upsurge of malaria.

Artemisinin-based combination therapies have contributed to lower mortality rates, but, unfortunately, decreased clinical efficacy of artemisinin derivatives has already been reported in Southeast Asia [3]. Due to the ability of the mutated parasite to employ a quiescence mechanism, it is able to survive the short exposition time to dihydroartemisinin (DHA), the active metabolite of artemisinin, which has a short half-life, resulting in a slower rate of parasite clearance and recrudescence in patients [4].

Natural products have an incalculable therapeutic potential for antimalarial drug discovery. The source of many currently used drugs is inspired or directly derived from nature [5]. Poupartones are alkyl cyclohexenones derivatives isolated from the leaves of *Poupartia borbonica* Gmel. Previous studies showed the antimalarial potential of this kind of compound. Poupartones are highly active against *P. falciparum*, and previous *in vivo* studies on mice showed a growth inhibition of 69.5% at day 7 post-infection at 15 mg/kg/day [6]. Unfortunately, even if the mice treated showed a decrease in parasitemia, they died prematurely. A preliminary testing on zebrafish larva demonstrated high contact toxicity as well as hints of cardiac toxicity, observed by a binocular microscope. For such compounds with promising activity, but potential toxicity, new drug delivery systems have to be developed with two main priorities: (1) to eliminate the parasite with maximum specificity and (2) to lower the adverse side effects of the drug compound.

iRBCs can be an interesting target for a controlled delivery system. In fact, iRBCs are known to express proteins on their surface that are not expressed in non-infected RBCs, thus presenting themselves as targets. These protein complexes, called knobs, are present in iRBC surfaces and allow effective cytoadhesion. This phenomenon is important for the parasite's survival, because it prevents elimination by splenic clearance. iRBC sequestration appears after 16–20 h post-invasion, when iRBCs are able to display these adhesive properties. Because iRBCs are sequestered in different organs, such as the brain and the lung, it leads to multiple problems, including hypoxia, hypoglycemia, and microvascular dysfunctions resulting in severe malaria, as mentioned previously [7]. The knob contains different proteins, including *P. falciparum* erythrocyte membrane 1 (PfEMP1) proteins, responsible for the adhesion phenomenon resulting in the rosetting of iRBCs [8].

Glycosaminoglycans, like heparin, are able to inhibit PfEMP1 proteins and, in this way, could prevent rosetting. Furthermore, its

ability to bind this specific erythrocyte membrane protein constitutes a good opportunity for the targeting strategy of nanocarriers [9, 10]. Heparin is also able to bind parasite proteins, such as the circumsporozoite (CS) protein. The CS protein is present on the sporozoites' and merozoites' cell surface and plays a role in hepatocyte invasion [11]. This approach represents a two-in-one opportunity to improve the efficiency of a targeting approach strategy, which was exploited with success by Marques et al. [10] in the development of liposomes loaded with chloroquine and coated with heparin.

Liposomes are small vesicles made of concentric spheres of lipids. They are biocompatible and allow for the controlled release of both hydrophilic and hydrophobic drugs. Compared to other nanoparticle systems, they demonstrated better capability to enhance the selectivity index of drugs by prolonging systemic circulation time and minimizing toxicity and immunogenicity [12]. *In vivo* assays performed with liposomes of artemisinin demonstrated a much longer blood circulation time than free artemisinin [13], hence demonstrating a good strategy for the development of therapeutics to treat parasitic diseases. Because this approach is very advantageous, several liposome formulations have been developed for malaria treatment, such as enclosing curcuminoids, chloroquine, and artemisinin derivatives, among others, which are described in the review of Aditya et al. [14].

Consequently, this strategy was applied to poupartone B, which revealed high antiplasmodial activity, but also toxicity, in previous studies [15]. This compound was selected because of the high bioactive potential of this class of compounds, which can be demonstrated further through nanocarrier technology. Indeed, the alkyl cyclohexanone derivatives isolated from some Anacardiaceae, such as *Lannea rivaie*, *Lannea welwitshii*, *Lannea edulis*, *Taparira guianensis*, *Taparira obtuse*, and, more recently, from the mushroom *Hygrophorus abieticola*, demonstrated antimalarial, antibacterial, fungicidal, and anticancer activities [15–19]. The aim of this study was to demonstrate the applicability of this formulation in the delivery of an isolated natural compound while minimizing its toxicity and improving its selectivity index, even on a partially artemisinin-resistant strain.

Results and Discussion

Liposome formulations combined with poupartone B (1.0 mg for 20.0 mM lipids) and 100 µg/mL heparin presented the most interesting parameters in terms of size, charge, and polydispersity index (PDI). The liposome size was 183 ± 22 nm and 256 ± 20 nm before and after heparin addition, respectively, LSA revealing a size augmentation due to the heparin attachment. The PDI was lower than 0.1 and, as a result, suitable for medical use [20]. The zeta potential was 16.6 ± 1.7 mV before the addition of heparin and decreased to 11.8 ± 1.4 mV after heparin electrostatic bonding. The fact that the charge remained positive, making this a cationic liposome, is an advantage, because these are preferably endocytosed by targeted cells as they are more attracted by the negatively charged cell membrane, improving the cellular concentration of the molecule of interest [20, 21].

The pre-validation of the analytical procedure allows for verifying the method's ability to accurately quantify poupartone B in the

liposome formulation. Linearity, trueness, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) [22] were evaluated and are presented in ► **Table 1**.

The accuracy acceptance limits were set at 15%, as per usual for isolated natural compounds. The upper and lower β -expectation tolerance limits never exceeded the acceptance limits (► **Fig. 1**). Considering these results, the HPLC method was found to fulfill its objective, that is the accurate quantification of poupartone B in the liposomal matrix.

Liposomes were diluted ten times before injection in HPLC. Using a calibration curve, the formulation was found to contain $304 \pm 3 \mu\text{g/mL}$ ($797.46 \pm 8 \mu\text{M}$) of poupartone B in the 20 mM of liposomes. The high activity of the compound allows for the use of a low concentration of product.

Heparin quantification

The quantification of heparin on the liposomes surface was performed indirectly, since the quantity incorporated was below the detection limit of several tested techniques. The alcian blue method [23] was tested, but it was not sensitive enough to obtain repeatable results. Hence, the method used was ^1H NMR performed on the lyophilized supernatants, which contained the heparin that was not incorporated in the liposome formulation. Maleic acid solution 5 mM in D_2O was used as a reference. The signal around 1.9 ppm is the proton signal from the acetyl group of the sugars in the heparin molecule. Through the integration of the maleic acid and the control of heparin, the average percentage of heparin was $25.78 \pm 0.63\%$. This corresponds to $25.78 \mu\text{g}$ of heparin on the liposomes surfaces (20 mM total lipids, 0.80 mM poupartone B) dispersed in 1.0 mL of PBS. Considering the IC_{50} of poupartone B ($1.81 \mu\text{M}$) [15], this quantity is thought to be adequate, as it stays within the interval of concentrations used by Marques et al. for targeting activities [10].

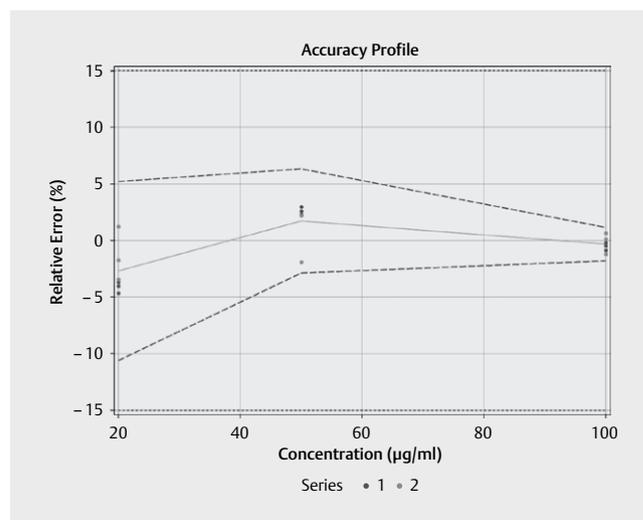
The formulation with poupartone B and heparin tends to be more active than the free poupartone B, both on the chloroquine-sensitive strain 3D7 as on the artemisinin partially resistant strain IPC 3445 (► **Table 2**).

This trend suggests liposome intracellular delivery of poupartone B to the iRBCs and that the improvement is due to the heparin. The decrease of toxicity on nonvascular human HeLa cells, which were used previously to highlight the toxicity of poupartone's compounds [15], reinforces the idea that this improvement of activity only occurred on iRBCs, and thus confirmed the applicability of heparin as a targeting ligand to iRBCs. These elements together are encouraging, increasing the selectivity index from two times (on IPC strain) to three times (on 3D7 strain) for the poupartone B/heparin liposome formulation. The formulation could be a good solution to avoid the compounds inherent toxicity.

Prolonged parasite clearance times with artemisinin derivatives were reported in Southeast Asia, suggesting the presence of an artemisinin resistance phenotype [3]. It was observed that the ring-stage parasites were able to develop a quiescent state, which was associated with a mutation in the Kelch 13 propeller domain [3]. In reality, the use of conventional *in vitro* methods to evaluate the IC_{50} of drugs cannot discriminate an artemisinin partially resistant strain from a susceptible one after exposure to DHA, the active metabolite [24]. In order to correlate survival of parasites *in vitro* with the

► **Table 1** Pre-validation results (k = number of series; n = number of replicates per series).

Trueness ($\mu\text{g/mL}$) ($k=2, n=3$)	Relative bias (%)	
20	-2.69	
50	-1.73	
100	-0.32	
Linearity		
Slope	1.502 ; 1.513	
Intercept	-2.946 ; -4.131	
r^2	0.9920 ; 0.9994	
Precision	Repeatability RSD (%)	Intermediate precision RSD (%)
20	1.7	2.4
50	1.7	1.9
100	0.7	0.7
LOQ ($\mu\text{g/mL}$)	20	
LOD ($\mu\text{g/mL}$)	5.3	



► **Fig 1** Accuracy profile obtained considering a linear regression for poupartone B; plain line: relative bias, dashed lines: β -expectation tolerance limits, dotted lines: acceptance limits, and dots: relative back-calculated concentrations.

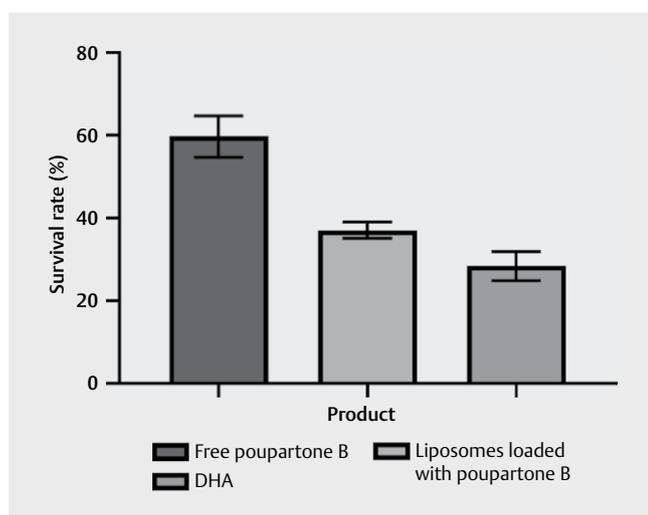
time of parasitic clearance observable *in vivo*, the ring-stage survival assay [25] was used where a short pulse of DHA treatment during the early ring stage of the erythrocyte phase allows for observing differences in susceptibility between the sensitive and resistant strain [25]. Synchronized young ring parasites were exposed to drugs during 6 h in correlation to the DHA's half-life. After the washing step, they were grown in medium for 66 h to evaluate recrudescence. The parasitic growth was assessed by microscopic analysis. The rate of parasite survival exposed to DHA, poupartone B, and the formulation containing poupartone B with heparin is shown in ► **Fig. 2**.

Poupartone B and the formulation were tested at the same concentration, corresponding to the IC_{50} of poupartone B, as estab-

► **Table 2** Antiplasmodial and cytotoxic activities of poupartone B, empty liposomes, and liposomes containing poupartone B with or without heparin.

Samples	<i>P. falciparum</i> IC ₅₀ (µg/mL) (n = 3) 3D7 IPC 3445	Cytotoxicity IC ₅₀ (µg/mL) (n = 2) HeLa	Selectivity index HeLa/3D7 HeLa/IPC 3445	Hemolysis (%)
Poupartone B	0.69 ± 0.20 0.40 ± 0.15	1.44 ± 0.34	2.09 3.60	< 1%
Formulation without poupartone B	NA NA	NA	NA	< 1%
Formulation poupartone B- liposome without heparin addition	0.86 ± 0.26 0.61 ± 0.19	3.88 ± 0.60 **	4.51 6.36	< 1%
Formulation poupartone B- liposome added with 1.3 µg heparin/mM liposome lipids	0.41 ± 0.01 † 0.35 ± 0.08	2.52 ± 0.02 **	6.15 7.20	< 1%
Artemisinin	0.004 ± 0.001	NT	-	NT
Doxorubicin	NT	0.085 ± 0.055	-	NT

NA = non-active; NT = not treated. † P value < 0.05 compared to the administration of the formulation without heparin; ** p value < 0.02 compared to the administration of poupartone B without any formulation.



► **Fig. 2** Survival rate of parasites in the RSA test (%) with a treatment dose of 2.6 µM poupartone B and liposomes containing 2.6 µM poupartone B with heparin. DHA (700 nM) was used as a control.

lished by a conventional method, of 1 µg/mL (2.6 µM). This way, approximately half of the parasite's population survives, and the test is able to establish the difference of survivability of the *Plasmodium* artemisinin partially resistant strain between the free poupartone B and the formulation.

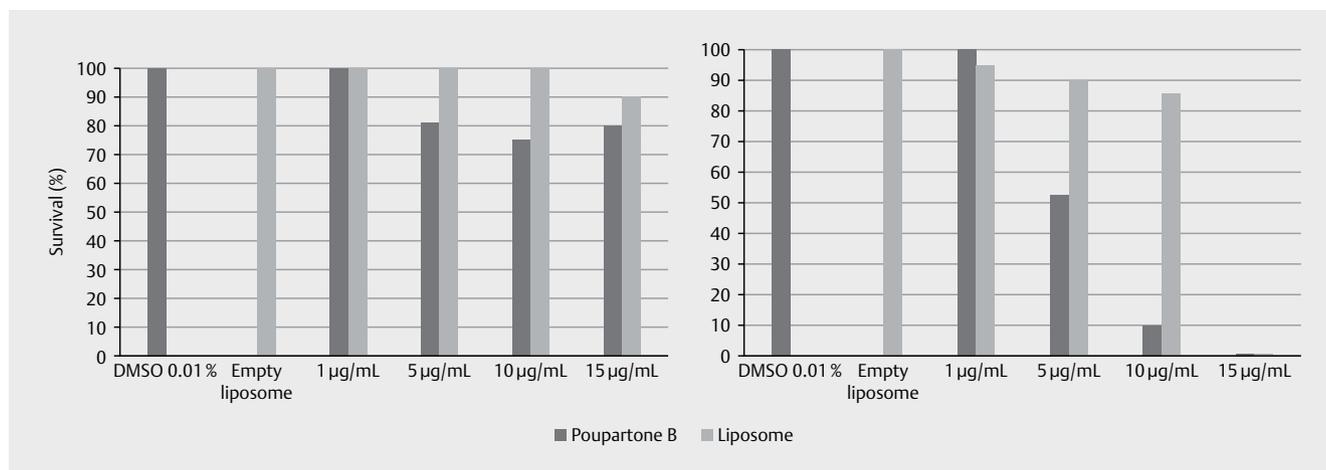
The formulation showed an approximately 1.6-fold increase in potency compared to poupartone B alone, supporting the fact that the liposome's formulation is able to improve the activity by concentrating poupartone B in red blood cells rapidly. As a whole, these results encourage further investigation, considering the liposomal system is able to deliver the compound in a short pulse time (< 6 h). DHA was used as a control, and confirmed the partial resistance of the strain, as the survival rate was evaluated at 28 ± 3.5%.

The zebrafish embryo acute toxicity test was used to observe the reduction of toxicity obtained by the nanovectorization. Zebrafish embryo and larvae are easily obtained in high quantities, and the transparency of the embryos and the possibility to use the

mutant's fluorescent embryos are convenient for investigating the causes of toxicity. Even if this model cannot replace rodents, it is very useful to qualitatively compare the toxicity of drugs in the early stages of research. Poupartone B and the liposome formulation were tested, and the petri dishes were observed at 24, 48, and 72 h post-fertilization (► **Fig. 3**), after the first treatment administration. At the 24 h mark, both populations were alive.

The survival rate at 10 and 5 µg/mL exposure concentrations differ greatly between the poupartone B and the formulation. With 5 µg/mL poupartone B, half of the larvae were dead at 72 h, which accounts for an LC₅₀ of 5 µg/mL. With 10 µg/mL the liposome's formulation, only 2 larvae died, thus the LC₅₀ was set between 10 and 15 µg/mL. At the end of the test, with 10 µg/mL of exposure concentration, more than 80% of the larvae were alive with the formulation while only 10% survived with the compound alone, which accounts for a major difference in survivability. All the population died at an exposure concentration of 15 µg/mL, which confirms the lethality of this dose, as previously highlighted [15]. Even if the survival rate was improved with the formulation, morphological alterations, such as the disappearance of somite, necrosis in the tail, smaller size, cardiac toxicity, and abnormal movements of the larvae, once outside the chorion, were observed by binocular microscopy in both populations, at the same degree.

In this study, a targeted nanovectorization approach based on the results of Marques et al. [10] was tested for the administration of a natural antimalarial cytotoxic compound, poupartone B. The results show a decrease of toxicity and an improvement in effectiveness. The RSA_{0-3h}, which is able to predict the potency of compounds against artemisinin-resistant parasites in malaria patients [24], demonstrated the potent activity of the heparin-targeted liposomes towards an artemisinin-resistant isolate compared to poupartone B alone. Furthermore, it confirmed that this formulation was able to kill resistant parasites, even with a short contact time. These results pave the way for further investigations on liposome formulation applied to alkyl cyclohexenone derivatives in the context of the DHA partial resistance observed in Asia. Cytotoxicity tests confirmed the decrease of toxicity by the formulation in both HeLa cells *in vitro* and in the zebrafish embryos model. Liposomes had already demonstrated their potential to decreased



► **Fig. 3** Survival (%) of the zebrafish at 48 and 72 h exposure to the control (DMSO 0.01%), liposomes containing heparin without poupartone B (empty liposome), a concentration range (1–15 µg/mL) of poupartone B (1–15 µg/mL), and liposomes containing the same quantity of poupartone B with heparin (1–15 µg/mL).

toxicity and improve effectiveness, and heparin has been highlighted for its ability to target iRBCs. This study contributes to highlighting the benefit of the combination of these strategies in terms of activity, especially against partially resistant *P. falciparum* strains. These approaches could represent an important advantage since the emergence of artemisinin resistance has become a major public health problem. This work also emphasizes the interest of an understudied class of natural compounds, the alkyl cyclohexanone derivatives, as potential bioactive substances. Objectively, even if poupartone B is too toxic to be a good candidate for becoming an antimalarial drug, this class of compound should be investigated in more depth, since it isn't clear the mechanism of action through which the compounds exert both antiplasmodial activity and toxicity. At this moment, the structure-activity relationship has not been clearly established. However, some hypotheses can be proposed considering the data present in the literature. It seems that this bioactive class of compounds frequently presents a ketone function [16, 18, 19, 26], and the toxicity appears to be related to the number of oxygen atoms. Some compounds of this group that demonstrated high bioactivities, without *in vitro* toxicity, contain less oxygen atoms than the others [26, 27]. Thus, poupartone B can be a good precursor for semisynthetic compounds with higher therapeutic potential and selectivity.

The discussed data indicates that a heparin-targeted nanovectorization system is efficient and could be successfully applied against artemisinin-resistant *P. falciparum*. Furthermore, the erythrocytic stage of malaria was the exclusive phase investigated. Other studies have demonstrated that heparin can also target different *P. falciparum* stages, such as the sexual stage in mosquitoes. This might pave the way for multiple approaches to proceed with these studies.

Nanotechnology will be a key player in the fight against the occurrence of drugs resistance, especially in the field of malaria, where new treatments are urgently needed.

Materials and Methods

General experimental procedures

Except where otherwise indicated, all lipid materials were purchased from Avanti Polar Lipids and all reagents were purchased from Sigma-Aldrich. Poupartone B was isolated from the leaves of male plants of *P. borbonica*, as previously described by our team (purity > 98% according to HPLC analysis) [15]. This anacardiaceae was collected in Langevin, Reunion Island, in June 2018 and identified by Hermann Thomas (Parc National de La Réunion). Voucher specimens (no. RUN 028F, TCN-P022F) were deposited in the Herbarium of the University of Reunion Island.

Liposomes preparation

Liposomes were prepared by the lipid film hydration method [28]. Different formulations (with and without bounded heparin) were performed based on the protocol described by Marques et al. [10]. Briefly, lipids (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate, DOTAP (4% molar), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC (76% molar), and cholesterol (20% molar) (ovine wool, > 98%) were dissolved with poupartone B in chloroform (20 mM total lipids with a poupartone B concentration of 2.6 mM). The organic solvent was removed by rotary evaporation under reduced pressure at 37 °C to yield a lipid film. The film was hydrated with 1.0 mL PBS (pH 7.4). Liposomes were formed by vortexing and downsized by extrusion through polycarbonate membranes (5 times on 400 nm, 3 times on 200 nm, and 3 times on 100 nm). Liposomes were purified 3 times by ultracentrifugation at 35 000 rpm at 4 °C during 2 h by cycle (Beckman Coulter, Optima L-90K). Heparin was electrostatically bound to cationic liposomes by the addition of proportional 100–150 µg heparin/mL PBS for 20 mM of lipid liposome preparation containing 4% DOTAP. The unbound heparin was removed by ultracentrifugation with the same conditions described for the purification and all supernatants were collected for NMR quantification. Liposomes size, PDI, and zeta potential were obtained by a dynamic light scattering technic

using Malvern Zetasizer (Nano ZS; Malvern Instruments). Encapsulated poupartone B was quantified by an HPLC-DAD method. Except where otherwise indicated, the term “formulation” is used to designate the liposomes containing poupartone B and bounded heparin.

Poupartone B quantification

Analytical HPLC was carried out on an Agilent 1100 Series at a flow rate of 1 mL/min. An RP select B LiChrospher 60 (250 × 4.6 mm) column was chosen. A mobile phase composed of formic acid 0.1 % in water and methanol was used in a gradient elution (40:60 to 0:100 v/v in 30 min). The UV-Vis detector was set at 254 nm. The HPLC-DAD method was validated. The following criteria were investigated: linearity, trueness, precision, accuracy, LOD, and LOQ. Pre-validation of the method was performed on two series with three independent replicates at three concentrations (20, 50, and 100 µg in the presence of 20 mM lipids such as in the liposome preparation) over a period of 2 days. Data processing was performed with Enoval 4.1 software.

When the formulation was analyzed, a calibration curve was performed for each quantification. Liposomes were diluted 10 times in MeOH and destroyed by ultra-sonication before being injected into HPLC for poupartone B quantification.

Heparin NMR quantification

¹H NMR spectra were recorded in D₂O on a Bruker Avance spectrometer operating at 500.13 MHz equipped with a cryoprobe. Maleic acid (purity > 99%; Sigma-Aldrich) was used as an internal standard for 1D experiment quantification. All spectra were calibrated with internal sodium 3-trimethylsilyl-2,2,3,3-d₄-propionate at the 0.00 ppm chemical shift. All the supernatants collected during the liposome preparation were gathered and lyophilized. The lyophilizate was dissolved in D₂O, and 700 µL of this solution were taken to quantify the heparin that had not bound to the liposomes. The amount of heparin bound to the liposomes was deduced by the difference between what was initially introduced and what was not bound.

In vitro antiplasmodial activities

Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum*, chloroquine-sensitive strain 3D7 (originally isolated from a patient living near Schipol airport in the Netherlands) [29], and artemisinin partially resistant strain IPC 3445 (originally isolated from a patient in western Cambodia in 2010) [30] were maintained following the procedure of Trager and Jensen [31]. Strains were obtained from ATCC, Bei Resources. The host cells were human red blood cells (A+). The culture medium comprised RPMI 1640 (Gibco, Fisher Scientific) containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine. The medium was supplemented with 1.76 g/L of glucose (Sigma-Aldrich), 44 mg/mL of hypoxanthine (Sigma-Aldrich), 100 mg/L of gentamycin (Gibco, Fisher Scientific), and 10 % human pooled serum (A+), as previously described [6, 15].

Liposome solutions were directly diluted in the medium; each test sample was applied in a series of eight 2-fold dilutions in a 96-well plate and tested in triplicate (concentration tested was calculated in µg/mL of poupartone B). The parasitemia was 2 %, and

the hematocrit was 1 %, as previously described [32]. Parasite growth was estimated after 48 h incubation by the determination of lactate dehydrogenase activity, according to the methods described by Makler et al. [33]. Artemisinin (purity > 98%; Sigma-Aldrich) at an initial concentration of 100 ng/mL was used as positive control in all experiments. IC₅₀ (half-maximal inhibitory concentration) values were calculated from sigmoidal curves.

Ring-stage survival assay (0–3 h)

Ring-stage survival assays were performed on artemisinin partially resistant strain IPC 3445 as previously described by Witkowski et al. [25] and Baumgärtner et al. [24].

Briefly, 0–3 h post-invasion ring stages were adjusted to 2 % hematocrit and 1 % parasitemia. Of this solution, 900 µL were added to 100 µL of solutions containing DHA (700 nM) (purity > 97%; Sigma-Aldrich), poupartone B (2.6 µM), or liposome formulation (2.6 µM of poupartone B). Tests were performed in 48-well plates and exposed for 6 h. Afterwards, cultures were transferred to 15 mL tubes and were centrifuged at 800 g for 5 min. The blood pellets were dispersed in medium (10 mL) in a cleaning step. After removing the drug, the red blood cells were dispersed with 1 mL of culture media. They were transferred to new wells for a 66-h incubation period. The survival percentage was established by comparing the number of viable iRBCs after explosion in thin blood smears to that of the drug-free DMSO incubation.

In vitro cytotoxic activity

Assays were performed on HeLa cells to evaluate the cytotoxicity potential of the formulation and the compound alone. Compounds were tested in 96-well microplates using the tetrazolium salt WST-1 (Roche Diagnostics Belgium) colorimetric assay based on the cleavage of the reagent by mitochondrial succinate-tetrazolium reductase in living cells. Tests were performed as previously described [15]. Briefly, cells were seeded 6000 per well in 200 µL of medium supplemented with adequate concentrations of the tested drugs. After 48 h of incubation, 10 µL of WST-1 were added to each well. After 30 min at 37 °C, the plates were shaken, and absorbance values were recorded at 450 nm. The absorbance values were expressed in percentage terms compared to untreated control cells. IC₅₀ values were calculated from graphs. The HeLa cells were obtained from the Laboratory of Medical Chemistry, GIGA, ULg.

In vitro hemolytic activity

A red blood cell suspension [A+, 10 % in PBS (v/v)] was incubated with poupartone B and liposomal formulation in triplicate. The final concentration was 5 µg/mL for poupartone B (with and without formulation) and the corresponding formulation without the active compound. After agitation at room temperature for 1 h, the tubes were centrifuged for 5 min at 2000 rpm, and 150 µL of each supernatant were transferred to a 96-microwell plate to measure the absorbance (OD) at 550 nm with a microplate reader. The positive control was Triton X-100 1 % (v/v) and PBS as the negative control. The percentage of red blood cell lysis (H) was calculated as follows: $H = (OD_{550 \text{ nm sample}} - OD_{550 \text{ nm PBS}}) / (OD_{550 \text{ nm Triton X-100 1 \% (v/v)}} - OD_{550 \text{ nm PBS}})$, as previously described [15].

Selectivity index

The selectivity index was obtained as the ratio of IC₅₀ between the cytotoxic activity on HeLa cells and 3D7 or IPC 3445 parasitic activities, respectively.

Zebrafish embryos acute toxicity test

Adult zebrafish (*Danio rerio*) were maintained while fulfilling the criteria of the Ethical Committee for the Use of Laboratory Animals at the University of Liège. They were maintained at 28 °C on a 14-h day/10-h night period, and fertilized eggs were collected, washed with sterile water, and placed in petri dishes. Embryos were collected, and their chorions were not removed. Previously, in another study, our team determined the LC₅₀ (half-maximal lethal exposure concentration) for poupartones [15]. Consequently, the narrower geometric series was performed without first carrying out the logarithmic concentrations series test [34] to avoid wasting fish uselessly.

Poupartone B was dissolved in DMSO solution and was diluted with the medium used for the zebrafish. The exposure concentrations of poupartone B were between 1 and 15 µg/mL, both individually and in the formulation, with full knowledge from previous studies that at a concentration of 15 µg/mL of the compound is completely lethal in the zebrafish model [15]. The DMSO's final highest concentration was 0.4%. The liposome formulations were directly dissolved in the medium used for zebrafish. Twenty embryos were used per condition in a 6-well plate. Each well contained 5 mL of the treatment dose, which was replaced once daily for 3 days. The embryos were observed each day until 72 h post-fertilization. Wells with 20 embryos were used as the control for the solvent (DMSO, 0.4%), the formulation (liposome + heparin), and poupartone B at 15 µg/mL.

Statistical analysis

Statistical significance between the compound and the formulation was set at $p < 0.05$ and analyzed with Student's *t*-test in GraphPad Prism.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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