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Research Paper

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Improvement of the pharmacokinetic characteristics of liposomal doxorubicin using CD47 biomimickry

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Abstract

Objectives In view of their biodegradability, biocompatibility, encapsulation efficiency and targeted release, as well as low toxicity, liposomes are being widely used in the context of drug delivery. However, the efficiency of such drug delivery systems might face limitations by macrophage-mediated clearance (CL), which reduces circulation half-life ($T_{½}$). This problem can be resolved through surface functionalization via poly (ethylene glycol) (PEG) in the process of PEGylation. However, the use of PEG might have its own disadvantages. Accordingly, the main purpose of this study was to produce novel stealth nanoliposomes using CD47 mimicry peptide [namely self-peptide (SP)] as an alternative to PEG for minimizing macrophage-mediated CL and enhancing circulation $T_{½}$.

Methods At first, doxorubicin (Dox)-containing liposomes [i.e.liposomal Dox (LD)] were coated with different concentrations of SP (viz. SP-LD) (0.5%, 1% and 2%). In addition, PEG-functionalized LD (i.e. PLD) was fabricated as a standard control group. Then, various types of liposomal formulae were injected into a population of mice, assigned to six groups (four mice per group) for biodistribution. After sacrificing these animals in prespecified time points (namely 0.5, 6, 12, 24, 48, 72, 96 and 168 h), serum, liver, spleen, heart, kidney and lung samples were collected to estimate the encapsulated drug content in different groups through measuring intrinsic autofluorescence signal of Dox.

Key findings The tissue distribution results in the liver, spleen, heart, kidney and lung samples indicated a significant difference between the SP-LD and the PLD groups. Furthermore, the examination of Dox content, 6 h after administration, showed a growth rate of 28% in Dox content in the SP group compared with the PLD one. Subsequently, these values were, respectively, 63% and 75% at 24 and 48 h.

Conclusions The results of tissue distribution and serum kinetic analysis correspondingly revealed that the use of the SP could augment the circulation time of Dox in comparison with PEG, and it could additionally minimize the tissue accumulation of the drug, which is normally the cause of drug-induced toxicity. The use of the SP on nanoliposomes could prolong the circulation of $T_{½}$ and diminish the tissue accumulation of LD. These findings are relevant for improving therapeutic efficacy and reducing the toxicity of liposomal drugs.

Keywords: CD47; mimicry peptide; self-peptide; nanoparticles; macrophage

Introduction

Nanoparticles (NPs) are regarded as popular drug carriers, thanks to their capacity to improve pharmacokinetics and to control drug release. Among these ultrafine particles, nanoliposomes are the most widely used in the context of drug delivery.[1, 2] Besides, liposomes are reportedly capable of solubilizing drugs and thus increasing their delivery to target cells and organs.[3] The penetration rate of NPs into non-target cells and organs also declines the effective dose of drugs in affected locations of the body, thereby limiting the therapeutic levels of nanodrugs. In addition, the transfer of NPs to non-target tissues can cause serious toxicity. In the liver, lymph nodes and spleen, NPs are largely trapped as they are infused by the mononuclear phagocytic system (MPS) containing phagocytic cells, especially resident macrophages.[4] As well, macrophage uptake occurs following the opsonization of NPs by plasma proteins, such as immunoglobulins, albumins, complement components and apolipoproteins.[5] To resolve this problem, the surface of NPs can be functionalized by materials with shielding effects, including polyvinyl alcohol, poloxamer, polysaccharides, poly(amino acids)[6] and poly(ethylene glycol) (PEG), the latter being the most widely utilized. Intravascular PEG can further increase the circulation time of NPs. In this respect, PEGylation refers to PEG grafting on the surface of NPs, so that ethylene glycol units react tightly with water molecules to form hydrating layers,[7] thereby stopping MPS to adsorb and then clear the protein.

PEGylated liposome-based Doxil has been the first nanodrug delivery system approved by the Food and Drug Administration (FDA).[8] There is also much evidence for clinical purposes of doxorubicin (Dox) hydrochloride-entrapped polymeric micelle carrier natural killer (NK) 911, consisting of PEG copolymerized with Dox-conjugated poly (aspartate) and paclitaxel-entrapped micelle Genexol-PM, as a polymeric NP micelle formulation of paclitaxel, composed of PEG plus polylactic acid (PLA).[9] It should be noted that PEGylation has been reported to be in doubt regarding the beneficial effects because of the large size of PEG carrying NPs or liposomes that may reduce the likelihood of interactions between cells and NPs or liposomes, thereby preventing their penetration into target cells and organs. Additionally, the clearance (CL) of PEGylated drugs can be augmented from the bloodstream following their repeated use. As claimed, the antiPEG was reported in 1983 and induced successfully in animals even though the PEG was considered as a non-antigenic and poorly immunogenic material. These reports have thus far questioned the advantages of PEGylation in increasing the circulation time of NPs or liposomes. Accordingly, there is a pressing need for a new approach as an alternative to PEG.[10, 11]

As an integrin-related protein, CD47 is known as the main self-recognition marker on red blood cells (RBCs). The splenic red pulp macrophages can thus remove the $CD47^{-/-}$ RBCs rapidly from the blood.[12] The RBCs can be also evaded from macrophage phagocytosis by CD47 through interactions with signal regulatory protein alpha (SIRPα) as an inhibitory receptor on the membrane of macrophages. A CD47 analogue can be further produced by some viruses, including variola and vaccinia viruses, against the macrophages.[13, 14] Leukaemia cells and haematopoietic stem cells can similarly protect themself against macrophage uptake by CD47 up-regulation.[15] In this respect, a recent study had evaluated the interactions between polymeric NPs conjugated to CD47 and macrophages. The macrophage-mediated CL had been accordingly impeded by recombinant CD47-coated polystyrene NPs,[16] interestingly, even following NP opsonization. Indeed, circulation half-life ($T_{½}$) can be prolonged via interactions between CD47 and SIRPα as a new strategy to fabricate NPs evading macrophage-mediated CL.

Therefore, this study aimed to harness the antiphagocytic property of CD47 in the drug delivery system of nanoliposomal Dox (nanoLD) as a widely used chemotherapeutic agent in clinical practices. Since CD47 is a molecule weighing 70 kDa, it is difficult to use this large protein on liposome surface, so the utilization of CD47 mimicry peptide [i.e. self-peptide (SP)], which has the ability to interact with macrophages and to inhibit phagocytosis of liposomes, can be an appropriate alternative to PEG.[17, 18, 19] Accordingly, this study was designed to evaluate the ability of SP-coated liposome to reduce macrophage-mediated CL in comparison with PEGylated liposomal Dox (PLD) uptake.

Materials and Methods

Chemicals

The 1.69-kD SP (cgggCERVIGTGWVRC) with 95.08% purity was provided from ChinaPeptides Co. (Shanghai, China). As well, Dowex 50WX4-400, Dox and Lipoid Co. cholesterol (Chol) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Methoxypolyethylene glycol (Mw 2000), distearylphosphatidylethanolamine (mPEG2000-DSPE) and hydrogenated soya phosphatidylcholine (HSPC) were also obtained from the manufacturer. To prepare acidified isopropyl alcohol [isopropanol 90%/aqueous solution of hydrogen chloride (HCl) 0.075 M], HCl 1 M (7.5 ml), water (2.5 ml) and isopropanol (90 ml) were mixed together (Merck KGaA, Darmstadt, Germany). Moreover, Doxil was bought from Behestan Darou Co. (Tehran, Iran).

Conjugation of CD47 mimicry peptide to DSPE-PEG(2000) Maleimide

The peptide was conjugated to mPEG2000-DSPE through thioether bridge in cysteine residue thiol groups. The mPEG2000-DSPE and the peptide were, respectively, dissolved in chloroform dimethyl sulfoxide (DMSO), and then they were mixed together at 1:1 molar ratio. Next, the chloroform was deleted in the presence of argon gas. After that, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

(HEPES), as a buffering agent, was added to achieve the final volume of 1 ml, followed by ligation at 37°C for 24 h.

Determining coupling efficacy of peptides

To determine the processes of peptide–liposome coupling, three techniques, namely thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) were employed.

Preparation of liposomes

The extrusion process and thin-film hydration method were applied to formulate the liposome. Ammonium sulfate gradient method for remote loading was also used to encapsulate Dox in the liposomes.[20] Table 1 presents the nomenclature of liposomes and lipid composition. Thus, certain levels of lipids and chloroform were mixed in round-bottomed flasks. Then, a vacuum-equipped rotary evaporator was utilized to remove the solvent. The resulting product was freeze-dried to obtain thin films that were hydrated with 250 mm of preheated ammonium sulfate to reach 50-mm total lipid content. It was subsequently followed by sonication under an argon atmosphere at 60°C for 15 min, passing through polycarbonate nanopore filters, with pore sizes of 400, 200, 100 and 50 nm (Avestin, Canada). The medium was renewed by HEPES-buffered sucrose (10 mm of HEPES, 300 mm of sucrose and pH of 7.0) via dialysis cassettes (Pierce, Rockford, IL) with molecular weight cut-off of 12–14 kDa. Afterwards, the 0.2-ml aliquots of the liposomes were incubated in the presence of Dox (7 µmol of total phospholipid/1 mg of Dox) at a temperature of 20, 30, 40, 50, 60 and 70°C for 60 min. They were then cooled down up to the ambient temperature to explore the optimal temperature for Dox loading. The un-encapsulated Dox was removed by transferring the 25-µl aliquots to vials with Dowex resin (40 mg) and sucrose 10% (1 ml). The optimal temperature was considered to perform the bulk Dox loading of the liposomes. Dox incubation was further carried out at a temperature of 60°C with dipalmitoylphosphatidylcholine/Chol-L and 1,2-distearoyl-sn-glycero-3-phosphocholine/Chol-L and at a temperature of 50°C with egg phosphatidylcholine (EPC)-L, EPC/Chol-L and dimyristoylphosphatidylcholine/Chol-L. The other liposomes were excluded because of the unreasonable efficiency of Dox loading. As well, the liposomes were centrifuged in the presence of columns, previously equilibrated by cation exchange resin at 1700*g* for 10 min to remove unincorporated Dox. As previously explained, the liposomes were labelled by fluorescence through the augmentation of 0.2 mol percentage of the perchlorate salt of DiD (D307) as a lipophilic tracer to the chloroform-mixed lipids, without the dialysis phase.

Characterization of liposomes

A dynamic light scattering device (Nano-ZS, Malvern, the UK) was used to characterize the liposomes in terms of size, zeta-potential and polydispersity index (PDI). Bartlett phosphate assay was further performed to measure the concentration of the phospholipid. A spectrofluorimeter (470-nm ex/590-nm em, PerkinElmer LS45) was further employed to obtain the liposome Dox content versus Dox calibration curve in the acidified isopropanol. Thus, the vials with Dowex resin (40 mg) and sucrose 10% (1 ml) were appended by 25-µl liposomes and shaken for 1 min. Then, the resulting supernatant (0.1 ml) was poured into 2-ml acidified isopropanol (90% isopropanol/0.075 M HCl) to measure the Dox fluorescence. Additionally, spectrofluorimetry (600-nm ex/665-nm em) was performed to measure the liposome Dox concentration in the presence of the acidified isopropanol. It should be noted that the pre-purification or post-purification Dox levels were determined to calculate Dox encapsulation efficiency. The encapsulated Dox percentage was computed via the following equation:

$$\%Dox\ encapsulated = \frac{\text{Dox encapsulation after purification}}{\text{Dox encapsulation before purification}} \times 100$$

Animal study

The weight of the mice, aged 4 weeks and obtained from Pasteur Institute of Iran (Tehran, Iran), was between 25 and 30 g. They were housed with free access to animal food and water. The Ethics Committee of Mashhad University of Medical Sciences (Mashhad, Iran) approved the study protocol (date: 20 December 2017; approval code: IR.MUMS.fm.REC.1396.633). Following anaesthetization by intraperitoneal injection of 115 mg/kg ketamine and 11.5 mg/kg xylazine (Alfasan, Woerden, the Netherlands), the animals were assigned to six groups (four mice per group) for biodistribution. The tail vein was selected to inject each liposomal formula, including 0.5% LD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD, 2% SP-PLD and 2% SP-PLD equivalent volumes of about 200-uL dextrose (5%) with single dose of 15 mg/kg Dox equivalent. The blood samples were taken from the heart after deeply anaesthetizing among all groups at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h (four animals at each time point). Then, the animals were sacrificed for tissue examinations.

Biodistribution study

After weighing, the dissected spleen, kidneys, lungs, heart and liver organs were transferred into a 2-ml polypropylene microvials (Biospec, OK, USA) with 1 ml acidified isopropanol in the presence of zirconia beads. A MiniBeadbeater-1 (Biospec, OK, USA) was further used to homogenize the microvials. The coagulated blood at 4°C was then centrifuged at 22000*g* for 10 min to collect the sera that were then diluted with 1 ml acidified isopropanol. The drug was extracted after overnight storage of all homogenized tissues and serum samples at 4°C. Spectrofluorimetry (470 nm ex/ 590 nm em) was additionally utilized to analyse Dox content present in the

Table 1 Characterization of various liposomes

Liposome	Liposome composition (molar ratio)	*Z*-average size (nm)	PDI	Dox loading efficacy (%)
Liposomal doxorubicin	HSPC, Chol (62: 38)	107 ± 0.9	0.01 ± 0.01	98 ± 1.4
PLD	HSPC, Chol, mPEG2000-DSPE (61.5: 38: 0.5)	110 ± 1.1	0.12 ± 0.02	97 ± 1.9
0.5% SP-LD	HSPC, Chol, SP-mPEG2000-DSPE (61.5: 38: 0.5)	111 ± 1.4	0.04 ± 0.02	97 ± 1.3
1% SP-LD	HSPC, Chol, SP-mPEG2000-DSPE (61: 38: 1)	112 ± 1.5	0.09 ± 0.04	96 ± 2.9
2% SP-LD	HSPC, Chol, SP-mPEG2000-DSPE (56.2: 38.3: 5.3)	106 ± 1.8	0.11 ± 0.02	97 ± 2.4

supernatant of the centrifuged samples. The serial dilutions were prepared from the tissue Dox and the serum extracts of the control mice to plot the calibration curve.

Pharmacokinetics

The plasma was collected through immediate centrifugation of the blood samples at 1078*g* for 10 min, and then Dox content was measured in different groups. The area under the curve (AUC) was correspondingly divided by the area under the momentum curve to compute the mean residence time (MRT). The PKSolver as an add-in program in Microsoft Excel was consequently applied to compute the pharmacokinetic parameters.[21]

Statistical analysis

GraphPad Prism5 (GraphPad Software, San Diego, CA, USA) was employed for statistical analyses. As well, Dox content as milligram of drug or metabolite per millilitre of plasma or grams of the kidney, heart, liver, lung and spleen was reported as mean ± standard error of the mean (SEM), and then analysed using analysis of variance (ANOVA), Bartlett's test and Student–Newman–Keuls (SNK) test.

Results

Characterization of liposomes

As presented in Table 1, the particle size obtained for different liposomes was about 110 nm (PDI < 0.01), and the liposome size was not affected by SP. The data are expressed as mean ± SEM for three separate experiments.

The number of the peptide molecules in each liposome was obtained in accordance with the factors below. The concentration of the phospholipids was 13.279 mm in the liposome stock. The number of the lipid molecules was 8 × 104 in each liposome with the mean size of 110 nm. As well, the number of liposomes was 1 × 1014 in 1 ml, and the total peptide content was 0.4 mm. Moreover, the number of the peptide was 150, 300 and 600 ligands in each liposome.

DOX encapsulation efficiency was reported to be higher than 96% in all the samples concerned. According to leakage stability tests, there was no significant difference in Dox release from peptide-modified or non-modified liposomes during 48-h incubation at a temperature of 37°C in exposure to fetal calf serum 30%. The least Dox leakage was also seen for liposomes and more than 90% of the encapsulated Dox was still encapsulated.

Biodistribution study

The intrinsic autofluorescence signal of Dox was calculated to measure the biodistribution of all liposomes in different mice tissue samples.

Liver

Figure 1 shows the results of biodistribution in the liver at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h. As illustrated in Figure 1, the decrease in Dox content in the SP-LD group was significantly different at 0.5, 6, 24, 48 and 74 h compared with that in the PLD groups ($P < 0.01$). As a result, a significant difference was observed between the PLD and LD groups ($P < 0.001$) at the mentioned time points. In addition, the SP subgroups established

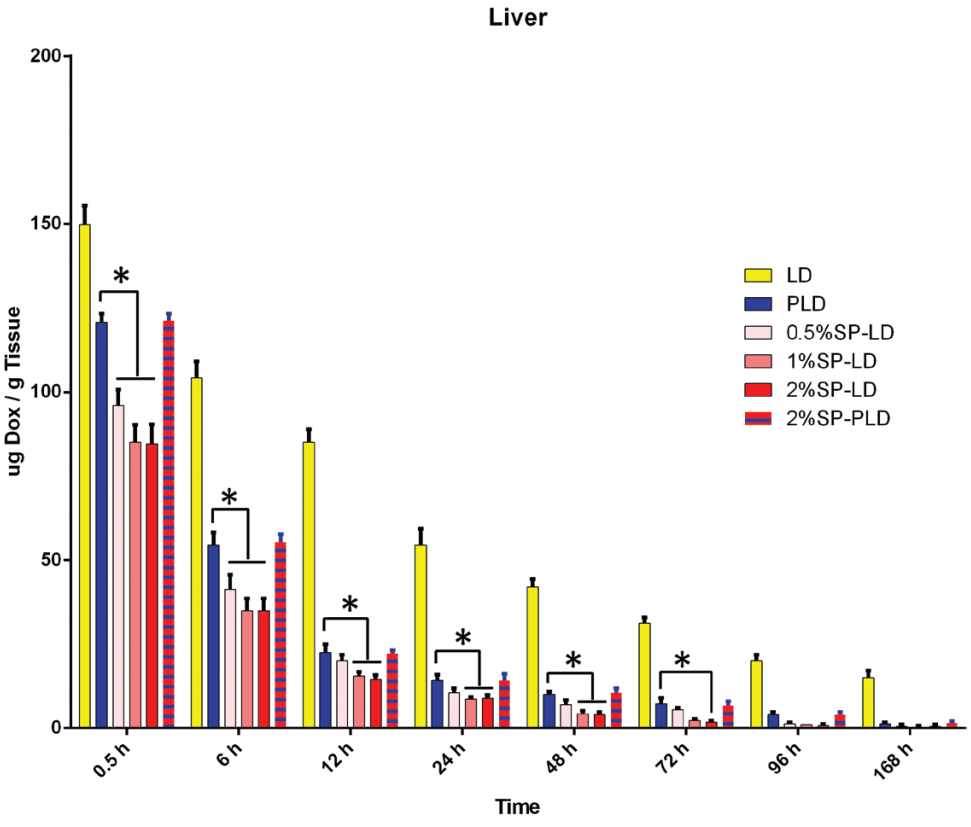

Figure 1 Dox content in the liver at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg Dox equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

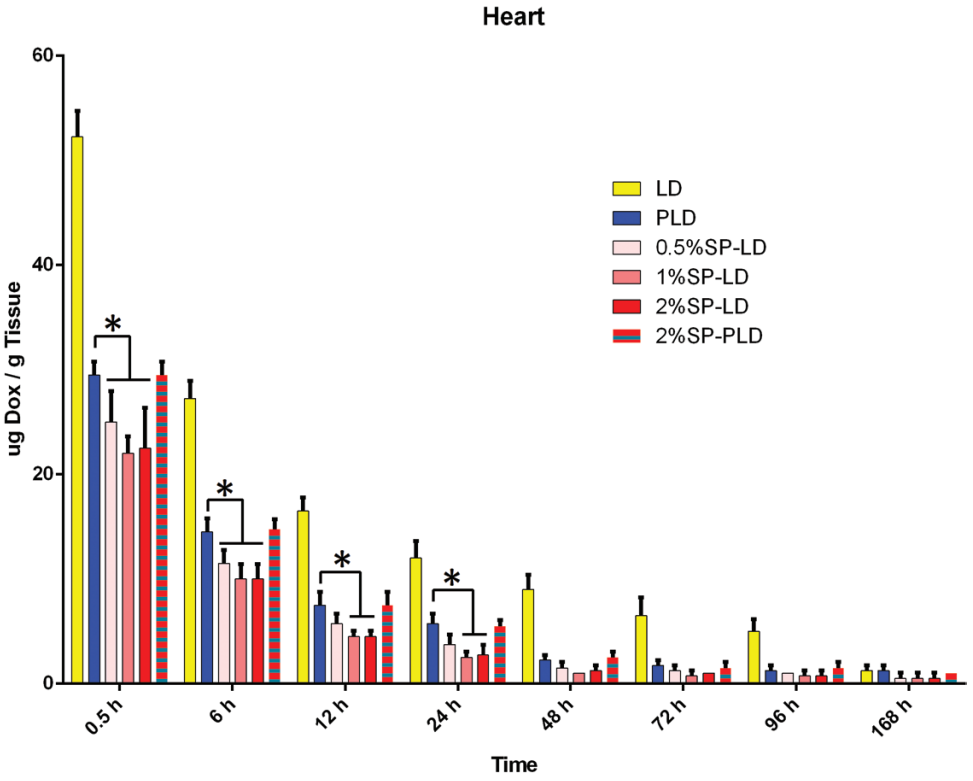

Figure 2 Dox content in the heart at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg Dox equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

a significant difference between the groups of SP-LD 0.5% and 2%, as well as between the groups of 0.5% and 1% ($P < 0.01$), but no significant difference was found between the groups of 1% and 2%.

Heart

The results of biodistribution in the heart at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h are illustrated in Figure 2. Accordingly, there was a significant difference at 0.5, 6, 12 and 24 h following injection between the SP-LD and the PLD groups ($P < 0.01$). As can be seen, the Dox content in the SP-LD group in the heart was reduced by half compared with that in the PLD group.

Spleen

Figure 3 illustrates the results of biodistribution in the spleen at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h. As observed, there was a significant difference at 0.5, 6, 12, 24, 48 and 72 h after injection between the SP-LD and the PLD groups ($P < 0.01$). At 48 and 72 h, Dox content in the SP-LD group also decreased by about one-third compared with that in the PLD group. In the spleen, as with other tissues, the results indicated no significant difference between the groups of the SP-LD 1% and 2%.

Lung

The results of biodistribution in the liver at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h are illustrated in Figure 4. As seen in Figure 4, the reduction in Dox content in the SP-LD group was much higher than that in other groups. As a result, a significant difference was reported between the SP-LD and the PLD groups at 0.5, 6, 12 and 24 h following injection ($P < 0.01$). In addition, the SP subgroup showed a significant difference between the groups of 0.5% and 2% ($P < 0.01$) as well as between the groups of 0.5% and 1%, but no significant difference was observed between the groups of 1% and 2%.

Kidney

Figure 5 shows the results of biodistribution in the kidney at the time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h. According to Figure 5, the decrease in Dox content in the SP-LD group was much higher than that in other groups. The results also demonstrated a significant difference between the SP-LD group and the PLD groups at 0.5, 6, 12, 24 and 48 h after injection ($P < 0.01$). In addition, there was a significant difference between the groups of 0.5% and 2% in the SP subgroup ($P < 0.01$) as well as between the groups of 0.5% and 1%, but no significant difference was observed between the groups of 1% and 2%.

Pharmacokinetics

The results of the biodistribution of mice (Figure 6) indicated that the serum Dox content at 0.5 h following injection was not significantly different between the 1% SP-LD, 0.5% SP-LD and SP-PLD and PLD groups. Comparison of Dox content at 6 h between the SP (1% SP-LD and 2% SP-LD) and PLD groups additionally demonstrated an increase of 28% in Dox content in the SP group ($P < 0.01$). Subsequently, the rate of this increase was, respectively, by 63% and 75% at 24 and 48 h ($P < 0.01$).

However, there was no significant difference in the SP subgroups of 1% and 2%. In addition, no significant difference was observed between the two groups of SP-PLD and PLD. Table 2 outlines other pharmacokinetic parameters, including concentration maximum (C_{max}), AUC, CL, MRT and $T_{½}$.

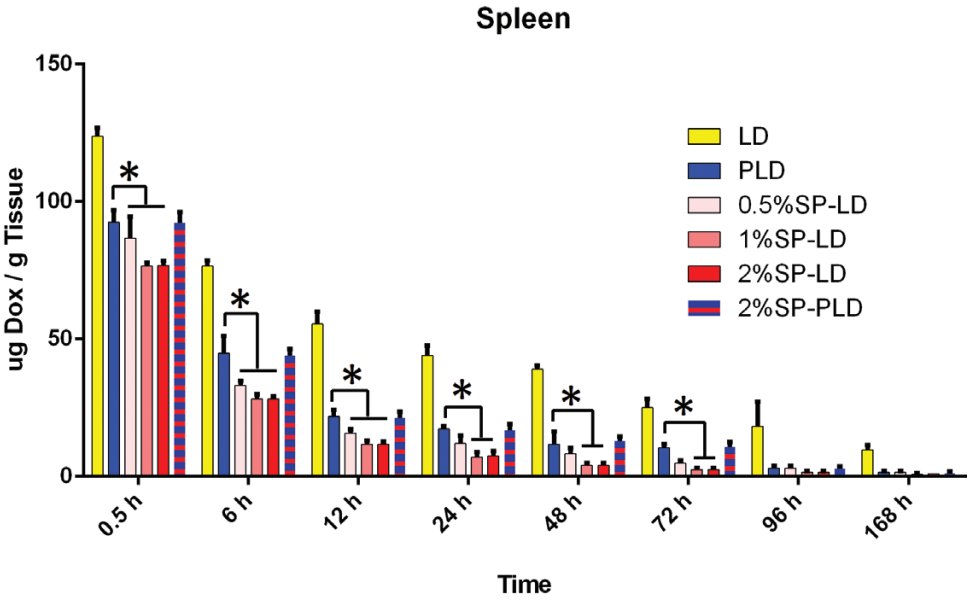

Figure 3 Dox content in the spleen at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg Dox equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

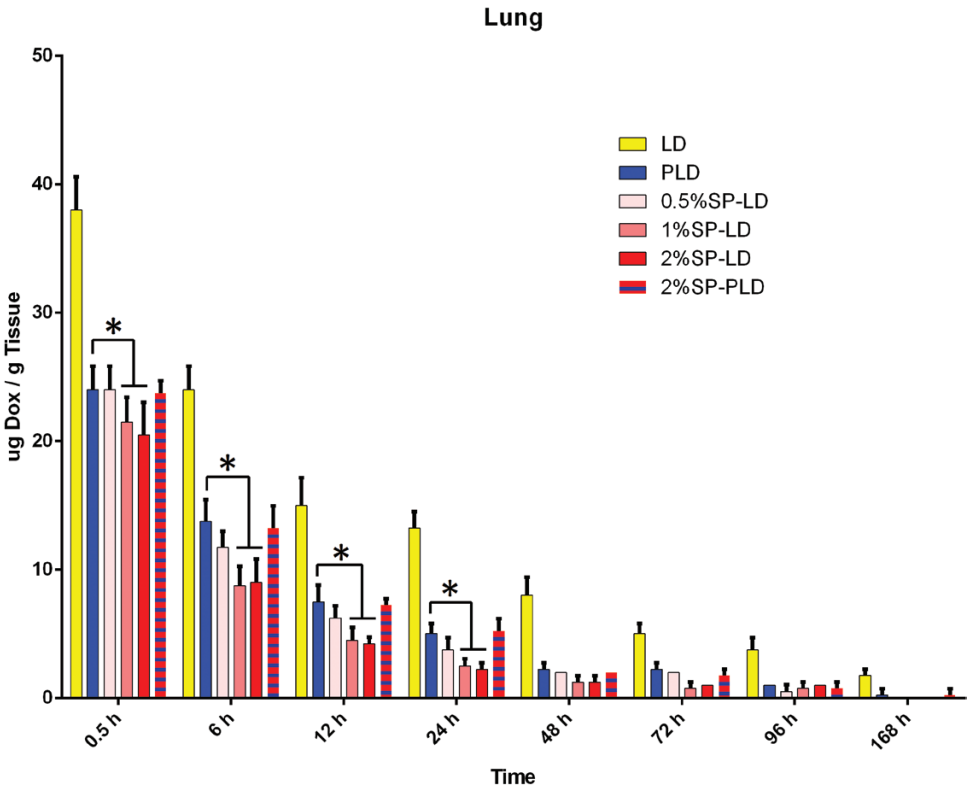

Figure 4 Dox content in the lung at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg Dox equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

Discussion

PEGylation has been recognized as an effective measure to enhance the circulation time of drugs and drug carriers. Nevertheless, PEG can cause immediate and delayed hypersensitivity. In addition, it reduces the amount of cell uptake and disrupts the endosomal escape process. Therefore, it is essential to find appropriate alternatives capable of performing the role of PEG without any complications. In this respect, CD47 is one of the surface proteins on RBCs that sends the 'Don't eat me' signal once exposed to the SIRPα receptor on the surface of macrophages and prevents macrophage-mediated CL. Therefore, this study tested the impact of CD47 mimicry peptide incorporation into the surface Dox-containing liposomes on the pharmacokinetics of the drugs.

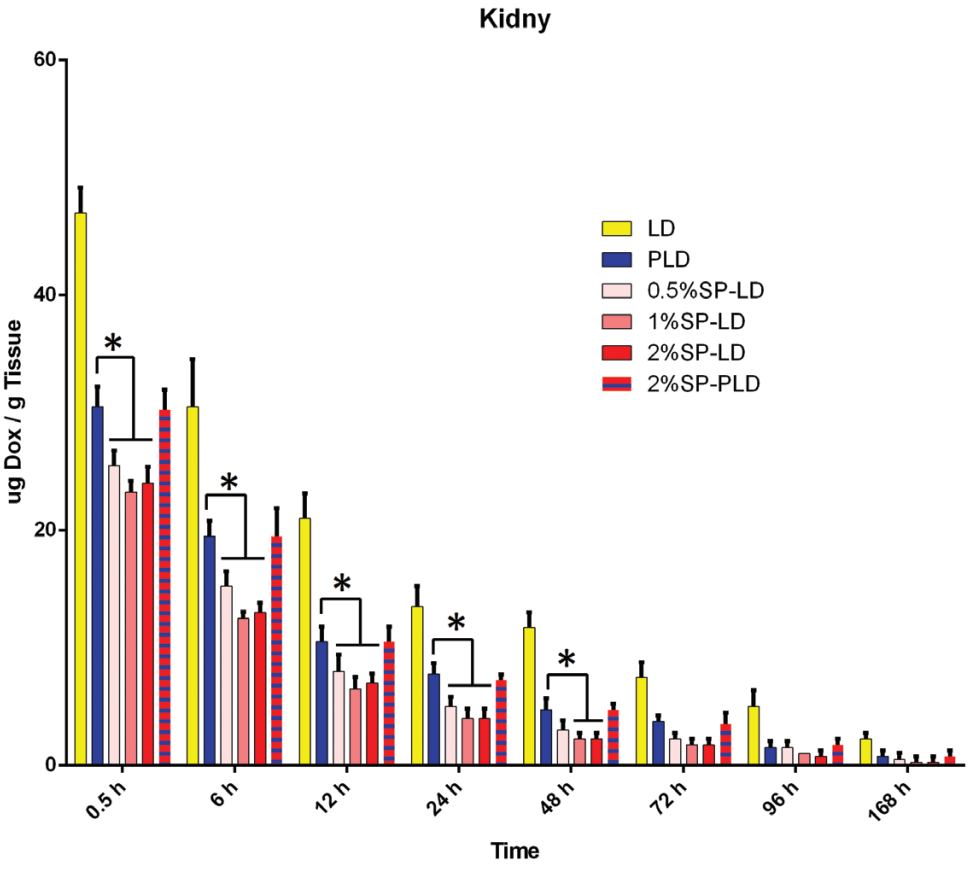

Figure 5 Dox content in the kidney at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg DOX equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

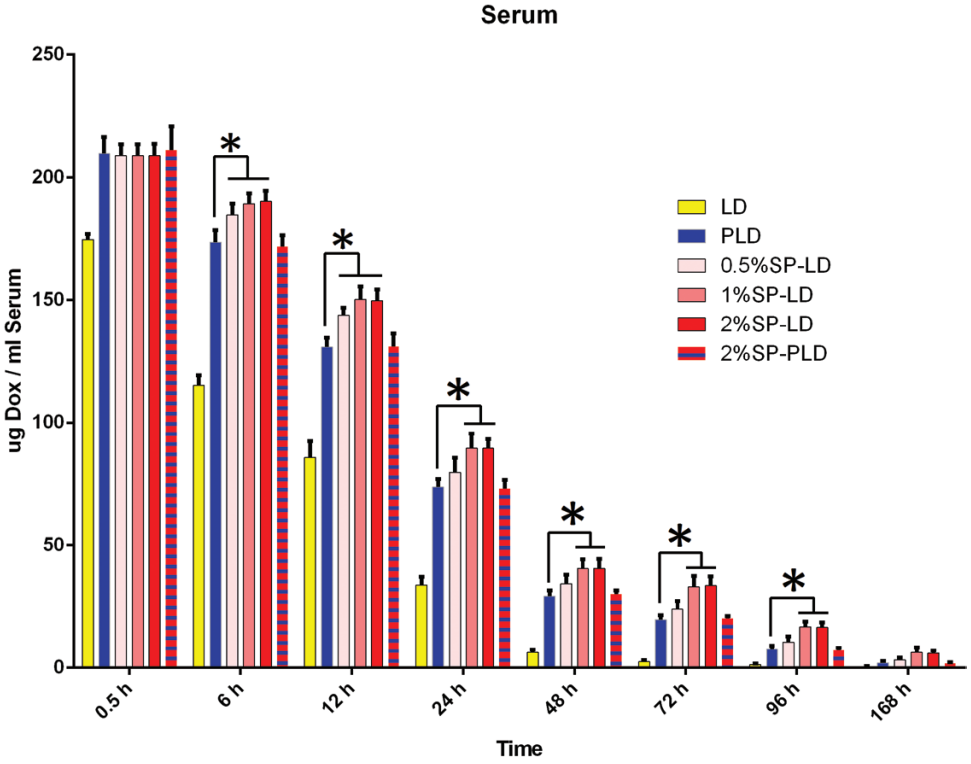

Figure 6 Plasma Dox content at different time points of 0.5, 6, 12, 24, 48, 72, 96 and 168 h after taking LD, PLD, 0.5% SP-LD, 1% SP-LD, 2% SP-LD and 2% SP-PLD at 15 mg/kg Dox equivalent. Each bar shows the mean of four measurements. * Indicates a significant difference ($P < 0.01$).

Table 2 Plasma pharmacokinetic parameters after intravenous injection (15 mg/kg) of LD, PLD, 1% SP-LD, 2% SP-LD and 2% SP-PLD to C26 colon carcinoma tumour-bearing mice ($n = 4$)

Formulation	C_{max} (ug/ml)	AUC (μg/ml×h)	Cl (mg/kg)/(μg/ml)/h	MRT (h)	$T_{1/2}$ (h)
LD	174.5	2873.2	0.00520	16.9	29.3
PLD[1]	209.75	5794.5	0.00255	31.4	28.1
0.5% SP-LD	174.5	2889.7	0.00284	16.9	29.3
1% SP-LD[2]	208.75	7518	0.00189	46.7	43.1
2% SP-LD[2]	208.75	7510.5	0.00190	45.9	42
2% SP-PLD	211	5773.5	0.00256	30.7	27

[1]A significant difference with LD ($P < 0.01$).
[2]A significant difference with LD, PLD and 2% SP-PLD ($P < 0.01$).

The results obtained from HPLC and NMR demonstrated that the yield of linkage between SP and mPEG2000-DSPE was approximately 100%. Subsequently, Dox loading into liposomes was performed well with a yield of >95%, and then Dox-containing liposomes with surface SP were prepared. The results of this study confirmed that SP-LD had a significantly higher circulation time compared with PLD, and the accumulation of the former had markedly reduced in tissues such as the liver and the spleen.

The results of tissue biodistribution in different tissues showed a significant difference between SP-LD and PLD in the liver, spleen and lung tissues. A greater accumulation of PLD was additionally found in these tissues. The reason for this was that the reticuloendothelial system (RES) had taken up PLD due to having tissue macrophages, but SP-LD was less phagocytosed and thus less likely to enter into RES tissues. These antiphagocytic effects were consistent with the results reported by Tsai *et al*.,[16] wherein that had used streptavidin-biotin to link CD47 to polystyrene beads and then had exposed these CD47-coated beads to the human monocytic cell line THP-1 and observed that phagocytosis rate had decreased to 50% relative to the control sample (CD47-free polystyrene). In another study, Bruns *et al*.[22] had reported the antiphagocytic effects of CD47 receptor in artificial antigen-presenting cells (aAPC). In this study, they had succeeded in designing 'Don't eat me' aAPCs using CD47-immunoglobulin (Ig) on classical aAPCs.

In this study, the results of biodistribution in the liver tissue demonstrated that Dox content at 72 h after injection reduced by one-third in the formulation of 1% SP-LD ($C_{t=72}$ = 2.25 ug Dox/g tissue) compared that of PLD ($C_{t=72}$ = 7.25 ug Dox/g tissue). In the spleen tissue, Dox content also decreased to one-quarter in the formulation of 1% SP-LD ($C_{t=72}$ = 2.25 ug Dox/g tissue) at 72 h following injection relative to that of PLD ($C_{t=72}$ = 10.5 ug Dox/g tissue). Comparing these values at the same time point in the lung tissue also revealed reduction to one-third in 1% SP-LD ($C_{t=72}$ = 0.75 ug Dox/g tissue) compared with PLD ($C_{t=72}$ = 2.25 ug Dox/g tissue).

Of note, another benefit of inhibiting macrophage-mediated phagocytosis is augmenting the circulation time of SP-LD formulation and thus increasing the circulation time of drugs and better treatment outcomes. For example, the level of 1% SP-LD formulation ($C_{t=96}$ = 16.75 ug Dox/ml plasma) was more than 2-fold at 96 h after injection compared with PLD formulation (Ct = 7.75 ug Dox/ml plasma). The circulation time was also more accurately investigated for liposomal formulations by calculating parameters, such as large AUC, slow CL rate, small volume of distribution (VoD) and long elimination $T_{½}$. The value of $T_{½}$ for 1% SP-LD formulation ($T_{½}$ = 43.1 h) showed an increase of more than 50% compared with PLD formulation ($T_{½}$ = 28.1 h). In addition, the CL rate of 1% SP-LD formulation [0.00189 (mg/kg)/(μg/ml)/h] was decreased by up to 34% compared with PLD formulation [0.00255 (mg/kg)/(μg/ml)/h], indicating a slower CL of SP-LD formulation from blood flow relative to PLD formulation. However, the C_{max} of the drug in the bloodstream was approximately the same for SP-LD and PLD formulations (208 μg/ml). In other pharmacokinetic parameters, such as AUC and MRT, the superiority of SP-LD was noted compared with PLD.

In fact, the study results demonstrated the role of SP in reducing the macrophage-mediated phagocytosis of LD, which could potentially lead to enhanced tumour accumulation of Dox. This has been clearly supported by our recent findings in tumour-bearing mice treated with SP-LD, suggesting enhanced tumour accumulation, reduced tumour size and increased survival of SP-LD compared with PLD.[23] In addition, the toxicity in the non-target tissues decreased as Dox accumulation in non-target tissues diminished. In this study, 150, 300 and 600 peptides were estimated to be, respectively, present on the surface of 0.5% SP-LD, 1% SP-LD and 2% SP-LD formulations. Comparing different SP groups showed that the surface concentration of 1% was the most optimal because less than this value (150 peptides) could have a less antiphagocytic effect while more SP (600 peptides) had no additive value. Accordingly, the identical order of magnitude was seen for the theoretical density of SP on liposome (350 molecules/mm^2) under physiological conditions on the human RBCs (250 molecules/mm^2).[24]

Another important point in this study was comparable biodistribution and pharmacokinetic findings between SP-PLD and PLD (0.5%, 1% and 2%) formulations, wherein no significant difference was found among them. This observation was probably due to the small size of SP, which allowed its shielding by PEG, thereby leading to the neutralization of the effects of SP by PEG. Therefore, it can be concluded that the combination of SP and PEG does not give any extra benefit in terms of biodistribution and pharmacokinetic features of liposomes.

Conclusions

One of the most important problems facing NP-based drug delivery systems is rapid macrophage-mediated CL of NPs in the bloodstream and accumulation of NPs in RES tissues. In this study, the antiphagocytic effects of CD47 were utilized using CD47 mimicry peptide with a similar to native CD47. Incorporating this peptide into the surface of liposomes consequently reduced the rate of liposome uptake by macrophages. As a result, the circulation time of nanoliposomes was 1.5 times higher than that of the PEGylated formulation. Another benefit of this SP-based drug delivery system was reduced accumulation of drug-containing liposomes in the spleen, liver, heart and kidney tissues. Therefore, given the antiphagocytic effects of SP, this peptide might serve as an appropriate alternative to

PEG. Given the promising results of this research, these SP-decorated nanoliposomes can be utilized in experimental tumour models and pharmacological effects of SP-LD versus PLD can be further investigated. In addition, the capacity of CD47 to reduce phagocytosis can be very useful for other nanoparticulate drug-delivery platforms. Furthermore, the application of CD47-modified biomaterials can be assumed as a great potential for medical devices, such as hip replacements, endovascular stents and cardiopulmonary bypass circuits.

Authors Contributions

M.R.J. and A.S. conceived and designed the study. S.M.G. and M.H. conducted the experiments and analysed the data. S.M.G. prepared the first draft of the manuscript. All authors critically revised the first draft and approved the final version.

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Conflict of Interest

None declared.

Ethical Approval

In-vivo studies were approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

Data Availability Statement

The data associated with this study can become available by the corresponding author upon a reasonable request.

References

1. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. *Int J Nanomedicine* 2008; 2: 133. https://doi.org/10.2147/ijn.s596
2. Kumari A, Yadav SK, Yadav SC *et al.* Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids Surf B Biointerfaces* 2010; 1: 1–18. https://doi.org/10.1016/j.colsurfb.2009.09.001
3. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* 2005; 2: 145–60. https://doi.org/10.1038/nrd1632
4. Patel HM, Moghimi SM. Serum-mediated recognition of liposomes by phagocytic cells of the reticuloendothelial system – the concept of tissue specificity. *Adv Drug Deliv Rev* 1998; 1–2: 45–60. https://doi.org/10.1016/s0169-409x(97)00131-2
5. Tenzer S, Docter D, Kuharev J *et al.* Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nat Nanotechnol* 2013; 10: 772–81. https://doi.org/10.1038/nnano.2013.181
6. Guo S, Huang L. NANOPARTICLES escaping RES and endosome: challenges for siRNA delivery for cancer therapy. *J Nanomater* 2011;2011: 1–12. https://doi.org/10.1155/2011/742895
7. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003; 3: 214–21. https://doi.org/10.1038/nrd1033
8. Barenholz Y. Doxil(R)--the first FDA-approved nano-drug: lessons learned. *J Control Release* 2012; 2: 117–34. https://doi.org/10.1016/j.jconrel.2012.03.020
9. Sutton D, Nasongkla N, Blanco E *et al.* Functionalized micellar systems for cancer targeted drug delivery. *Pharm Res* 2007; 6: 1029–46. https://doi.org/10.1007/s11095-006-9223-y
10. Hong RL, Huang CJ, Tseng YL *et al.* Direct comparison of liposomal doxorubicin with or without polyethylene glycol coating in C-26 tumor-bearing mice: is surface coating with polyethylene glycol beneficial? *Clin Cancer Res* 1999; 11: 3645–52.
11. Zhang P, Sun F, Liu S *et al.* Anti-PEG antibodies in the clinic: current issues and beyond PEGylation. *J Control Release* 2016; 244: 184–93. https://doi.org/10.1016/j.jconrel.2016.06.040
12. Oldenborg PA, Zheleznyak A, Fang YF *et al.* Role of CD47 as a marker of self on red blood cells. *Science* 2000; 5473: 2051–4. https://doi.org/10.1126/science.288.5473.205
13. Lindberg FP, Lublin DM, Telen MJ *et al.* Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem* 1994; 3: 1567–70.
14. Parkinson JE, Sanderson CM, Smith GL *et al.* The vaccinia virus A38L gene product is a 33-kDa integral membrane glycoprotein. *Virology* 1995; 1: 177–88. https://doi.org/10.1006/viro.1995.9942
15. Jaiswal S, Jamieson CH, Pang WW *et al.* CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* 2009; 2: 271–85. https://doi.org/10.1016/j.cell.2009.05.046
16. Tsai RK, Discher DE. Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J Cell Biol* 2008; 5: 989–1003. https://doi.org/10.1083/jcb.200708043
17. Liu Y, O'Connor MB, Mandell KJ *et al.* Peptide-mediated inhibition of neutrophil transmigration by blocking CD47 interactions with signal regulatory protein alpha. *J Immunol* 2004; 4: 2578–85. https://doi.org/10.4049/jimmunol.172.4.2578
18. Gheibi Hayat SM, Bianconi V, Pirro M *et al.* Stealth functionalization of biomaterials and nanoparticles by CD47 mimicry. *Int J Pharm* 2019; 569: 118628 PMID: 31421198. https://doi.org/10.1016/j.ijpharm.2019.118628
19. Hayat SMG, Bianconi V, Pirro M *et al.* CD47: role in the immune system and application to cancer therapy. *Cell Oncol (Dordr)* 2020; 43: 19–30 PMID: 31485984. https://doi.org/10.1007/s13402-019-00469-5
20. Woodle MC, Papahadjopoulos D. [9] Liposome preparation and size characterization. *Methods Enzymol* 1989; 171: 193–217. https://doi.org/10.1016/s0076-6879(89)71012-0
21. Zhang Y, Huo M, Zhou J *et al.* PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Meth Prog Bio* 2010; 3: 306–14. https://doi.org/10.1016/j.cmpb.2010.01.007
22. Bruns H, Bessell C, Varela JC *et al.* CD47 enhances in vivo functionality of artificial antigen-presenting cells. *Clin Cancer Res* 2015; 9: 2075–83. https://doi.org/10.1158/1078-0432.CCR-14-2696
23. Gheibi Hayat SM, Jaafari MR, Hatamipour M *et al.* Harnessing CD47 mimicry to inhibit phagocytic clearance and enhance anti-tumor efficacy of nanoliposomal doxorubicin. *Expert Opin Drug Deliv* 2020; 7: 1049–58. https://doi.org/10.2174/0929866527666200413100120
24. Tsai RK, Rodriguez PL, Discher DE *et al.* Self inhibition of phagocytosis: the affinity of 'marker of self' CD47 for SIRPalpha dictates potency of inhibition but only at low expression levels. *Blood Cells Mol Dis* 2010; 1: 67–74. https://doi.org/10.1016/j.bcmd.2010.02.016