International Journal of Nanomedicine

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Enhanced Pharmacokinetics of Celastrol via Long-Circulating Liposomal Delivery for Intravenous Administration

Bo Wang[1,2], Jiquan Shen[2], Changjian Zhou[2], Xinggao Wang[2], Shuanghu Wang[3], Ruixing Hou[1]

[1]Department of Orthopaedics, Suzhou Ruihua Orthopedic Hospital Affiliated Suzhou Medical College of Soochow University, Suzhou, Jiangsu, 215000, People's Republic of China; [2]Department of Orthopaedics, the Sixth Affiliated Hospital of Wenzhou Medical University, the People's Hospital of Lishui, Lishui, Zhejiang, 323000, People's Republic of China; [3]Central Laboratory of the Sixth Affiliated Hospital of Wenzhou Medical University, the People's Hospital of Lishui, Lishui, Zhejiang, 323000, People's Republic of China

Correspondence: Shuanghu Wang, Central Laboratory of the Sixth Affiliated Hospital of Wenzhou Medical University, the People's Hospital of Lishui, Lishui, Zhejiang, 323000, People's Republic of China, Email wangshuanghu@lsu.edu.cn; Ruixing Hou, Department of Orthopaedics, Suzhou Ruihua Orthopedic Hospital Affiliated Suzhou Medical College of Soochow University, Suzhou, Jiangsu, 215000, People's Republic of China, Email hrx2020@suda.edu.cn

Background: Rheumatoid Arthritis (RA) involves prolonged inflammation of the synovium, damaging joints and causing stiffness and deformity. Celastrol (Cel), derived from the Chinese herbal medicine *Tripterygium wilfordii* Hook F, offers immunosuppressive effects for RA treatment but is limited by poor solubility and bioavailability.

Purpose: In this study, long-circulating Cel-loaded liposomes (Cel-LPs) were used to increase the pharmacokinetics of Cel, thereby improving drug delivery and efficacy for the treatment of RA.

Methods: Cel-LPs were prepared and administered orally and intravenously to compare the elimination half-life of drugs and bioavailability of Cel. Cel-LPs were prepared using the lipid thin-layer-hydration-extrusion method. Human rheumatoid arthritis synovial (MH7A) cells were used to investigate the compatibility of Cel-LPs. The pharmacokinetic studies were performed on male Sprague-Dawley (SD) rats.

Results: The Cel-LPs had an average size of 72.20 ± 27.99 nm, a PDI of 0.267, a zeta potential of −31.60 ± 6.81 mV, 78.77 ± 5.69% drug entrapment efficiency and sustained release (5.83 ± 0.42% drug loading). The cytotoxicity test showed that liposomes had excellent biocompatibility and the fluorescence microscope diagram indicated that liposome entrapment increased intracellular accumulation of Rhodamine B by MH7A cells. Furthermore, the results exhibited that Cel-LPs improved the pharmacokinetics of Cel by increasing the elimination half-life ($t_{1/2}$) to 11.71 hr, mean residence time ($MRT_{(0-\infty)}$) to 7.98 hr and apparent volume of distribution (Vz/F) to 44.63 L/kg in rats, compared to the Cel solution.

Conclusion: In this study, liposomes were demonstrated to be effective in optimizing the delivery of Cel, enabling the formulation of Cel-LPs with prolonged blood circulation and sustained release characteristics. This formulation enhanced the intravenous solubility and bioavailability of Cel, developing a foundation for its clinical application in RA and providing insights on poorly soluble drug management.

Keywords: celastrol, rheumatoid arthritis, liposomes, oral and intravenous delivery, pharmacokinetic

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by joint pain, stiffness and swelling caused by inflammation of the synovium.[1] In addition, RA is induced by dysregulation of pro-inflammatory and anti-inflammatory mediators caused by an abnormal immune response.[2,3] Accumulating evidence indicates that the invasion and destruction of articular bone and cartilage by this aggressive front of tissue leads to stiffness and deformity of the joints.[4,5]

Early targeted intervention can effectively alleviate the deterioration of the disease. Currently, the first-line treatment options include nonsteroidal anti-inflammatory drugs (NSAIDs), adrenocortical hormones, disease-modifying anti-

Received: 8 March 2024
Accepted: 25 May 2024
Published: 11 June 2024

© 2024 Wang et al. This work is published and licensed by Dove Medical Press Limited. The full terms of this license are available at https://www.dovepress.com/terms.php and incorporate the Creative Commons Attribution – Non Commercial (unported, v3.0) License (http://creativecommons.org/licenses/by-nc/3.0/). By accessing the work you hereby accept the Terms. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed. For permission for commercial use of this work, please see paragraphs 4.2 and 5 of our Terms (https://www.dovepress.com/terms.php).

rheumatic drugs (DMARDs), and biological agents, among others. The drugs are often poorly tolerated and cause numerous adverse reactions.[6,7] Therefore, new effective treatments and drug preparations are urgently required to address the enormous medical needs and achieve continuous clinical remission for most patients.

Celastrol (Cel) is an immunosuppressive agent derived from the Chinese herbal medicine *Tripterygium wilfordii* Hook F. Cel is one of the most promising drugs used in the treatment of chronic inflammation and RA. Growing evidence indicates that Cel may hinder macrophages from developing into pro-inflammatory via modulating the NF-κB and Notch 1 signaling pathways, resulting in reduced pro-inflammatory cytokines and RA progression.[8,9] The low water solubility, low oral bioavailability, narrow therapeutic window and high incidence of systemic adverse reactions limit further clinical use of Cel.[10] Therefore, this method delivers these substances simultaneously with sustained release.

Nanocarrier liposomes are useful in biomedicine and pharmaceutical sciences due to their excellent biocompatibility, high entrapment, hydrophilic cargo and surface modification. Liposomes with multiple functions have been used to treat a variety of diseases.[11–14] Previous studies have revealed that intravenous injection of Cel-LPs can achieve greater efficacy while reducing systemic adverse reactions due to the prolonged circulation of PEG-modified liposomes.[15,16]

This study aimed to develop long-circulating Cel-LPs to maximize the efficacy of Cel in the treatment of RA. The physicochemical properties of Cel-LPs were successfully characterized. The fluorescence probe Rhodamine B (RhoB) was encapsulated in liposomes and observed under a fluorescence microscope in human rheumatoid arthritis synovial (MH7A) cells. We next evaluated the pharmacokinetics of Cel-LPs in healthy male Sprague-Dawley (SD) rats following oral and intravenous administration.

Materials and Methods

Reagents and Materials

Celastrol (A0106-1g, MW:450.61, purity ≥ 99%) was purchased from Chengdu Must Bio-Technology Co., Ltd. Cholesterol (Chol, C104032-5g) and Soy lecithin (SL, L105732-25g) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd, China. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol)-2000] (DSPE-PEG2000) was purchased from Shanghai Yare Biotechnology Co., Ltd. Rhodamine B (RhoB) was purchased from Beijing Inno Chem Science & Technology Co., Ltd. All other chemicals including methanol, acetonitrile, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) were of analytical grade and used without further purification. The MH7A cells were purchased from Guangzhou Jennio Biotech Co., Ltd and cultured in DMEM high glucose medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Twenty-four SPF male Sprague-Dawley (SD) weighing 220–250 g rats were obtained from the Animal Laboratory of Wenzhou Medical University (animal license number: SYXK2021-0020 (Zhejiang, China). The rats were housed indoors with an alternate cycle of light and dark for 12 hr. All animal experiments were approved by the Animal Care and Use Committee of Wenzhou Medical University (No. xmsq2021-0409). We followed the GB/T35892 Guidelines for Ethical Review of Animal Welfare and the 3R principles of laboratory animal welfare.

Optimization and Preparation of Cel-LPs

Cel-LPs and blank liposomes were prepared using the lipid thin-layer-hydration-extrusion method described previously.[17,18] The solvent mixture of chloroform and methanol (15 mL, 2:1 v/v) was used to accurately dissolve reagents such as 100 mg SL, 16.67 mg Chol, 8.33 mg DSPE-PEG2000 and 10 mg Cel. The solvent was removed after ultrasonic treatment by rotary evaporation at 45 °C. We then hydrated the thin film with PBS (or double-distilled water) under ultrasound for 30 min. A liposome Extruder (Model: LiposoFast-1, Avestin, Inc., Canada) was used to extrude large vesicles through a microporous membrane for 13 cycles to yield monodispersed uniform-size liposomes. The same method was performed to prepare blank liposomes and Rhodamine B-loaded liposomes.

Method Validation for in vivo and in vitro Analysis of Cel

HPLC Analysis of Cel

We first analyzed the content of Cel using a UPLC-MS/MS system equipped with an ACQUITY I Class UPLC and an XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, United States). There are two parts to the UPLC system: a Binary Solvent Manager (BSM) and a Sample Manager with a Flow-Through Needle (SM-FTN). The treated sample (2 μL was injected into an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, Waters Corp., Milford, MA, USA). The mobile phase comprised 0.1% formic acid and acetonitrile and flowed at 0.4 mL/min. The analysis was carried out at 45°C for 3.5 min.

Test of Specificity

The Cel was weighed and dissolved in methanol after being accurately weighed. HPLC was used to detect the solution and the chromatogram was recorded based on these conditions.

Establishment of the Standard Curve

The standard sample reserve solution was diluted in methanol to 1 mg/mL. We prepared a series of in vivo standard plasma samples with the concentrations of 500, 250, 100, 50, 25, 10, 5, 2.5 and 1 ng/mL by diluting standard reserve solution into 45 μL of rat blank plasma. The peak area was determined sequentially after injecting 150 μL of the internal standard solution (Midazolam). A ratio of peak area to mass concentration was applied to the Cel in vivo standard curve. Three samples were analyzed for each concentration. A series of standard samples were prepared in vitro with concentrations of 20, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1 μg/mL after the same treatment and then the in vitro standard curve of Cel was determined.

Calculation of Precision and Accuracy

The standard sample reserve solution and in vitro and in vivo quality control samples were prepared at concentrations of 0.3, 3 and 9 μg/mL. The 150 μL of internal standard solution was added to samples for three-day continuous measurements.

Stability Test

The stability index measures the stability of drugs in each step including short-term stability, long-term stability and automatic sampler stability. The two quality control samples with different concentrations were treated as follows: placed at room temperature for 6 h, 4°C for 24 h and −80°C for 7 d. The internal standard solution was used to analyze six samples of each concentration following each treatment.

Physicochemical Characterization

Characterization of Cel-LPs

The particle size, polydispersity index (PDI) and zeta potential of the freshly prepared blank liposomes and Cel-LPs were measured by DLS using a Zetasizer Nano ZS at 25°C. The samples were diluted with double-distilled water and all measurements were repeated three times. The surface morphology and internal structure particle size of Cel-LPs were observed using a transmission electron microscope (TEM). We applied diluted Cel-LPs to a copper grid (300mesh) coated with nitrocellulose while 2% phosphotungstic acid solution was applied as a negative stain. The samples were photographed using a TEM following drying.

Determination of Encapsulation Efficiency (EE) and Drug Loading (DL) of Cel-LPs

The EE and DL of Cel-LPs were determined by centrifugal ultrafiltration. An ultrafiltration centrifuge tube (MWCO: 3 kDa, micropore, Merck & Co., Inc., USA) was used to separate free Cel from Cel-LPs and the free Cel in the filtrate was analyzed by HPLC. Briefly, 1 mL of freshly prepared Cel-LPs suspension was diluted with 10 mL of methanol. After adequate demulsification and filtration, 150 μL of the filtrate was used to measure the concentrations of Cel and record C (micrograms per liter). Meanwhile, 1 mL of Cel-LPs suspension was adequately centrifuged for 30 min under 3000 r/min in an ultrafiltration centrifugal tube. The filtrate was absorbed and fixed to 10 mL in methanol and 150 μL solution was taken to measure Cel and record C' (mg/mL). M (mg/mL) represents the ratio between the weight of the total lipids and the volume of Cel-LPs. The EE and DL of Cel-LPs were calculated as follows:

EE (%) = (C - C') / C × 100%

DL (%) = (C - C') / (M + C) × 100%

In vitro Release of Cel from Cel-LPs

The dialysis bag method was used with minor modifications to determine the release of Cel-LPs in vitro at 37°C in PBS (pH 5.5, 6.8 and 7.4) containing 0.5% Tween 80 (v/v).[13] Briefly, freshly prepared Cel-LPs were transferred to the previously activated dialysis bags (Model: MD34, MWCO: 8–14 kDa. Beijing Solarbio Science & Technology Co., Ltd., China), which were placed in the 0.5% PBST (pH 6.8 and 7.4, 40 mL) at 37 ± 1°C under stirring at 100 r/min, repeated 3 times. 1 mL receiving 0.5% PBST medium was absorbed in regular intervals (0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 48, 72 and 96 h) and 1 mL fresh 0.5% PBST medium was supplied each time to maintain sink conditions. HPLC was used to analyze the content of Cel in the receiving medium.

Cytotoxicity Test

The cytotoxicity of blank liposomes and Cel-LPs was evaluated using the Cell Counting Kit-8 (CCK-8) according to the supplier's directions. Briefly, the MH7A cells were seeded at 5×10^3 cells per well in 96-well plates in DMEM containing 10% FBS overnight. Afterwards, 10 μL of CCK-8 solution was added to each well and incubated at 37°C for 3 hr. The absorbance (OD) was measured at 450 nm by a Multiskan FC microplate reader (Thermo Fisher Scientific (China) Co., Ltd). The results of each experiment were derived from three parallel measurements. The cell viability was evaluated using the following equation:

Cell viability (%) = [(As - Ab) / (Ac - Ab)] × 100%

As (drug addition): absorbance of medium with cells, CCK-8 solutions, and drug solutions.

Ac (0 addition): absorbance of medium with cells, CCK-8 solution but no drug solution.

Ab (blank): absorbance of medium with CCK-8 solution but no cells and drug solution.

Cell viability: cell proliferation or cytotoxicity.

Cellular Uptake of Liposomes

The uptake of Cel-LPs by MH7A cells was observed by a fluorescence microscope (Model: EVOS M5000, Thermo Fisher Scientific (China) Co., Ltd). We used RhoB-LPs instead of Cel-LPs as fluorescent probes. The MH7A cells were seeded in 12-well plates and incubated overnight at 37°C and 5% CO_2. In the next step, monomer RhoB or RhoB-LPs in DMEM were separately added to each well in equal concentrations (RhoB or RhoB-LPs were dissolved in PBS). The cells were adequately washed with PBS, fixed with 4% polyformaldehyde fix solution, stained with 4.6-diamidino-2-phenylindole dihydrochloride (DAPI) and observed under a fluorescence microscope.

Pharmacokinetics Study

Healthy twenty-four male SD rats (average weighing 220–250 g) were randomly assigned to each of four groups: Cel-LPs IV group, Cel IV group, Cel-LPs PO group and Cel PO group. Cel solution (10 mg/kg, Cel dissolved in 5% DMSO, 65% PEG and 30% normal saline) and Cel-LPs solution (12.5 mg/kg, equivalent to 10 mg/kg Cel solution, Cel-LPs dissolved in PBS) were administered separately to rats in the Cel PO group and Cel-LPs PO group. The sublingual veins of rats were injected with Cel solution (2 mg/kg) and Cel-LPs solution (2.5 mg/kg, equivalent to 2 mg/kg Cel solution) after anesthesia. Then, the blood samples were collected from the tail vein (300 μL) at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 48 hr following injection. Next, the blood samples were centrifuged at 4000 r/min. The plasma was absorbed into the newly designated EP tube and stored at −20°C. The internal standard solution was added to a 50 μL plasma sample and vortexed for one min. The upper layer was centrifuged for 5 min at 13,000 r/min and analyzed using the sensitive LC-MS/MS method.

Statistical Analysis

Statistical analysis of the pharmacokinetic parameters was performed using the non-AV model to analyze the pharmacokinetic parameters including the maximal plasma concentration (C_{max}), maximum plasma time (T_{max}), apparent

volume of distribution ($V_{z/F}$), AUC, elimination half-life ($t_{1/2}$), plasma clearance (CL) and MRT. DAS was used to analyze component concentration changes over a while. The curves for plasma drug concentration time were plotted with GraphPad Prism (Version 8.0.1; GraphPad Software Inc., San Diego, CA, USA). Statistical data analyses were performed utilizing the one-way ANOVA using SPSS (version 22; SPSS Inc., Chicago, IL, USA). All the experimental data were expressed as the mean ± standard deviation (mean ± SD) and *P*-values of less than 0.05 were considered significant.

Results and Discussion

Researchers and clinicians have always struggled to optimize the effect of drugs with poor water solubility. Liposomes have been widely used as a drug delivery system for nearly half a century.[19] Liposomes are biocompatible and biodegradable carriers that are derived from natural compositions.[20] Studies have demonstrated that liposomes exert significant biocompatibility, nontoxicity, sustained release and long half-lives and can carry hydrophobic (contained in lipid bilayers) and hydrophilic drugs.[21,22] A variety of liposomes have been developed since AmBisome® was introduced in 1994.[11,23]

Phosphatidylcholine and Chol have inherent characteristics that limit the stability, efficiency, bioavailability and circulation of liposomes in vivo.[24] SL exhibited significant rigidity/fluidity than other saturated phospholipids, which could increase the permeability of liposomes.[25] Liposomes typically contain SL, one of the most widely used phospholipids. Past studies revealed that elevated concentrations of Chol increased the stability of liposome bilayers and reduced drug leakage.[26] The delivery efficiency of drugs must be improved by appropriately modifying liposomes. Researchers have found that modification of liposomes with DSPE-PEG forms a hydration film on the surface, which may increase the hydrophilicity of liposomes and reduce binding capacity and elimination by the reticuloendothelial system and mononuclear macrophage system, thereby prolonging circulation time in vivo.[27,28]

Therefore, liposomes were modified with DSPE-PEG2000 to enhance entrapment efficiency, bioavailability and circulation time of the drug delivery system. The cells were encased in liposomes to maximize medication distribution in the body by limiting drug loss in circulation or gathering in non-interesting sections, reducing the nonspecific side effects and toxicity of Cel, along with enhancing its efficacy and therapeutic index.

Method Validation and LC-MS/MS

The chromatogram of the Cel standard solution was obtained according to the above HPLC conditions (Figure 1A). The peak of Cel and midazolam appeared in 2.62 and 1.09 min, respectively. The following standard curve equations were calculated based on the peak area and mass concentration of Cel: y=0.0058936 * x + 0.00397721 (r^2=0.998351, in rat plasma) and y=3.37958 * x + 0.0600251 (r^2=0.995598 in PBS). Based on the findings, there were good linear relationships between the concentration and peak area of Cel in the range of 1~500 ng/mL (in rat plasma) and 0.1~10 μg/mL (in PBS).

Table 1 displays the precision (expressed by RSD) and accuracy (expressed by RE) of Cel in rat plasma and PBS calculated from the peak area of the chromatogram. There was a significant difference between the RSD% and RE% of less than 15% and 115%, respectively. In addition, Table 2 shows the peak areas of the chromatograms calculated for Cel in rat plasma and PBS. The above results met all requirements for determining the content of the study.

Optimization and Physicochemical Characterization of Cel-LPs

Characterization of Cel-LPs

Liposomes were obtained by the lipid thin-layer-hydration-extrusion method. The lipid membrane was first deposited on the substrate by rotary evaporation and then hydrated with PBS solution. Liposomes are usually reduced in size and layer structure through homogenization, ultrasound, extrusion and freeze-thaw cycles.[29] Blank liposomes were prepared by following the above method. Cel-LPs were prepared using the same method (Cel-LPs had the same concentration of lipids as Cel solution). Cel solution (Cel dissolved in DMSO) appeared transparent reddish brown in Figure 1B, blank liposomes were milky white and Cel-LPs had a reddish-brown appearance similar to the Cel solution. The liposomes present in the liquid produced the liquid to be slightly murky, indicating that Cel exists within the liposomes.

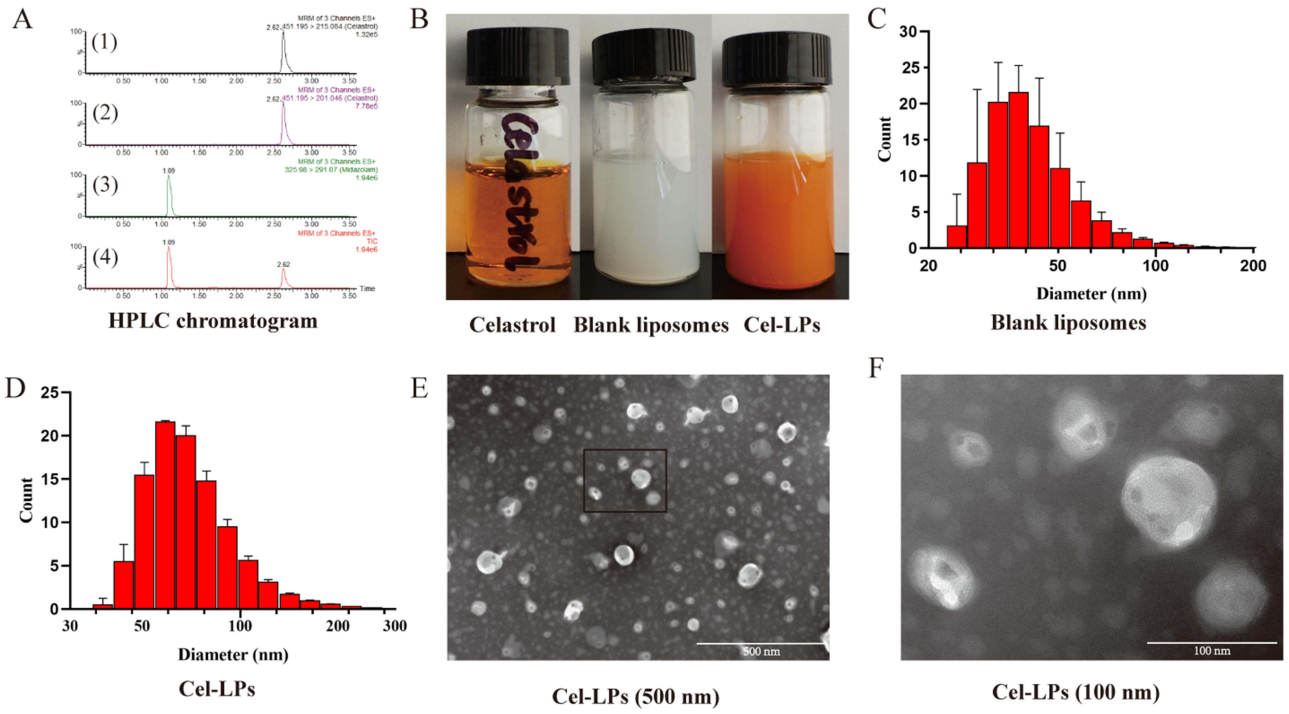

Figure 1 The preparation and characterization of Cel-LPs. (**A**) The HPLC chromatogram of Celastrol, (**B**) The appearance of Celastrol solution, Blank liposomes and Cel-LPs, (**C**) and (**D**) Size distribution of Blank liposomes and Cel-LPs, (**E**) and (**F**) Transmission electron microscopic image of Cel-LPs.

As shown in Figures 1C and 1D, compared with blank liposomes, the particle size of Cel-LPs encapsulated with Cel was increased. The average particle and the overall standard deviations of blank liposomes and Cel-LPs were 46.35 ± 17.86 nm and 72.20 ± 27.99 nm, respectively. Their particle sizes ranged from 50 to 200 nm, an ideal range for liposomes, due to their high membrane permeability and inability to be swallowed.[16,30] Accumulating evidence shows that the negative surface charge on liposomes can reduce the risk of swallowing by preventing liposomes from aggregating.[31,32] Table 3 shows that Cel-LPs had a zeta potential of −31.60 mV, whereas blank liposomes had a zeta potential of −17.00 mV. Cel-LPs possess higher negative charges, which supports their extended circulation time. Studies have indicated that a significant amount of liposomes with strong negative zeta potentials develop negative charges in aqueous pH 7.4, which increases their physical stability. The stability of a colloidal suspension depends on the surface charge of particles, which promotes particle repulsion and prevents the accumulation of particles.[33] In Figures 1E and 1F,

Table 1 Inter- and Intra-Day Precision and Accuracy of Celastrol in Rat Plasma and PBS (n=6)

		Intra-Day		Inter-Day	
	Nominal Concentration	**Precision RSD (%)**	**Accuracy RE (%)**	**Precision RSD (%)**	**Accuracy RE (%)**
In Rat Plasma (ng/mL)	3	8.31	111.3	7.53	107.53
	30	9.26	106.52	5.59	108.33
	300	6.71	110.79	11.09	109.65
In PBS (μg/mL)	0.3	6.14	102.33	7.38	106.11
	3	6.13	107.5	11.88	108.94
	9	3.54	104.08	3.14	105.54

Abbreviation: PBS, phosphate buffered saline.

Table 2 Stability of Celastrol in Rat Plasma and PBS (n=6)

		Intra-Day		Inter-Day	
	Nominal Concentration	**Precision RSD (%)**	**Accuracy RE (%)**	**Precision RSD (%)**	**Accuracy RE (%)**
In Rat Plasma (ng/mL)	3	8.31	111.3	7.53	107.53
	30	9.26	106.52	5.59	108.33
	300	6.71	110.79	11.09	109.65
In PBS (μg/mL)	0.3	6.14	102.33	7.38	106.11
	3	6.13	107.5	11.88	108.94
	9	3.54	104.08	3.14	105.54

Abbreviation: PBS, phosphate buffered saline.

Table 3 Summary of the Physicochemical Characteristics of Blank Liposomes and Cel-LPs

Sample	**Particle Size (nm)**	**PDI**	**Zeta Potential (mV)**	**Encapsulation Rate (%)**	**Drug Loading (%)**
Blank liposomes	46.35 ± 17.86	0.212	−17.00 ± 16.60	–	–
Cel-LPs	72.20 ± 27.99	0.267	−31.60 ± 6.810	78.77% ± 5.69%	5.83 ± 0.42%

Notes: Data represent mean ± SD, n = 3.
Abbreviation: PDI, polydispersity index.

TEM images confirmed that liposomes were round and oval with uniform particle size and smooth surfaces. Moreover, the layers of the liposome membrane were observed.

Drug Entrapment Efficiency and Loading Efficiency

Liposome loading performance can be evaluated using EE and DL, which directly correlate with the entrapment and absorption of nanoparticles. Previous studies indicate that high EE (more than 70%) means the drug is effective and less likely to cause adverse reactions.[34] The hydrophobic lipophilic drug Cel is actively dispersed between lipid bilayers in liposomes.[35] The EE and DL of the prepared long-circulating Cel-LPs were 78.77 ± 5.69% and 5.83 ± 0.42%, respectively (Table 3), indicating an efficient drug loading for disease treatment. Chen et al developed Cel-loaded galactose-modified PEGylated liposomes and Cel-loaded PEGylated liposomes using the film dispersion method with encapsulation efficiencies of 90% and 97%, respectively.[36] Therefore, liposomes encapsulating Cel have a higher encapsulation efficiency.

In Vitro Drug Release

The solubility of Cel in water is very low, approximately 0.3 μg/mL. PBS was diluted with 0.5% Tween 80 to simulate the conditions for drug release in vivo. Figure 2A shows the release of Cel-LPs under different pH buffer conditions in vitro. The results indicated that drug release was rapid in the initial stage with 10.51 ± 1.41% (pH 5.5), 13.57 ± 1.52% (pH 6.8) and 9.19 ± 1.04% (pH 7.4) in the first 10 hr. After a slow-release period, a plateau was reached after 120 hr with the percentages of the cumulative release of 45.57 ± 4.92% (pH 5.5), 36.80 ± 2.20% (pH 6.8) and 19.93 ± 1.96% (pH 7.4), respectively. The percentages of release under pH 5.5 and 7.4 were slightly lower than pH 6.8 in the first 48 hr (Figure 2B). We found that the total percentage of release in pH 5.5 and 6.8 were still higher than pH 7.4 and pH 5.5 was the highest.

The entrapment of liposomal reduced drug release. Cel adsorbed on the liposome surface accelerated the release of the former, while drug diffusion from the stable lipid bilayer maintained a longer release cycle in the latter segment.[37] Liposome entrapment results in sustained release, which contributes to the effective treatment of diseases. This method of drug delivery facilitates rapid blood drug concentration and the recovery of patients from disease. The continuous release

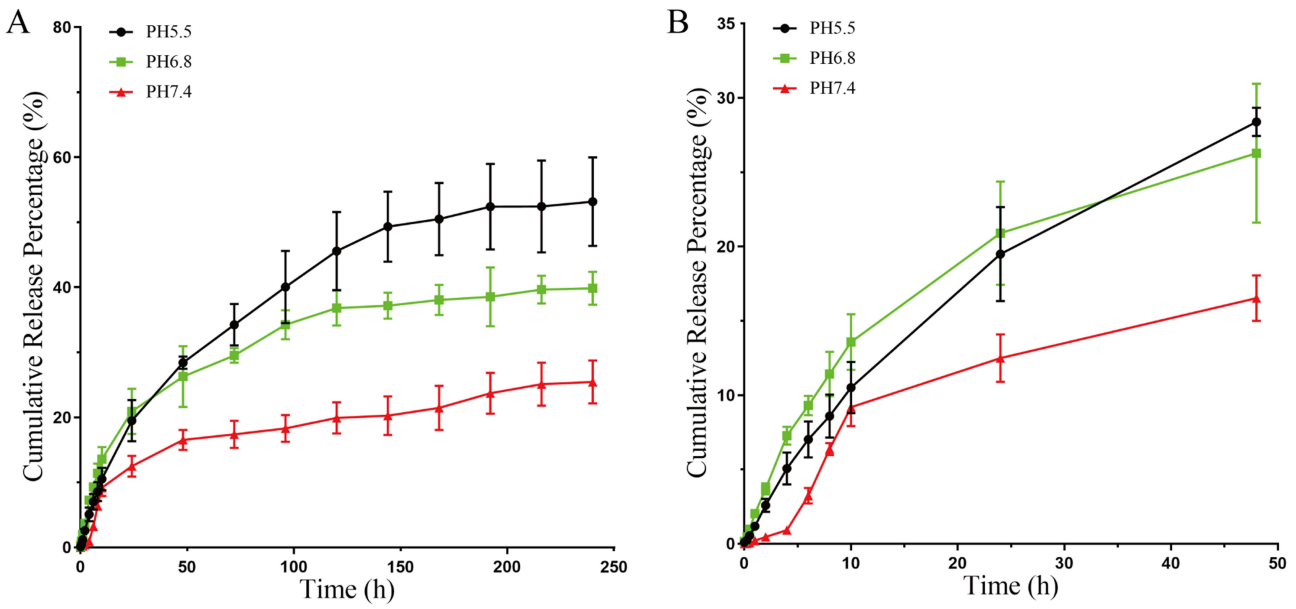

Figure 2 In vitro cumulative release percentage of Cel-LPs in pH 5.5, 6.8 and 7.4 at 37°C. (**A**) The all 240 hours, (**B**) The first 48 hours. (Data represent mean ± SD, n = 3).

further allows blood drug concentrations to remain stable. Our results indicated that the total release percentage of Cel-LPs was highest at pH 5.5, which could be more effective in acidic inflammatory conditions.

Cytotoxicity Test

Figure 3A presents the results. There was no cytotoxicity of blank liposomes on MH7A cells, although cell survival fluctuated slightly with increasing concentration and incubation period. It was observed that the long-cycle liposomes prepared were highly biocompatible. The cytotoxicity of Cel and Cel-LPs on MH7A cells was dose-dependent and time-dependent. MH7A cells were not significantly affected by Cel and Cel-LPs at the concentrations of 0.0625–0.5 μg/mL over a 24-hour incubation period. MH7A cells showed a significant reduction in viability after 24 hr when Cel-LPs concentrations reached 1 μg/mL. Nevertheless, Cel did not significantly affect the viability of MH7A cells at a concentration of 1 μg/mL. The high affinity of liposomes for membranes may have allowed Cel to enter cells more efficiently and have a stronger cytotoxic effect.[35] Additionally, the higher concentrations of Cel and Cel-LPs result in more excellent cytotoxicity effects on MH7A cells. After prolonging the incubation period to 48 h and 72 h, the cell survival rate of each group was significantly decreased. The survival rate of the cells treated with the same concentrations of Cel and Cel-LPs differed significantly ($P < 0.05$) following 48 or 72 h of incubation.

The Uptake of Liposomes by MH7A Cells

The uptake of RhoB and RhoB-LPs by MH7A cells was analyzed by fluorescence microscopy. The results showed that RhoB-LPs could significantly enhance intracellular fluorescence of the cells compared to RhoB solution (Figure 3B). Liposomes are microcapsules containing compact phospholipid bilayers that can fuse with other lipid bilayers to facilitate drug uptake. The results of the cytotoxicity tests were similar to those of the previous studies.[13]

Pharmacokinetics Study

The plasma concentrations of Cel were determined following oral and intravenous administration of Cel-LPs and free Cel to SD rats (Figure 4). The primary pharmacokinetic parameters in plasma are summarized in Table 4. The results indicated no significant differences in pharmacokinetic parameters between the Cel-LPs PO and Cel PO groups, with plasma concentrations increasing slowly. The liposomes may not be stable enough in the gastrointestinal tract. The

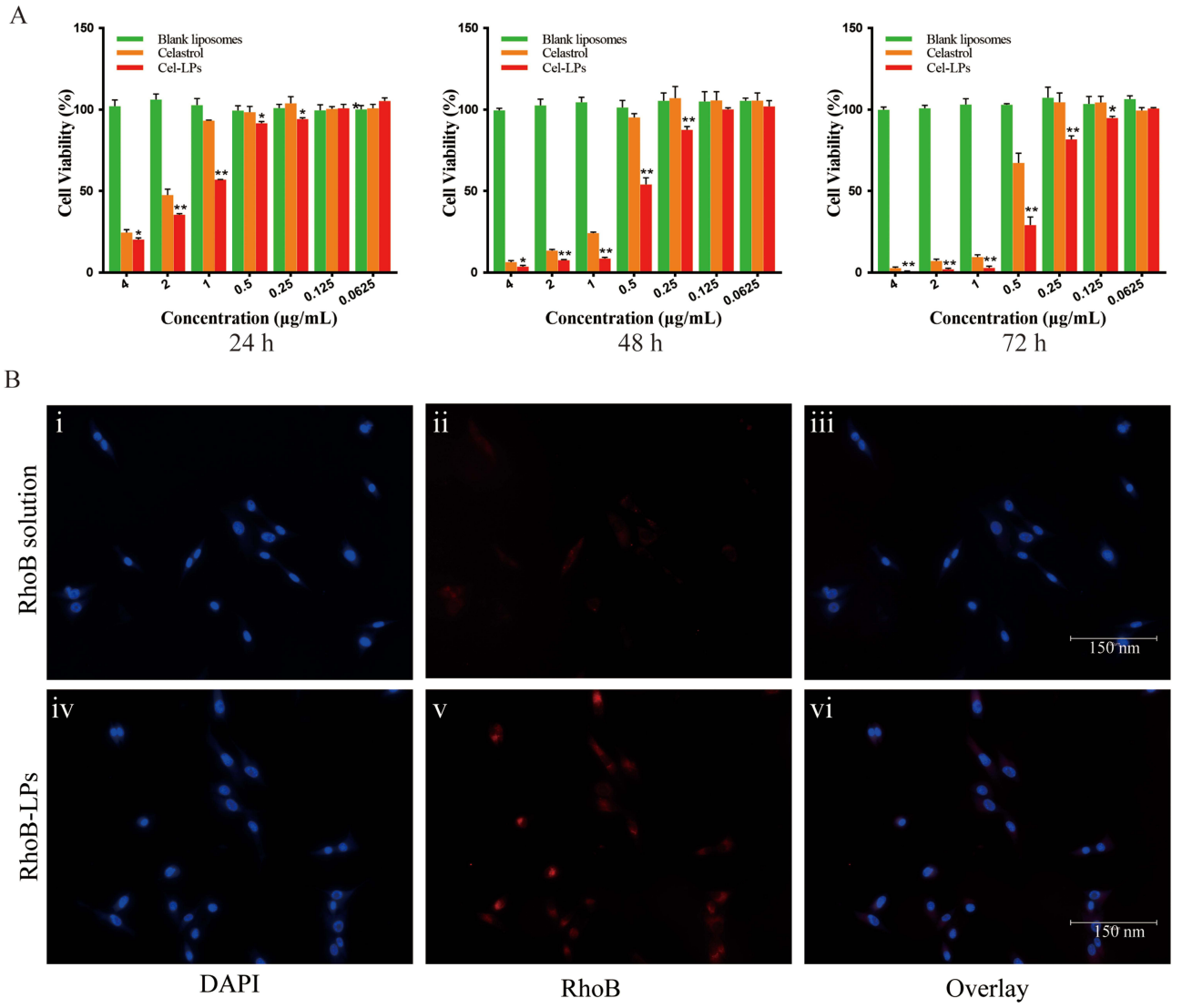

Figure 3 The viability and uptake of Cel-LPs in MH7A cells. (**A**) The viability of MH7A cells upon incubation with Celastrol solution, Blank liposomes and Cel-LPs for 24h,48h and 72h (Data represent mean ± SD, n = 3). *Results are significantly different (*P< 0.05, **P< 0.01), the Cel-LPs groups were compared with the Celastrol group. (**B**) Representative fluorescence microscopic images showing the uptake of liposomes by the MH7A cells. (Up) RhoB solution; (Down) RhoB-LPs. Fluorescent images of the cell nucleus stained with DAPI (i and iv), fluorescent images of the RhoB (ii and v), overlay (iii and vi).

degradation of Cel by enzymes or interactions with intestinal components may result in a premature release of Cel, which would not improve its pharmacokinetic parameters compared with those of the free drug.

Following intravenous injection, maximum blood concentrations of the two drugs were reached and subsequently decreased. A significant difference was noticed between the Cel IV and Cel-LPs IV groups in the first two hr ($P< 0.05$). Intriguingly, we found no significant statistical difference in blood concentrations between the Cel-LPs IV and the Cel IV groups after the third hr.

The $AUC_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$ were similar between the two groups, which indicates that the doses administered were identical in both groups. However, the Cel-LPs IV group had an $MRT_{(0\text{-}\infty)}$ of 7.98 ± 0.33 h, while the Cel IV group had 2.88 ± 0.18 h. A long period of low blood concentrations and a prolonged retention time were observed in the Cel-LPs IV group ($P<0.001$). A significant improvement was also observed in the $t_{1/2}$($P<0.001$). The results showed that the Cel-LPs IV group had 3.5 times longer $t_{1/2}$ than the Cel IV group (3.35 ± 1.50 h). There were also significant differences ($P<0.001$) in $MRT_{(0\text{-}t)}$ and $V_{z/F}$. Oral absorption of Cel-LPs and Cel was found to be greatly limited, while injection increased their absolute bioavailability. Furthermore, the Cel-LPs IV group showed significantly higher $MRT_{(0\text{-}t)}$ and Vz/

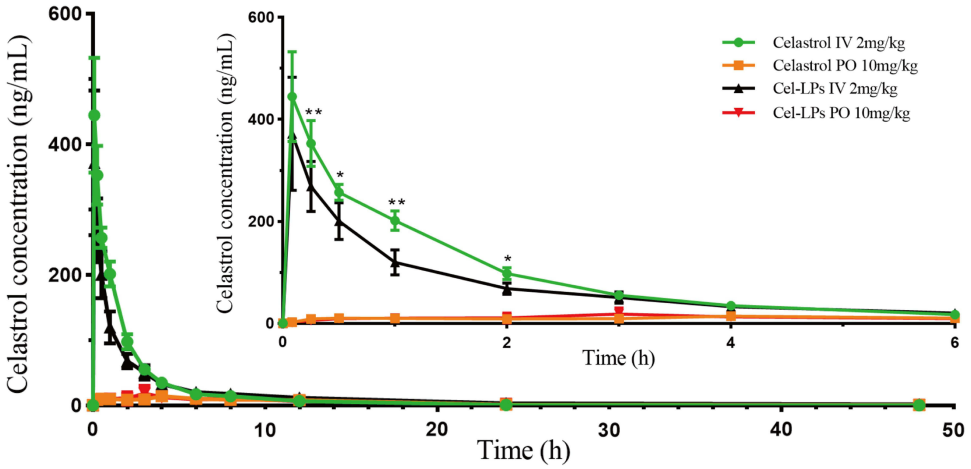

Figure 4 The plasma concentration-time profiles of Celastrol (Data represent mean ± SD, n = 6). *Results are significantly different (*P < 0.05, **P < 0.01). The Cel-LPs IV groups were compared with the Cel IV group.

F values than the Cel IV group by 2.2- and 3.4-fold, respectively. Intriguingly, our data indicated that other pharmacokinetic parameters had no significant difference (P>0.05). The encapsulation of liposomes prolonged the circulation time and facilitated sustained absorption of the two drugs following intravenous administration. The absolute bioavailability (F%) of Cel-LPs and Cel was 5.78 ± 1.07% and 5.36 ± 1.59%, respectively. We found that the administration of Cel-LPs increased bioavailability, prolonged drug retention time and maintained high blood concentrations, thereby promoting curative effects.

This experiment presents several limitations. The study was conducted on healthy animals, which may not be representative of patients with RA. The pharmacokinetic outcome of CIA rats requires further study. We conducted pharmacokinetic studies on six rats in each group. Our experiments require this number of subjects, but adding more could strengthen our credibility. Furthermore, the research does not address the long-term stability and ideal storage conditions of Cel-LPs. Therefore, liposomal formulations should be thoroughly explored and documented for practical use.

Table 4 The Main Plasma Pharmacokinetic Parameters of Celastrol in the Four Groups

Pharmacokinetics Parameters	IV group		PO Group	
	Cel-LPs IV Group	Cel IV Group	Cel-LPs PO Group	Cel PO Group
$AUC_{(0-t)}$ (ug/L*h)	711.27 ± 84.89	731.16 ± 56.78	212.33 ± 50.02	192.92 ± 64.19
$AUC_{(0-\infty)}$ (ug/L*h)	751.16 ± 76.94	734.87 ± 57.14	218.22 ± 49.51	199.39 ± 70.60
$MRT_{(0-t)}$(h)	6.13 ± 1.03$^{\#}$	2.74 ± 0.31	11.67 ± 1.59	10.87 ± 1.10
$MRT_{(0-\infty)}$ (h)	7.98 ± 0.33$^{\#}$	2.88 ± 0.18	13.09 ± 4.40	12.24 ± 2.50
$t_{1/2}$ (h)	11.71 ± 3.05$^{\#}$	3.35 ± 1.50	6.84 ± 4.30	7.03 ± 4.52
T_{max} (h)	0.12 ± 0.06	0.11 ± 0.07	3.25 ± 2.19	5.83 ± 3.25
$V_{z/F}$ (L/kg)	44.63 ± 12.88$^{\#}$	13.13 ± 5.81	450.04 ± 272.16	496.05 ± 251.23
$C_{Lz/F}$ (L/h/kg)	2.71 ± 0.25	2.74 ± 0.21	48.59 ± 14.95	55.97 ± 20.98
C_{max} (ug/L)	372.40 ± 53.31	445.97 ± 86.89	21.77 ± 10.84	15.80 ± 4.03

Notes:$^{\#}$Results are significantly different ($^{\#}P$< 0.001), the Cel-LPs IV groups were compared with the Cel IV group. Data represent mean ± SD, n = 6.

Abbreviations: AUC, area under the plasma concentration-time curve; MRT, mean residence time; $t_{1/2}$, elimination half-life; T_{max}, maximum plasma time; $V_{z/F}$, apparent volume of distribution; $C_{Lz/F}$, plasma clearance; C_{max}, maximal plasma concentration.

Conclusions

In this study, we successfully developed Cel-LPs with sustained release profiles. Liposomes encapsulated over 75% of Cel with a suitable size distribution window. Cel-LPs exhibited slow and continuous release profiles, which may allow the drug to maintain an adequate blood concentration during treatment. The fluorescence microscope indicated that Rhodamine-B-loaded liposomes enhanced the absorption of RhoB by MH7A cells. Further experiments showed that Cel-LPs were not toxic and increased the absorption of Cel. In addition, the pharmacokinetics of oral and intravenous administration indicated that Cel-LPs prolonged the half-life and retention time of Cel. In summary, this study has provided valuable insights into how poorly soluble drugs are managed and has increased the solubility and bioavailability of Cel through intravenous administration, laying the foundation for its clinical application in the treatment of RA.

Funding

This study was supported by the Medicine and Health Technology Funding Project of Zhejiang (2022RC303, 2022RC302, 2024KY580), and Lishui Technology Plan Project (2022GYX24, 2023SJZC060, 2023GYX04).

Disclosure

The authors report no conflicts of interest in this work.

References

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011;365(23):2205–2219. doi:10.1056/NEJMra1004965
2. Firestein GS, McInnes IB. Immunopathogenesis of Rheumatoid Arthritis. *Immunity*. 2017;46(2):183–196. doi:10.1016/j.immuni.2017.02.006
3. Tobon GJ, Youinou P, Saraux A. The environment, geo-epidemiology, and autoimmune disease: rheumatoid arthritis. *J Autoimmun*. 2010;35(1):10–14. doi:10.1016/j.jaut.2009.12.009
4. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature*. 2003;423(6937):356–361. doi:10.1038/nature01661
5. Uhlig T, Moe RH, Kvien TK. The burden of disease in rheumatoid arthritis. *Pharmacoeconomics*. 2014;32(9):841–851. doi:10.1007/s40273-014-0174-6
6. Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. *Lancet*. 2007;370(9602):1861–1874. doi:10.1016/s0140-6736(07)60784-3
7. Burmester GR, Feist E, Dörner T. Emerging cell and cytokine targets in rheumatoid arthritis. *Nat Rev Rheumatol*. 2014;10(2):77–88. doi:10.1038/nrrheum.2013.168
8. An L, Li Z, Shi L, et al. Inflammation-Targeted Celastrol Nanodrug Attenuates Collagen-Induced Arthritis through NF-kappaB and Notch1 Pathways. *Nano Lett*. 2020;20(10):7728–7736. doi:10.1021/acs.nanolett.0c03279
9. Gao Q, Qin H, Zhu L, Li D, Hao X. Celastrol attenuates collagen-induced arthritis via inhibiting oxidative stress in rats. *Int Immunopharmacol*. 2020;84:106527. doi:10.1016/j.intimp.2020.106527
10. Cascão R, Fonseca JE, Moita LF. Celastrol: a Spectrum of Treatment Opportunities in Chronic Diseases. *Front Med*. 2017;4:69. doi:10.3389/fmed.2017.00069
11. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Delivery Rev.* 2013;65(1):36–48. doi:10.1016/j.addr.2012.09.037
12. Skupin-Mrugalska P, Minko T. Development of Liposomal Vesicles for Osimertinib Delivery to EGFR Mutation-Positive Lung Cancer Cells. *Pharmaceutics*. 2020;12(10):939. doi:10.3390/pharmaceutics12100939
13. Xu H, Ji H, Li Z, Qiao W, Wang C, Tang J. In vivo Pharmacokinetics and in vitro Release of Imatinib Mesylate-Loaded Liposomes for Pulmonary Delivery. *Int J Nanomed*. 2021;16:1221–1229. doi:10.2147/ijn.S294626
14. Crommelin DJA, van Hoogevest P, Storm G. The role of liposomes in clinical nanomedicine development. What now? Now what? *J Control Release*. 2020;318:256–263. doi:10.1016/j.jconrel.2019.12.023
15. Kim CE, Lim SK, Kim JS. In vivo antitumor effect of cromolyn in PEGylated liposomes for pancreatic cancer. *J Control Release*. 2012;157(2):190–195. doi:10.1016/j.jconrel.2011.09.066
16. Gupta V, Gupta N, Shaik IH, et al. Liposomal fasudil, a rho-kinase inhibitor, for prolonged pulmonary preferential vasodilation in pulmonary arterial hypertension. *J Control Release*. 2013;167(2):189–199. doi:10.1016/j.jconrel.2013.01.011
17. Bangham AD. Membrane models with phospholipids. *Prog Biophys Mol Biol*. 1968;18:29–95. doi:10.1016/0079-6107(68)90019-9
18. Nguyen HT, Tran TH, Thapa RK, et al. Incorporation of chemotherapeutic agent and photosensitizer in a low temperature-sensitive liposome for effective chemo-hyperthermic anticancer activity. *Expert Opin Drug Delivery*. 2017;14(2):155–164. doi:10.1080/17425247.2017.1266330
19. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol*. 1965;13(1):238–252. doi:10.1016/s0022-2836(65)80093-6
20. Wissing SA, Kayser O, Müller RH. Solid lipid nanoparticles for parenteral drug delivery. *Adv. Drug Delivery Rev.* 2004;56(9):1257–1272. doi:10.1016/j.addr.2003.12.002
21. Al-Jamal WT, Kostarelos K. Liposomes: from a clinically established drug delivery system to a nanoparticle platform for theranostic nanomedicine. *Acc. Chem. Res.* 2011;44(10):1094–1104. doi:10.1021/ar200105p
22. Sawant RR, Torchilin VP. Liposomes as 'smart' pharmaceutical nanocarriers. *Soft Matter*. 2010;6(17):4026.

23. Hann IM, Prentice HG. Lipid-based amphotericin B: a review of the last 10 years of use. *Int J Antimicrob Agents*. 2001;17(3):161–169. doi:10.1016/s0924-8579(00)00341-1
24. Roux E, Stomp R, Giasson S, Pézolet M, Moreau P, Leroux JC. Steric stabilization of liposomes by pH-responsive N-isopropylacrylamide copolymer. *J Pharmaceut Sci*. 2002;91(8):1795–1802. doi:10.1002/jps.10172
25. Nguyen TL, Nguyen TH, Nguyen DH. Development and In Vitro Evaluation of Liposomes Using Soy Lecithin to Encapsulate Paclitaxel. *Int J Biomater*. 2017;2017:8234712. doi:10.1155/2017/8234712
26. Vemuri S, Rhodes CT. Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharm Acta Helv*. 1995;70 (2):95–111. doi:10.1016/0031-6865(95)00010-7
27. Crommelin D, Grit M, Talsma H, Zuidam NJ. Pharmacy I. Liposomes as Carriers for Drugs and Antigens: approaches to Preserve Their Long Term Stability. *Drug Dev Industrial Pharm*. 1994;20:4027.
28. Bittman R, Blau L. The phospholipid-cholesterol interaction. Kinetics of water permeability in liposomes. *Biochemistry*. 1972;11(25):4831–4839. doi:10.1021/bi00775a029
29. Furukawa RJBR, Ras BI, Conferenc EI. Experimental study and model of the physical chemical properties of liposome under ultrasonic irradiation. *Drug Dev Industrial Pharm*. 2010;584(1):843–848.
30. Harashima H, Sakata K, Funato K, Kiwada H. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. *Pharm Res*. 1994;11(3):402–406. doi:10.1023/a:1018965121222
31. Nahar K, Absar S, Patel B, Ahsan F. Starch-coated magnetic liposomes as an inhalable carrier for accumulation of fasudil in the pulmonary vasculature. *Int J Pharm*. 2014;464(1–2):185–195. doi:10.1016/j.ijpharm.2014.01.007
32. Sheng Y, Chang L, Kuang T, Hu JJRA. PEG/heparin-decorated lipid-polymer hybrid nanoparticles for long-circulating drug delivery. *Int J Med*. 2016;6(28):23279–23287.
33. Freag MS, Saleh WM, Abdallah OY. Self-assembled phospholipid-based phytosomal nanocarriers as promising platforms for improving oral bioavailability of the anticancer celastrol. *Int J Pharm*. 2018;535(1–2):18–26. doi:10.1016/j.ijpharm.2017.10.053
34. Anwer MK, Ali EA, Iqbal M, et al. Development of Sustained Release Baricitinib Loaded Lipid-Polymer Hybrid Nanoparticles with Improved Oral Bioavailability. *Molecules*. 2021;27(1):68. doi:10.3390/molecules27010168
35. Pattni BS, Chupin VV, Torchilin VP. New Developments in Liposomal Drug Delivery. *Chem. Rev.* 2015;115(19):10938–10966. doi:10.1021/acs.chemrev.5b00046
36. Chen X, Hu X, Hu J, Qiu Z, Yuan M, Zheng G. Celastrol-Loaded Galactosylated Liposomes Effectively Inhibit AKT/c-Met-Triggered Rapid Hepatocarcinogenesis in Mice. *Mol Pharmaceut*. 2020;17(3):738–747. doi:10.1021/acs.molpharmaceut.9b00428
37. Gupta N, Al-Saikhan FI, Patel B, Rashid J, Ahsan F. Fasudil and SOD packaged in peptide-studded-liposomes: properties, pharmacokinetics and ex-vivo targeting to isolated perfused rat lungs. *Int J Pharm*. 2015;488(1–2):33–43. doi:10.1016/j.ijpharm.2015.04.031

International Journal of Nanomedicine Dovepress

Publish your work in this journal

The International Journal of Nanomedicine is an international, peer-reviewed journal focusing on the application of nanotechnology in diagnostics, therapeutics, and drug delivery systems throughout the biomedical field. This journal is indexed on PubMed Central, MedLine, CAS, SciSearch®, Current Contents®/Clinical Medicine, Journal Citation Reports/Science Edition, EMBase, Scopus and the Elsevier Bibliographic databases. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.

Submit your manuscript here: https://www.dovepress.com/international-journal-of-nanomedicine-journal