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ORIGINAL RESEARCH

RETRACTED ARTICLE: In vitro and in vivo evaluation of novel NGR-modified liposomes containing brucine

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Abstract: In this study, a novel NGR (Asn-Gly-Arg) peptide-modified liposomal brucine was prepared by using spray-drying method. The surface morphology of the liposomes, encapsulation efficiency and particle size were investigated. The data showed that the addition of NGR did not produce any significant influence on brucine liposomes in terms of particle size or zeta potential. In addition, after 3 months of storage, no drastic changes such as visible aggregation, drug content changes or precipitation in the appearance of NGR-brucine liposomes occurred. The in vitro release results indicated that the release of brucine from NGR liposomes was similar to that of liposomes, demonstrating that the NGR modification did not affect brucine release. The in vitro drug-release kinetic model of NGR-brucine liposomes fitted well with the Weibull's equation. In vivo, NGR-brucine liposomes could significantly extend the bioavailability of brucine; however, there was no significant difference observed in the pharmacokinetic parameters between liposomes and NGR liposomes after intravenous administration. Antitumor activity results showed that NGR-modified liposomes exhibited less toxicity and much higher efficacy in HepG2-bearing mice compared with non-modified liposomes. The enhanced antitumor activity might have occurred because brucine was specifically recognized by NGR receptor on the surface of tumor cells, which enhanced the intracellular uptake of drugs.

Keywords: brucine, liposome, NGR, HepG2, in vivo, in vitro

Introduction

Brucine (CAS No 57-24-9) is an alkaloid and exists mainly in the seeds of *Strychnos nux-vomica* L. (Loganiaceae),[1] which is widely found in many southern Asian countries. Brucine itself is known as an anti-inflammatory and analgesic drug for relieving arthritic and traumatic pain.[2–4] Its main pharmacodynamic actions include relief of pain, reduction of swelling and promotion of circulation.[5] Strychinin and brucine are the two main active ingredients of the semen strychni. In addition, some research have indicated that strychinin can effectively inhibit the proliferation of several types of cancer cells, including glioma, breast cancer, colorectal cancer and others,[6–8] with an obvious inhibitory effect on liver cancer cells. Studies involving in vitro culture of hepatoma carcinoma cells have shown that strychinin could inhibit the proliferation of HepG2 and SMMC-7721 cells.[9–12] Unfortunately, the potential use of brucine is severely limited due to high incidence of side effects. Because it is strongly fat-soluble and easily distributed in the central nervous system (CNS) in the brain and other organs, it exerts severe CNS toxicity.[13,14] There is a narrow margin of safety between a therapeutic and a toxic dose. Thus, the key to reduce the toxicity and increase the effect of brucine is to increase the concentration of strychinin in its effect target and reduce its distribution in brain tissues to lower CNS toxicity.

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Colloidal drug delivery systems, such as liposomes, represent a mature technology with considerable potential for the entrapment of both lipophilic and lipophobic drugs.[15] Encapsulation or entrapment of drugs in liposomes results in distinct changes in the pharmacokinetic and pharmacodynamic properties of free drugs, and in some cases, causes an apparent decrease in toxicity and/or an increase in therapeutic efficacy.[16]

In recent years, the use of ligand–receptor-based system for targeted drug delivery has become a hot research topic. Use of tumors itself and receptors on newborn vascular endothelial cells as target, together with intravenous administration of targeted liposomes to promote active targeting, can effectively increase efficacy. NGR is a polypeptide which contains asparagine-glycine-arginine (Asn-Gly-Arg) sequence.[17] Tumor cells and tumor newborn vascular endothelial cells exhibit high expression of aminopeptidase N (APN; CD13). NGR can integrate with high specificity, inhibit the generation of tumor newborn blood vessels and thus inhibit the growth and transfer of tumors. APN is a membrane-bound, zinc-dependent metalloproteinase that plays a key role in tumor invasion and angiogenesis.[18]

In this study, a novel NGR peptide-modified liposom[illegible] brucine was prepared by using spray-drying method. Th[illegible] surface morphology of the liposomes, encapsul[illegible]n efficiency and particle size were investigated. The[illegible] formul[illegible]ions were characterized by in vitro release stu[illegible] The [illegible]al formulation providing sustained drug [illegible]lease [illegible] selected for in vivo study.

Materials and methods

Chemicals and reagents

Brucine was purchased [illegible]om Yanjian Biopharma Ltd., Co. (Shanghai, People's Republic of China). The chemical structure of brucine is sh[illegible]wn in F[illegible]ure 1. [illegible]al standard (IS) strychinin was purcha[illegible]d fro[illegible] Shanghai Institute of Biological Products (Sha[illegible]i, People's Republic of China). Soybean phosphatidylcholi[illegible] (SPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000) and cholesterol (CHOL) were obtained from Sinopharm Chemical Reagent (Shanghai, People's Republic of China). HepG2 was purchased from Genomeditech Biopharma Ltd., Co. (Shanghai, People's Republic of China). The NGR peptide was synthesized by Ningbi Kangbei Biochemical Co., Ltd. (Zhejiang, People's Republic of China). NGR-PEG-DSPE was synthesized according to previously reported method.[19,20] All other reagents were obtained from Sinopharm Chemical R[illegible]gent. M[illegible]anol and [illegible]cetonitrile (chromatographic grade) [illegible]ere obtained fr[illegible] EMD Millipore, Billerica, MA, USA. [illegible]ter f[illegible] high-performance liquid chromatography (HPLC) w[illegible] double-[illegible]stilled, and all other reagents were [illegible]alytical gra[illegible]

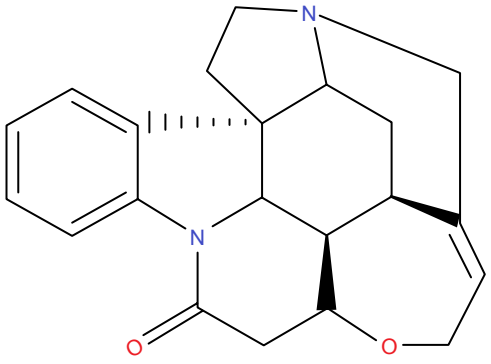

Figure 1 The chemical structure of brucine.

Prepa[illegible]on of li[illegible]somes

The [illegible]GR-modified liposomes containing brucine (NGR-bru[illegible]ne) were pr[illegible]pared by thin-film hydration method, as descri[illegible]ed previo[illegible]sly.[8] Briefly, a mixture of brucine, SPC, CHOL, P[illegible]DSPE and NGR-PEG-DSPE (the molar ratio of [illegible] PEG-DSPE:PEG-DSPE:CHOL:SPC was 5:5:30:60; [illegible]e weight ratio of lipid:brucine was 19:1; the modification [illegible]egree of NGR in NGR-brucine was about 0.5% [molar [illegible]atio %]) was dissolved in chloroform. Then, the solvent was evaporated using an RE52 rotary evaporator (Shanghai Yarong Biochemistry Instrument Company, Shanghai, People's Republic of China) in a round-bottomed flask at 40°C for about 40 min to obtain a solid film. This film was then flushed with nitrogen gas for 30 min and stored overnight in a desiccator to remove any traces of chloroform. After that, the thin film was hydrated in a 5% glucose solution by sonication in a water bath for 10 min to produce a suspension of liposomes. Then, the liposomes were freeze-dried for 72 hours. The dry powder was rehydrated and sonicated for 3 min prior to application. For the preparation of liposomes containing brucine, a similar procedure was carried out except that the NGR-PEG-DSPE was replaced by PEG-DSPE.

Characterization

Particle size and zeta potential of the liposomes were measured by the dynamic light scattering technique using a zeta potential/particle sizer (Beckman Coulter, Brea, CA, USA). All measurements were performed in triplicate, and the

values are represented as mean ± SD (n=3). The morphologies of liposomes were visualized by transmission electronic microscopy (TEM) (JEM-1200EX; JEOL, Tokyo, Japan). The samples were added to the surface of copper grids, and stained with phosphotungstic acid (1%, w/v). The accelerating voltage was set at 120 kV. The encapsulation efficiency was estimated from the following formula:

$$\text{Encapsulation efficiency} = \frac{\text{Actual amount of drug loaded in liposomes}}{\text{Theoretical amount of drug loaded in liposomes}} \times 100\%$$

HPLC analysis

The concentration of brucine in the prepared liposomes was determined by HPLC. Separation was carried out at 35°C using a reverse-phase C18 column (5 µm, 4.6×250 mm). The mobile phase consisted of acetonitrile and buffer (10 mm sodium heptane sulfonate and 20 mm potassium dihydrogen phosphate, pH adjusted to 2.8 with 10% phosphonic acid). The ratio of acetonitrile/buffer (v/v) was adjusted to 24:76. The detection wavelength was 264 nm, and a flow rate of 1.0 mL/min was employed. A sample volume of 20 µL was injected.

Storage stability studies

The NGR-brucine liposomes and non-targeted brucine liposomes were studied for stability at 4°C. These formulations were tested at regular time intervals to identify changes in particle size, zeta potential and drug content.

In vitro release

The in vitro release of NGR-brucine liposomes, brucine liposomes and free brucine was analyzed according to the published method. The liposome suspension (drug content: 2 mg) and free drug were placed in a dialysis bag with a molecular weight cut-off of 10,000 Da. The dialysis bag was suspended in 100 mL PBS (pH 7.4) which was incubated at 37°C under constant rotation at 500 rpm. At scheduled time intervals, aliquot samples were withdrawn and assayed for brucine content by HPLC as described above. The volume of dissolution medium was maintained at 100 mL throughout the experiment.

In vivo pharmacokinetic studies

Thirty Sprague Dawley rats were divided into three groups (10 rats per group). All experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the China National Institutes of Health (Shanghai, People's Republic of China), and legal approval was obtained from Tongji University School of Medicine. All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Three groups were given a single dose of 2.5 mg/kg brucine solution (dissolved in PBS), brucine liposomes and NGR-brucine liposomes, respectively. Blood samples were collected at 5, 10, 30, 60, 90, 120, 180, 240, 360, 480 and 720 min after the administration, and the plasma was separated by centrifugation. The brucine concentration in the plasma was quantitatively analyzed using the HPLC method.

Briefly, plasma samples (100 µL) were mixed with strychinin (50 µg/mL) IS dissolved in methanol (20 µL). To this mixture, 100 µL of aqueous ammonia was added, and the samples were vortexed. Then, 3 mL of n-hexane–dichloromethane–isopropanol (65:30:5, v/v/v) was added and vortexed for 2 min. After centrifugation for 5 min at 12,000 rpm, the supernatant was collected, and the organic solvent was eliminated under nitrogen gas stream at 50°C. Then, the mixture was resuspended with the mobile phase (200 µL). An aliquot of the supernatant (20 µL) was injected into the HPLC system after centrifugation.

Histology studies

The histopathological changes induced by brucine liposomes and NGR-brucine liposomes after pharmacokinetic studies were evaluated. Animals were anesthetized, and their livers, spleens and kidneys were dissected and washed with cold saline. The organs were pressed between filter pads, weighed and then fixed in 10% neutral formalin using standard techniques and stained with hematoxylin and eosin for histopathological examination. All tissue samples were examined and graded under light microscopy with 500× magnification.

In vivo antitumor activity

The HepG2 model was established as described before.[21] On the 8th day, the kunming mice were randomly assigned to four groups (12 animals per group): group 1 was administered a 5% glucose injection, group 2 was administered free brucine, group 3 was administered brucine liposomes and group 4 was administered NGR-brucine liposomes. The brucine formulations were all injected via the tail vein on days 8, 10, 12 and 14, at a dose of 15 mg/kg. The total dose of brucine administered in all treatment groups was 60 mg/kg. A digital caliper was used to measure the tumor

diameters, and tumor volumes (mm^3) were calculated using the following formula: tumor volume = length × width^2 ×0.5. Throughout the study, mice were weighed regularly in order to monitor the potential toxicities.

Statistical analysis

All data are presented as mean ± SD. One-way analysis of variance was used to determine significance among groups. Statistical significance was established at $P<0.05$.

Results

Characterization of NGR-brucine liposomes

Table 1 shows that the addition of NGR did not produce any significant influence on brucine liposomes in terms of particle size or zeta potential. The average particle size of brucine liposomes and NGR-brucine liposomes was 85.3±3.2 and 92.6±4.1 nm, respectively. The zeta potential of brucine liposomes and NGR-brucine liposomes was −16.2±3.5 and −16.5±3.3 mV, respectively. The encapsulation efficiency of brucine in liposomes and NGR-modified liposomes was 87.4%±3.1% and 89.6%±2.7%, respectively. The high encapsulation efficiency in the formulation might be related to the strong hydrophobicity of brucine. Table 1 also gives the stability data of the particle size of NGR-brucine liposomes stored at 4°C. After 3 months of storage, no dramatic change such as visible aggregation, drug content changes or precipitation in the appearance of NGR-brucine liposomes occurred. TEM images (Figure 2) showed that the liposomes dispersed well with a uniform shape.

In vitro release

The in vitro release of brucine from the free drug, liposomes and NGR liposomes was studied in PBS (Figure 3). Over time, brucine in liposomes was released much more slowly than free drug. Table 2 shows that the in vitro drug-release kinetic model of NGR-brucine liposomes fitted well with the Weibull's equation: $\ln(1/(1-Q))= -2.154 \ln t + 1.12$ (r=0.9829).

Pharmacokinetics

The pharmacokinetic parameters studied in rats given 2.5 mg/kg of brucine as free drug, encapsulated in liposomes and encapsulated in NGR liposomes (2.5 mg brucine equivalent dose) are listed in Table 3. Figure 4 shows the mean plasma brucine concentration versus time curves, corresponding to the intravenous administration of free drug, liposomes and NGR liposomes, respectively. As shown in Figure 4, after a single injection of brucine injection, the plasma drug concentration quickly reached the maximum (1,029.2±129.1 ng/mL) in 5 min, and then it decreased rapidly and remained at around 15% of the C_{max} value 2 h later, which implied a rapid in vivo elimination of brucine in rats. In the case of intravenous administration, the in vivo profile of liposomes was smoother than brucine-injected group. The $t_{1/2}$ and area under the curve$_{0-\infty}$ of liposomes and NGR liposomes were 2.28- and 2.45- and 2.65- and 3.13-fold higher compared with free drug. Thus, it was reasonable to conclude that the liposomes could significantly extend the bioavailability of brucine in vivo; however, there was no significant difference in the pharmacokinetic parameters

Table 1 The particle size and zeta potential of NGR-brucine liposomes before and after storage at 4°C (n=3)

Preparations	Particle size (nm)	Zeta potential (mV)	Encapsulation efficiency (%)	Polydispersity index
NGR-brucine liposomes				
Day 0	92.6±4.1	−16.5±3.3	89.6±2.7	<0.39
Brucine liposomes				
Day 0	85.3±3.2	−16.2±3.5	87.4±3.1	<0.38
NGR-brucine liposomes				
Day 30	93.2±3.3	−14.6±2.7	88.4±3.1	<0.41
Brucine liposomes				
Day 30	86.7±1.9	−15.7±2.8	86.7±2.9	<0.42
NGR-brucine liposomes				
Day 60	94.1±3.5	−15.1±3.1	87.7±3.9	<0.44
Brucine liposomes				
Day 60	87.6±3.4	−15.9±3.4	86.4±1.6	<0.43
NGR-brucine liposomes				
Day 90	94.3±3.9	−14.7±5.2	87.2±4.3	<0.46
Brucine liposomes				
Day 90	88.5±2.8	−15.3±1.9	85.9±4.2	<0.45

Abbreviation: NGR, Asn-Gly-Arg.

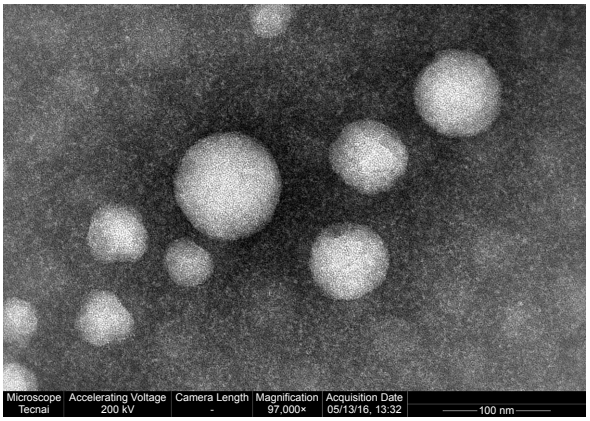

Figure 2 Transmission electron microscopy image of NGR-modified brucine liposomes (magnification 97,000×).
Abbreviation: NGR, Asn-Gly-Arg.

observed between liposomes and NGR liposomes after intravenous administration.

In vivo antitumor activity

As shown in Figure 5, both brucine liposomes and NGR-brucine liposomes significantly inhibited the growth of the HepG2 tumors in mice. However, NGR-modified liposomes could more effectively inhibit tumor growth than non-modified liposomes, starting from day 13. The tumor volumes of NGR-modified group were smaller than those of non-modified group. The tumor inhibition rate of NGR-modified liposomes was higher than that of non-modified liposomes ranging from 74.9%±5.1% to 64.2%±6.4%. Changes in the body weights of tumor-bearing mice are presented in Figure 6. The average body weights of mice injected with 5% glucose injection significantly increased after tumor cell implantation, while the weight of mice treated

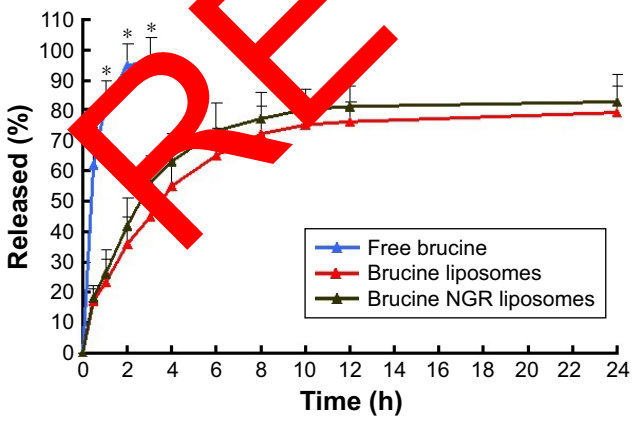

Figure 3 The release profile of free brucine, brucine liposomes and NGR-modified brucine liposomes (n=6).
Note: *P<0.05, free brucine vs brucine liposomes or NGR-modified brucine liposomes.
Abbreviation: NGR, Asn-Gly-Arg.

Table 2 Dissolution kinetic parameters of brucine from NGR-modified liposomes (n=3)

Model	Formulations	
	Equation	Correlation coefficient (r)
Zero-order equation	$Q = 6.12t - 0.89$	0.9431
First-order equation	$\ln(1 - Q) = 5.27t - 1.01$	0.9152
Higuchi	$Q = 4.312t_{1/2} - 2.212$	0.9672
Weibull's equation	$\ln(1/(1 - Q)) = -2.154 \ln t + 1.12$	0.9829

Abbreviation: NGR, Asn-Gly-Arg.

with NGR-modified liposomes did not change significantly and the non-modified liposome group showed a moderate increase in weight during the experiment.

Histological studies

The histopathological examination of the liver, spleen and kidney was carried out to identify any damage done to the tissues. Micrographs of the liver, spleen and kidney were taken following their incubation with brucine formulations (Figure 7). No sign of damage such as the appearance of epithelial necrosis and sloughing of epithelial cells was detected.

Discussion

In vitro release

Both liposomes and NGR-modified liposomes showed an initial fast release of brucine within the first 4 h followed by a relatively sustained release. The burst release may be attributed to rapid diffusion of brucine from the surface of liposomes. The subsequent sustained release was due to the slow diffusion of brucine from the core of hydrophobic carrier. The in vitro release results indicated that the release of brucine from NGR liposomes was similar to that of liposomes, demonstrating that the NGR modification did not affect brucine release. After adding targeting materials, the speed of release of drug from the liposomes did not reduce

Table 3 Pharmacokinetic parameters of brucine after intravenous administration of free drug, liposomes and NGR liposomes to rats (n=6)

Parameter	Intravenous administration		
	Free drug	Liposomes	NGR-modified liposomes
$t_{1/2}$ (min)	36.2±6.5	82.6±8.5*	88.9±7.9*
AUC_{0-t} (μg·min/mL)	56.1±8.1	119.6±10.1*	135.3±26.5*
$AUC_{0-\infty}$ (μg·min/mL)	62.5±13.8	165.9±16.4*	195.7±28.6*
MRT (min)	32.7±4.6	72.4±5.7*	85.4±8.2*
CL (L/kg/min)	0.14±0.04	0.06±0.01*	0.02±0.01*

Note: *P<0.05, vs free drug.
Abbreviations: CL, clearance; NGR, Asn-Gly-Arg; MRT, mean residence time; AUC, area under the curve; $t_{1/2}$, biological half-life.

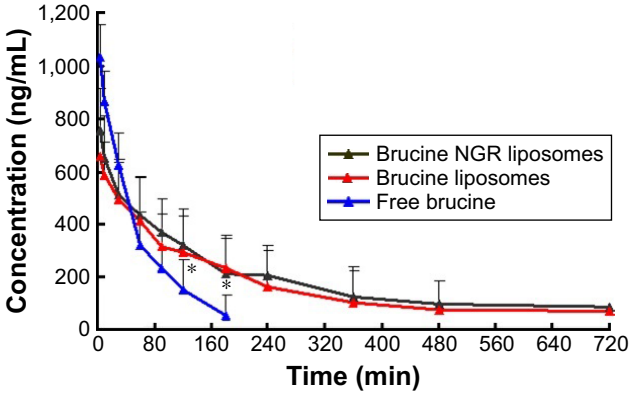

Figure 4 Concentration–time curve of brucine in different formulations: free brucine, brucine liposomes and NGR-modified brucine liposomes (n=6).
Note: *$P<0.05$, free brucine vs brucine liposomes or NGR-modified brucine liposomes.
Abbreviation: NGR, Asn-Gly-Arg.

obviously, with a possible reason being that the quantity of added targeting material did not obviously increase the steric hindrance of the liposomes.

Pharmacokinetics

Lipid carrier systems are ideal for drug delivery because they can alter the pharmacokinetics of the associated therapeutics. Compared with the liposomes group, in the solution grou the release of brucine was instantaneous due to its mode ate oil–water partition coefficient in vivo; after intravenous administration into blood, brucine could rapidly ter sues through the biofilm. As the phospholipid terial o lipo- somes group was added with DSPE-PEG kn n f its lon recycling time, its structure could pr uce steri indrance and liposomes were not easily sw lo d by macr ages.

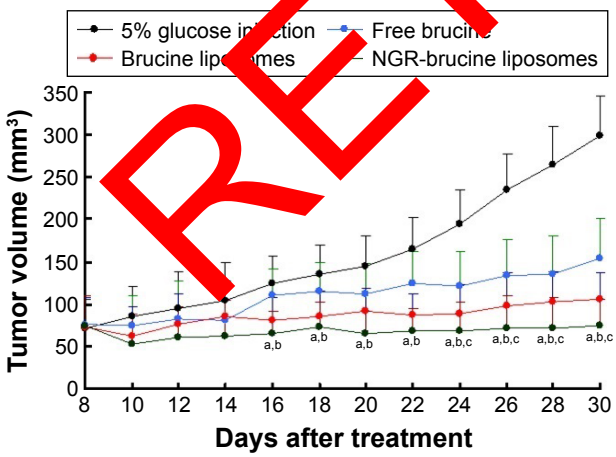

Figure 5 HepG2 xenograft tumor growth inhibition by brucine in different formulations.
Notes: Data = mean ± SD (n=12). [a]$P<0.05$, NGR-modified brucine liposomes vs 5% glucose injection; [b]$P<0.05$, NGR-modified brucine liposomes vs free brucine; [c]$P<0.05$, NGR-modified brucine liposomes vs brucine liposomes.
Abbreviation: NGR, Asn-Gly-Arg.

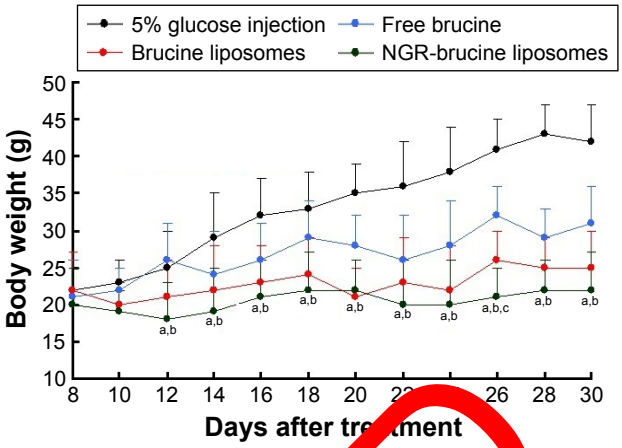

Figure 6 Animal body weights.
Notes: The body weights of treated mals we continuously onitored to investigate systemic cytotoxicity of b cine in differen mulat s. Data = mean ± SD (n=12). [a]$P<0.05$, NGR-mo ed brucine osomes glucose injection; [b]$P<0.05$, NGR-modified brucine somes ee brucine; [c]$P<0.05$, NGR-modified brucine liposomes vs bruci iposo s.
Abbreviation: NGR, A Gly-Arg.

Thus, the drug could y for a relatively long time in blood circula ith no leaka from liposomes into tissues, and hen , the initial concentration of drug in the liposomes group was igher than t solution group. The targeting effect of NGR ptide-mo ified liposomes was determined by evaluat- ng the bin g capacity of target head (NGR polypeptide) an tor (CD13, high expression in tumor cells and tumor ewborn vascular endothelial cells) as well as the stability of rug during target-searching process. If the drug leaked dur- ng blood circulation from liposomes, it could easily enter the tissues because of its lipophilic nature. Thus, the main target of this research was to evaluate the stability of target materials added to liposomes. According to the pharmacokinetic results, the area under the curve of liposomes was obviously higher than that of solution group, and the mean residence time of 0.5% NGR-modified liposomes was obviously longer than unmodified liposomes, which showed good stability during transfer from targeted liposomes to target area.

In vivo antitumor activity

Overall, the antitumor activity results showed that the NGR-modified liposomes exhibited less toxicity and much higher efficacy in HepG2-bearing mice compared with non-modified liposomes.[22] The enhanced antitumor activity might have occurred because brucine was specifically recognized by NGR receptor on the surface of tumor cells, which enhanced the intracellular uptake of drugs. NGR-modified liposomes exhibited high efficiency and low toxicity in the present study, which is expected to be considered in the development of other drug delivery systems. Thus, NGR-modified

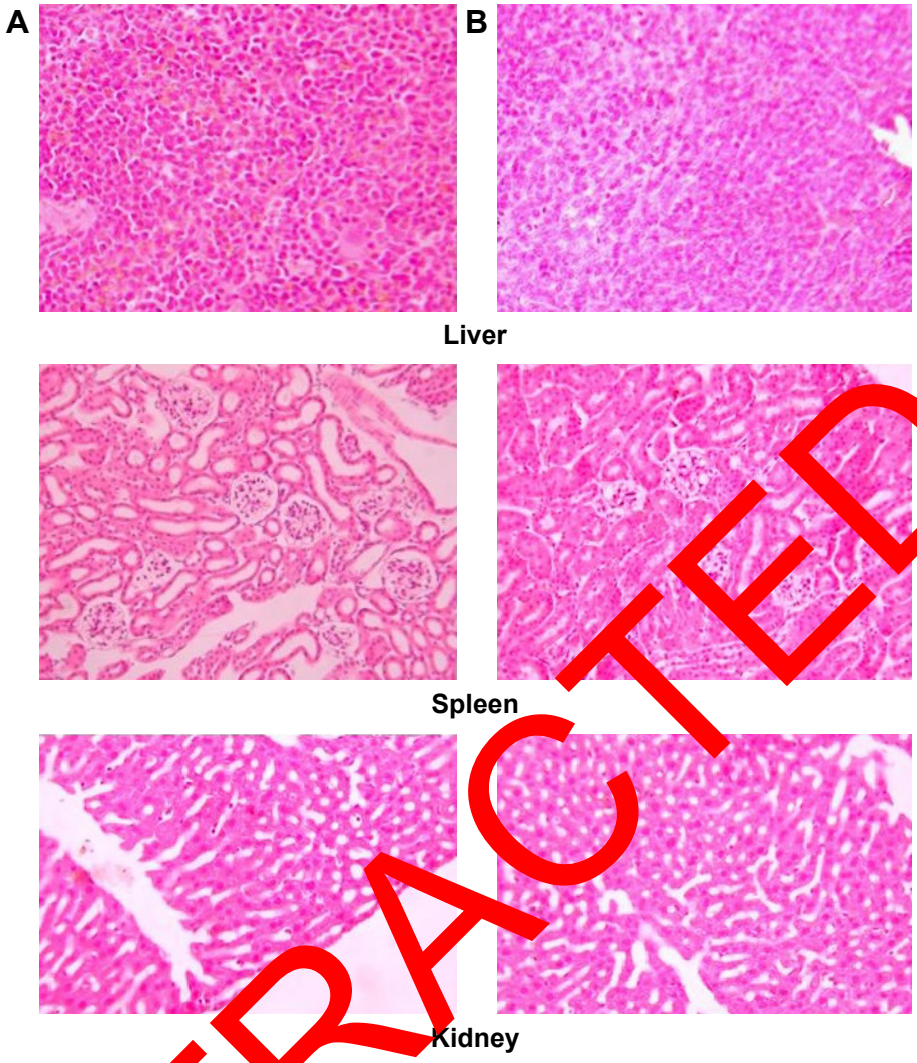

Figure 7 Histopathological studies of the liver, spleen and kidney: (**A**) brucine liposomes and (**B**) NGR-modified brucine liposomes (magnification ×5,000).
Abbreviation: NGR, Asn-Gly-Arg.

liposomes establish a platform to convert highly toxic active substance to an ideal candidate drug.

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Disclosure

The authors report no conflicts of interest in this work.

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