Macrophage membrane- and cRGD-functionalized thermosensitive liposomes combined with CPP to realize precise siRNA delivery into tumor cells

Jingxue Nai,1,2,5 Jinbang Zhang,1,3,5 Jiaxin Li,1,3 Hui Li,1,3 Yang Yang,1 Meiyuan Yang,1 Yuli Wang,1 Wei Gong,1 Zhiping Li,1 Lin Li,4 and Chunsheng Gao1,3

1State Key Laboratory of Toxicology and Medical Countermeasure, Department of Pharmaceutics, Beijing Institute of Pharmacology and Toxicology, Beijing, China; 2Wuhan University School of Pharmaceutical Sciences, Wuhan, China; 3Pharmaceutical College of Henan University, Kaifeng, China; 4State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China

INTRODUCTION

Despite the success of small interfering RNAs (siRNAs) in clinical settings, their fast clearance and poor delivery efficiency to target cells still hinder their therapeutic effect. Herein, a new treatment system was constructed by combining thermosensitive liposomes with the macrophage membrane, tumor-targeting cyclic Arg-Gly-Asp peptide, a cell-penetrating peptide, and thermotherapy. The constructed system was found to be thermosensitive and stable; the proteins were inherited from the macrophage membrane. This new system combined with thermotherapy displayed the least uptake by macrophages, the greatest uptake by HepG2 cells, the most obvious HepG2 cell apoptosis, and the strongest inhibition of Bcl-2 mRNA and Bcl-2 protein in HepG2 cells. Moreover, 24 h after system administration in tumor-bearing mice, the most prominent distribution of siRNA was observed in tumors, while almost no siRNA was found in other organs. The strongest inhibition of Bcl-2 mRNA, Bcl-2 protein, and tumors was found in mice that had received the proposed system. In summary, when using the constructed system both in vitro and in mice, less uptake by the reticuloendothelial system, greater accumulation in tumor cells, and improved therapeutic efficacy were observed. Therefore, this new system can deliver siRNA selectively and efficiently, and it is a promising therapeutic candidate for precise tumor-targeted therapy.

Small interfering RNA (siRNA)—short double-stranded RNA of 21–23 nucleotides—has proved efficient in potent and highly specific gene silencing, and now several RNAi therapeutics have been approved for the market.1–3 However, the desirable therapeutic efficacy of siRNA depends on the effective delivery carriers because siRNA plays a therapeutic role only in the cytoplasm and can barely pass through the cell membrane by itself.1–4

Therefore, many nanocarriers have been prepared to deliver siRNA.5–9 Among these, lipid-based vectors are considered one of the most suitable carriers because several of their products have already been applied in clinical settings. Despite these great achievements, the therapeutic activity of these systems usually depends on passive and random drug leakage,10 which seriously affects the delivery efficiency of siRNA into target cells. To ensure the satisfactory delivery efficiency of anti-tumor siRNA into target cells, scientists are focusing their efforts in two directions: the first is in improving the targeting ability of carriers to tumor tissues, and the second is in promoting the efficiency of siRNA delivery into the cytoplasm of target cells. To increase their tumor-targeting ability, PEGylated targeting ligands are commonly used to decorate these carriers.11 However, it is still challenging to realize ligand-conjugated nanovehicles specific to different tumors in patients because tumor heterogeneity and interpersonal differences are inevitable.11 To further reinforce targeting ability, the macrophage membrane was introduced into the delivery system in this study. Disguising the nanoparticles with the cell membrane can help transfer membrane proteins from source cells onto the nanoparticle surface, thereby providing many distinct advantages, such as reduced reticuloendothelial system (RES) clearance, prolonged blood circulation, and improved accumulation at specific pathological sites.10–13 The macrophage membrane is considered especially suitable to camouflage tumor-targeting nanoparticles because the macrophage can target, identify, and phagocytose cancer cells.11 Even when nanocarriers arrive at tumor sites, siRNA still has a long way to go before entering into the cytoplasm of tumor cells. First, after it is liberated near the tumor tissues, siRNA can go into the cytoplasm by penetrating the cell membrane. Second, siRNA can also be delivered into the cytoplasm with the aid of nanocarriers, which are
engulfed by tumor cells, can escape from lysosomes, and can finally release siRNA in the cytoplasm. We believe that the first mode of siRNA delivery listed above is more controllable and effective than the second one because it is difficult to control the efficiency of cell engulfing, lysosome escaping, and intact siRNA liberation into the cytoplasm. Therefore, the purpose of our study was to acquire a new siRNA delivery system that could release siRNA near the tumor tissues, make siRNA efficiently penetrate the cell membrane, and achieve precise siRNA delivery into the cytoplasm of target cells.

To acquire the controllable drug release, nanoparticles sensitive to stimuli were considered. Among them, the external environment-triggered carriers were considered more applicable in the clinical setting because endogenous stimuli always vary to a large degree. Thermosensitive liposomes (TSLs), whose lipid materials undergo a gel-to-liquid crystalline phase transition upon heating in a mild hyperthermia range, were selected as the basic vectors because they enable encapsulated drugs to be released immediately and completely. We also believe that TSLs combined with tumor-targeting materials can improve the insufficient drug concentration at the target site occurring in the phase III study of ThermoDOX, involving the TSLs containing doxorubicin developed by Celsion.

To guarantee the membrane-penetrating efficiency, siRNA was further designed to be conjugated with cell-penetrating peptide (CPP), which has a potential membrane-penetrating ability. Accordingly, a multifunctional delivery system (CPP coupled siRNA [C-siRNA]/MTSLR) combining TSLs with the macrophage membrane, the tumor-targeting cyclic Arg-Gly-Asp (cRGD) peptide, and the CPP was designed to realize the precise delivery of siRNA into tumor cells.

C-siRNA/Macrophage membrane combining with TSL-cRGD (C-siRNA/MTSLR) was constructed as shown in Figure 1A, and the proposed illustration of C-siRNA/MTSLR is shown in Figure 1B. After injection, C-siRNA/MTSLR first escaped from the RES uptake and gathered near the tumor thanks to the macrophage membrane proteins and cRGD. Then, theranostics was exerted on the tumor region, and the C-siRNA/MTSLR phase was transferred, and most of
the C-siRNA was released near the tumor. Thereafter, free C-siRNA entered into the tumor cells and was digested by enzymes in the cytoplasm. The liberated free siRNA finally exerted its therapeutic effect. Hence, the markedly enhanced intracellular delivery and satisfactory therapeutic efficacy of siRNA were expected for this system because it best exploited the advantages of TSLs, the macrophage membrane, cRGD, CPP, and thermotherapy, and it bypassed their disadvantages.

To validate the actual delivery efficacy of this multifunctional delivery system, Bcl-2 siRNA was selected as the model siRNA because it can selectively inhibit the expression of the Bcl-2 protein and induce regression of many solid tumors; RAW 264.7 mouse macrophage cells were used as model macrophages to ensure homology with mice in the following evaluation. After construction, C-siRNA/MTSLR was characterized and evaluated in vitro in mice with HepG2 cells (human hepatocellular carcinomas) or HepG2 tumors as models, respectively.

RESULTS

Characterization of C-siRNA/MTSLR

For the convenience of comparison, different formulations such as C-siRNA encapsulated in TSLs (C-siRNA/TSLs), cRGD-decorated C-siRNA/TSLs (C-siRNA/TSLRs), macrophage membrane-disguised C-siRNA/TSLs (C-siRNA/MTSLs), and C-siRNA/MTSLRs were fabricated and characterized.

The constructed nanoparticles regularly appeared round under both transmission electron microscopy (TEM) and atomic force microscopy (AFM), as shown in Figures 2A and 2B. The particle sizes of the macrophage membrane, C-siRNA/TSL, C-siRNA/MTSL, and C-siRNA/MTSLR (Figure 2C) were 265.6 ± 1.9 nm, 86.9 ± 0.5 nm, 105.3 ± 1.9 nm, and 108.2 ± 0.3 nm, respectively, with a dispersity in index of 0.258 ± 0.005, 0.201 ± 0.008, 0.212 ± 0.005, and 0.195 ± 0.001, respectively. The zeta potentials of the purified macrophage membrane, C-siRNA/TSL, C-siRNA/MTSL, and C-siRNA/MTSLR were −22.7 ± 0.1 mV, −8.2 ± 0.5 mV, −18.9 ± 0.4 mV, and −15.0 ± 0.3 mV, respectively. The encapsulation efficiencies of siRNA were 89.56% ± 1.03%, 87.32% ± 0.95%, and 86.49% ± 2.31% for C-siRNA/TSL, C-siRNA/MTSL, and C-siRNA/MTSLR, respectively.

To verify the thermosensitivity of C-siRNA/MTSLR, the phase transition temperatures (Tm) of C-siRNA/TSL and C-siRNA/MTSLR were determined with differential scanning calorimetry (DSC), as shown in Figure 2D. There was an obvious phase transition for both formulations, and the melting endothermic peak existed at approximately 42°C in both DSC curves. The temperature-triggered in vitro release of siRNA from C-siRNA/TSL and C-siRNA/MTSLR was also evaluated, as shown in Figure 2E. During 10 min at 42°C, about 80% of siRNA was released from both C-siRNA/TSL and C-siRNA/MTSLR, whereas no more than 20% of the drug was liberated from either C-siRNA/TSL or C-siRNA/MTSLR during 60 min at 37°C.

To guarantee the capacity of immune escaping and tumor targeting mediated by the natural proteins from the macrophage membrane, the protein profile, specific proteins, and the protein secondary structure of the purified macrophage membrane and different nanoparticles were detected, and the results are displayed in Figures 2F–2H. The results show that the protein composition in the macrophage membrane remained intact in both C-siRNA/MTSL and C-siRNA/MTSLR, while no protein signal was detected in C-siRNA/TSLR. Specific proteins including CD47, integrin α4, and integrin β1 were displayed in the purified macrophage membrane and macrophage-membrane-coated nanoparticles but not in C-siRNA/TSLR.23 Far-ultraviolet circular dichroism (CD) spectra spectroscopy was used to evaluate the conformational changes of proteins. As shown in Figure 2H, there was a positive band at 195 nm and a negative band at 212 nm in the CD spectra of all of the samples, and no significant shift in the peaks or obvious changes in peak intensity were observed.

The colloidal stability of C-siRNA/MTSLR and its dilutions with phosphate-buffered saline (PBS) or Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) were monitored by a Turbiscan Lab expert. As shown in Figure 2I, less than 3% of the variations in both transmission and backscattering were observed during 24 h for all of the samples.

In vitro cellular uptake

The uptake of nanoparticles by HepG2 cells was analyzed with both confocal laser scanning microscopy (CLSM) and flow cytometry microscopy (FCM). As shown in Figure 3A, the most pronounced fluorescence was observed in preheated C-siRNA/MTSLR (C-siRNA/MTSLR-H) and free C-siRNA, followed by different unheated formulations, including C-siRNA/MTSL, C-siRNA/MTSLR, C-siRNA/TSL, and TSLs containing free siRNA (siRNA/TSLs). In contrast, there was almost no cyanine 5 (cy5) fluorescence in cells treated with free siRNA. Similar quantitative results were also obtained by flow cytometry (Figure 3B). The groups with C-siRNA/MTSLR-H or free C-siRNA showed the highest fluorescence intensity, followed by C-siRNA/MTSLR, C-siRNA/MTSL, C-siRNA/TSL, and siRNA/TSL in a decreasing order. Significant differences in fluorescence intensity were found between C-siRNA/MTSLR-H and C-siRNA/MTSLR, C-siRNA/MTSLR and C-siRNA/MTSL, C-siRNA/MTSL and C-siRNA/TSL, and free C-siRNA and free siRNA.

The cellular uptake of different formulations by mouse macrophage RAW 264.7 cells was also visually observed by CLSM (Figure 3C) and further quantified by FCM (Figure 3D). The fluorescence in cells

Figure 2. Characteristics of various nanoparticles

(A) TEM images. (B) AFM images. (C) Particle size distribution. (D) DSC thermograms of C-siRNA/MTSLR and C-siRNA/TSL. (E) Temperature-triggered release profiles of siRNA from C-siRNA/MTSLR and C-siRNA/TSL at 37°C and 42°C, respectively. The data are represented as mean ± SD. (F) Protein profiles determined by SDS-PAGE electrophoresis assay. (G) Identification of specific proteins including CD47, integrin α4, and integrin β1 analyzed by WB assay. (H) Protein secondary structure determined by CD. (I) Colloidal stability analysis of C-siRNA/MTSLR and its dilutions at 37°C.
Figure 3. Cellular evaluation of various formulations

(A) Cellular uptake evaluation of various nanoparticles into HepG2 cells by CLSM. Hoechst 33258 for nuclear staining (blue) and Cy5 fluorescence (red) were recorded. (B) Cellular uptake evaluation of various nanoparticles into HepG2 cells by FCM. (a) p < 0.05 versus Control; (b) p < 0.05 versus free siRNA; (c) p < 0.05 versus C-siRNA; (d) p < 0.05 versus siRNA/TSL; (e) p < 0.05 versus C-siRNA/TSL; (f) p < 0.05 versus C-siRNA/MTSL; (g) p < 0.05 versus C-siRNA/MTSLR. (C) Cellular uptake evaluation of various nanoparticles into RAW 264.7 cells by CLSM. Hoechst 33258 for nuclear staining (blue) and Cy5 fluorescence (red) were recorded. (D) Cellular uptake evaluation of various nanoparticles into RAW 264.7 cells by FCM. (a) p < 0.05 versus C-siRNA/TSL. (E) Cytotoxicity of various nanoparticles against HepG2 cells at different times. (a) p < 0.05 versus Control; (b) p < 0.05 versus C-siRNA/MTSL. (F) Cell apoptosis induced by siRNA in different formulations. Early apoptotic cells are reflected in the lower right quadrant; late apoptotic cells are exhibited in the upper right quadrant; necrotic cells are presented in the upper left quadrant; and healthy cells are displayed in the lower left quadrant. (G) The level of Bcl-2 mRNA in HepG2 cells treated with various samples. (a) p < 0.05 versus Control; (b) p < 0.05 versus free siRNA; (c) p < 0.05 versus C-siRNA; (d) p < 0.05 versus siRNA/TSL; (e) p < 0.05 versus C-siRNA/MTSL; (f) p < 0.05 versus C-siRNA/MTSLR; (g) p < 0.05 versus C-siRNA/MTSLR-H. (H) The level of Bcl-2 protein expression in HepG2 cells treated with various samples. Data in (B), (D), (E), and (G) are represented as mean ± SD.

Note: the samples in these experiments were not treated with heat except for those marked with “H.”
treated with C-siRNA/MTSL or C-siRNA/MTSLR was weaker than that in cells treated with C-siRNA/TSL. Moreover, FCM analysis showed that the cellular uptake of C-siRNA/TSL in RAW 264.7 cells had a 2-fold higher signal compared with that of nanoparticles modified with the macrophage membrane.

**In vitro cytotoxicity**

The cytotoxicity of the nanoparticles on HepG2 cells was evaluated by cell viability with the Cell Counting Kit-8 (CCK-8) assay. All groups, except those treated with the control, cRGD-decorated MTSL containing the negative control siRNA (C-si.NC/MTSLR), and free siRNA, inhibited the proliferation of HepG2 cells in a time-dependent manner (Figure 3E). The strongest proliferation inhibition was found in the groups treated with C-siRNA or C-siRNA/MTSLR-H at different times.

**In vitro cell apoptosis**

As shown in Figure 3F, the minimal apoptosis was observed in the control group and in the groups treated with free siRNA or C-siN.C/MTSLR. The highest apoptosis rate was found in cells treated with free C-siRNA or C-siRNA/MTSLR-H. The proportion of apoptosis decreased in the following order: C-siRNA/MTSLR-H, C-siRNA/MTSLR, C-siRNA/MTSL, C-siRNA/TSL, and siRNA/TSL.

**In vitro gene silencing**

To verify the gene-silencing effect of siRNA, the Bcl-2 messenger RNA (mRNA) level was analyzed by quantitative real-time PCR, and Bcl-2 protein expression was detected by western blot (WB) analysis. As shown in Figure 3G, there was no obvious Bcl-2 suppression in cells treated with preheated C-si.NC/MTSLR (C-si.NC/MTSLR-H) or free siRNA, while obvious Bcl-2 suppression was found in most other formulations. The efficiency of Bcl-2 suppression in cells decreased in the following order: C-siRNA/MTSLR-H, C-siRNA/MTSLR, C-siRNA/MTSL, C-siRNA/TSL, and siRNA/TSL. As displayed in Figure 3H, downregulation of Bcl-2 protein was found in the cells treated with siRNA in different formulations except for free siRNA, and the lowest Bcl-2 protein expression level was observed in cells treated with free C-siRNA or C-siRNA/MTSLR-H.

**In vivo distribution**

Obvious differences in siRNA distribution were found in mice that were given various nanoparticles, as shown in Figure 4. No fluorescence signal was observed in mice treated with physiological saline. The fluorescence dispersed systematically soon after administration, accumulated gradually in the bladder and kidneys, and finally disappeared completely at 24 h in mice that had been given free siRNA. C-siRNA seemed to be distributed in the whole body without evident tumor targeting and then eliminated just like free siRNA in mice. Compared with the undetected fluorescence signal in tumors of mice that had received free siRNA or free C-siRNA, visible fluorescence was observed in tumors of mice that had received different nanoparticles, and the fluorescence intensity in tumors increased in the following order: siRNA/TSL, C-siRNA/MTSLR without thermal treatment (C-siRNA/MTSLR-U), C-siRNA/TSL, C-siRNA/MTSL, and C-siRNA/MTSLR. No fluorescence was detected in any isolated tissue or organ in mice that had received physiological saline or
free siRNA. In contrast to free siRNA, weak fluorescence was found in the liver, spleen, and kidneys, and the fluorescence was also detected in tumors in mice treated with siRNA/TSL or C-siRNA/TSL. There was no fluorescence observed in the liver, spleen, and kidneys, while a stronger fluorescence was observed in tumors for nanoparticles decorated with the macrophage membrane, regardless of formulation. The most distinguished fluorescence in tumors was found in mice treated with C-siRNA/MTSLR combined with thermotherapy.

In vivo anti-tumor efficacy

The tumor volume in HepG2 tumor-bearing mice during the whole treatment period is shown in Figure 5A. There was no tumor inhibition found in mice treated with free siRNA or free C-siRNA, compared with the control group. Evident tumor inhibition was observed in mice treated with C-siRNA/TSL, while siRNA/TSL showed almost no anti-tumor efficacy. The tumor inhibition of different formulations increased in the following order: siRNA/TSL, C-siRNA/MTSLR-U, C-siRNA/TSL, C-siRNA/MTSL, and C-siRNA/MTSLR. Similar results were found in the isolated tumors, as shown in Figure 5B. There was no apparent reduction in body weight (Figure 5C) or any obvious changes in emotional behavior in the mice treated with various nanoparticles.

The gene silencing of siRNA in mice is displayed in Figures 5D and 5E. The expression levels of Bcl-2 mRNA and Bcl-2 protein in mice were not identical to those in vitro for siRNA in different formulations, except for free C-siRNA.

A histological analysis of the tumor sections (hematoxylin and eosin staining [H&E staining]) showed that the tumor cells appeared integral and that the karyokinesis was obvious in the control group; the vigorous growth of the tumor cells was observed in the group treated with free siRNA, free C-siRNA, or C-si.NC/MTSLR. Moreover, the shrinkage and destruction of the cellular nucleus and even the local necrosis of tumor cells were found in groups treated with siRNA encapsulated in nanocarriers, and the most conspicuous changes emerged in the group treated with C-siRNA/MTSLR combined with thermotherapy.

Brown granules, indicating the positive platelet-endothelial cell adhesion molecule 1 (CD31), were not observed in mice treated with C-siRNA/MTSLR, while at least three CD31-positive cells were found...
in groups treated with free siRNA, free C-siRNA, or C-siNC/MTSLR (Figure 5G).

Apoptosis of tumor cells was also analyzed with a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) assay, and the results are shown in Figure 5H. The green fluorescence, indicating cell apoptosis, was observed in all of the groups treated with the therapeutic siRNA encapsulated in different nanoparticles, and the strongest one appeared in the group treated with C-siRNA/MTSLR combined with thermotherapy.

**In vivo safety evaluation**

Routine blood indexes, serum biochemical indexes, and the main organs were examined, and the results are shown in Figure 6. There were no significant differences in all of the indexes from Figures 6A and 6B, and no pathological changes were observed in any of the organs from Figure 6C.

**DISCUSSION**

C-siRNA/MTSLR was acquired by introducing cRGD and the macrophage membrane into TSLs. It seems that the insertion of cRGD had no influence on the nanoparticle characteristics except on the slightly increased zeta potential, while the addition of the macrophage membrane obviously increased the particle size and markedly decreased the zeta potential of the nanoparticles. The thermosensitivity of the nanoparticles and the temperature-triggered drug release were not influenced by the addition of the macrophage membrane or cRGD because C-siRNA/MTSLR had almost the same Tm and similar drug release profiles as TSLs.23,24 The membrane proteins of C-siRNA/MTSLR corresponded with those of the macrophage membrane, indicating that C-siRNA/MTSLR might escape the uptake by RES and possess a tumor-targeting ability.11 The results from colloidal stability experiments indicate that both C-siRNA/MTSLR and its dilutions, especially in DMEM containing FBS, were stable during 24 h, indicating that C-siRNA/MTSLR is stable in vivo.

In the experiments of cellular uptake by HepG2 cells, almost no free siRNA was ingested into HepG2 cells; this was considered reasonable because it has been reported that it is almost impossible for free siRNA to enter into the cells by itself because of its negative charge, hydrophilicity, and high molecular weight.25,26 In contrast to free siRNA, considerable free C-siRNA was found in the cytoplasm of HepG2 cells, which was attributed to the powerful cell-penetrating ability of CPP.27 Nevertheless, free C-siRNA was not expected to demonstrate powerful tumor-cell-penetrating ability in vivo because of its non-specificity. The massive uptake of siRNA in C-siRNA/TSL, C-siRNA/MTSLR, and C-siRNA/MTSLR was also considered to be mainly dependent on the powerful cell-penetrating ability of CPP after the hyperthermia-triggered drug release. Less siRNA was observed to enter into HepG2 cells after treatment with C-siRNA/MTSLR without hyperthermia compared with heated C-siRNA/MTSLR, which may be related to the masking of CPP by the nanoparticles. Fluorescence was stronger in HepG2 cells treated with C-siRNA/MTSLR compared with those treated with C-siRNA/MTSLR, which may be related to the selective and specific binding of cRGD with integrin αvβ3 overexpression in HepG2 cells.28–30 The fluorescence intensity was also greater for C-siRNA/MTSLR compared with C-siRNA/TSL, which may be because of the active targeting ability of the macrophage membrane, as its surface proteins could recognize the tumor endothelium.11 Taken together, exerting hyperthermia on thermosensitive carriers, decoration by cRGD, coating by the macrophage membrane, and modification of siRNA with CPP can significantly improve siRNA uptake into HepG2 cells. For RAW 264.7 cells, the uptake of siRNA in macrophage-membrane-disguised nanoparticles was significantly lower than that in common TSLs, indicating that the macrophage membrane plays an important role in cellular uptake, and the coating of TSLs with the macrophage membrane may decrease the clearance by RES. This was attributed to the inherited proteins from the macrophage membrane, such as CD47, which could prevent macrophage-mediated phagocytosis in the RES and thereby prolong the half-life.11

The results of cytotoxicity, cell apoptosis, and gene silencing are consistent with those of the cellular uptake by HepG2 cells. C-siN.C/MTSLR did not lead to obvious cytotoxicity, cell apoptosis, or Bcl-2 gene silencing, which means the exhibited cell-proliferation inhibition, cell apoptosis, and Bcl-2 suppression of different formulations originated from siRNA. The similar results of cytotoxicity, cell apoptosis, and gene silencing with cellular uptake suggest that free and intact siRNA mediates Bcl-2 suppression, cell apoptosis, and cell-proliferation inhibition other than C-siRNA, nanocarrier-encapsulated siRNA, or fluorescein.

The results of the distribution indicate that the encapsulation by TSLs could really protect siRNA from rapid degradation and that thermotherapy improved the accumulation of siRNA at tumor regions. Moreover, the decoration with CPP further enhanced the accumulation of siRNA in the tumors, owing to its powerful membrane-penetrating ability. The non-specific uptake by RES was obviously reduced, and more obvious tumor targeting was observed after the coating of TSLs with the macrophage membrane, especially at 1 h. The existence of tumor-targeting cRGD further reinforced the aggregation of nanoparticles toward the tumor regions. Therefore, it is considered reasonable that the most evident tumor targeting was found in mice treated with C-siRNA/MTSLR under the combined actions of TSLs, the macrophage membrane, cRGD, CPP, and thermotherapy. This is consistent with the conclusions speculated from the results in vitro. However, the distribution in the whole body without evident tumor targeting and fast elimination similar to free siRNA was observed for free C-siRNA, which is reasonable owing to the non-specificity of C-siRNA. The most obvious tumor inhibition and the strongest gene silencing were observed in mice treated with C-siRNA/MTSLR. This is consistent with the distribution of siRNA in tumors, so the combined actions of TSLs, the macrophage membrane, cRGD, CPP, and thermotherapy were confirmed.
Figure 6. Safety evaluation of different nanoparticles in mice

(A) Photographs of main organs stained with H&E. (B) Serum biochemical indexes. (C) Results of blood test. Data in (B) and (C) are represented as mean ± SD.
The safety of C-siRNA/MTSLR was confirmed, considering that there were hardly any changes in body weight or mental health after multiple doses and there was a normal hemogram, as well as a lack of pathological results after a single dose.

Conclusions
A multifunctional tumor-targeting nanocarrier system combining TSLs with the macrophage membrane, cRGD, CPP, and thermotherapy for siRNA delivery was successfully developed in this study. The constructed system displayed an appropriate particle size, good colloidal stability, an ideal heat-triggered siRNA release, and satisfactory macrophage membrane features. More importantly, this multifunctional nanocarrier achieved markedly enhanced siRNA delivery into tumor cells, striking gene silencing, and satisfactory tumor inhibition efficacy both in vitro and in vivo on the premise of safety. In summary, this system could make full use of the site-specific, drug-liberating ability of TSLs combined with thermotherapy, the self-recognizing and tumor-recognizing ability of the macrophage membrane, the tumor-targeting ability of cRGD, and the potent cell-membrane-penetrating ability of CPP. Moreover, the system could bypass the absence of the drug-enriching ability toward the target site of TSLs combined with thermotherapy, the poor tumor-cell-engulfing ability of the macrophage membrane, weak specificity to tumors of cRGD caused by tumor heterogeneity and interpersonal differences, and the non-specificity of CPP. Therefore, this new system has great potential for siRNA delivery.

MATERIALS AND METHODS

Materials
C.PP (C5RMMKWK-Cys) was custom-synthesized by Shanghai GL Biochem (Shanghai, China). DSPE-PEG2000, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), and monostearoyl phosphatidyl choline (MSPC) were purchased from Shanghai Advanced Biochem (Shanghai, China). DSPE-PEG2000-cRGD was synthesized by Xi’an ruixi Biological Technology (Xi’an, China). Lyophilized Bcl-2 siRNA (sense strand: 5’-GUGAUAAGAUCAAUUdTdT-3’, antisense strand: 5’-AAUGGAUGUACUCAUCAGdTdT-3’), negative control Bcl-2 siRNA (sense strand: 5’-UGUGAAGGACUUAACUGdTdT-3’, antisense strand: 5’-GAGUUUAAGUCAAUCACGdTdT-3’), thiol-modified Bcl-2 siRNA, and cy5-labeled siRNA (cy5-siRNA) were all bought from GenePharma (Shanghai, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). DMEM and FBS were acquired from Gibco, Invitrogen (Carlsbad, CA, USA). Antibodies of integrin α4, integrin β1, CD47, CD31, Bcl-2, goat anti-rabbit immunoglobulin G (IgG) horseradish peroxide (HRP), and HRP-labeled goat/anti-rabbit IgG were bought from Abcam (Cambridge, UK).

RAW264.7 cells and HepG2 cells were both obtained from the Cell Resource Center (IBMS, CAMS/PUMC). Male KM mice and nu/nu nude mice (weighing 18–20 g) were purchased from SPF Biotechnology (Beijing, China). All animal experiments were performed according to the code of ethics defined by the Animal Care and Use Ethics Committee at the Beijing Institute of Pharmacology and Toxicology.

Synthesis of C-siRNA
The C-siRNA was synthesized by coupling siRNA with CPP via disulfide bonds, as reported.22,23 Briefly, thiol-modified Bcl-siRNA (sense strand: HS-5’-GUGAAGAUAGAUCAUCUUddTdT-3’, antisense strand: 5’-AAUGGAUGUACUCAUCAGdTdT-3’, CPP, and diamide were dissolved in a hydroxyethyl piperazine ethanesulfonic acid (HEPES) buffer solution (10 mM HEPES, 1 mM ethylene diamine tetraacetic acid, pH 8.0) with a final equimolar concentration of 6 μM, and stirred in the dark for 1 h at 40°C. Afterward, the formed products were purified via an ultrafiltration centrifuge tube with a molecular weight cutoff of 3,000 Da. The final product was confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS; Ultraflex b, Bruker Dal-tonics, Billerica, MA, USA). The synthetic route and mass spectra of C-siRNA (sense strand: CPP-S-S-5’-GUGAAGAUAGAUCAUCUUdTdT-3’, antisense strand: 5’-AAUGGAUGUACUCAUCAGdTdT-3’) are shown in Figure S1.

Isolation of macrophage membrane
To obtain the purified macrophage membrane, RAW 264.7 cells were first harvested gently with a rubber scraper and then further separated by centrifugation at 300 × g for 5 min. The precipitate was resuspended in an ice-cold Tris-magnesium buffer (TM buffer, pH 7.4, 0.01 M Tris and 0.001 M MgCl₂) at a concentration of 2.5 × 10⁷ cells/mL and broken into pieces ultrasonically. After incubation at 4°C for 30 min, the suspension was subsequently centrifuged at 10,000 × g for 10 min. The supernantant was centrifuged again at 100,000 × g for 30 min. The acquired precipitate was finally collected, identified, quantified, and stored at –20°C for use.23,31

Preparation of different nanoparticles
TSLs were composed of DPPC, MSPC, and DSPE-PEG2000 (165:5.5:5, molar%) and were obtained as reported in the literature, with some modifications.22,23,28 In brief, the lipids were first dissolved in chloroform, and a thin film formed after the evaporation of the solvent under vacuum by a rotator RE-2000 (Ya Rong Biochemical Instrument Factory, China). The formed film was then hydrated with HEPES solution (20 mM HEPES, 150 mM NaCl, pH 7.0) containing C-siRNA at 45°C for 60 min, and the suspension was extruded through a polycarbonate membrane (200 nm pore size) using an extruder machine (Emulsiflex-C3, Avetin, Ottawa, ON, Canada). The free drug was finally removed by dialysis. Thus, TSLs with a homogeneous shape and uniform size were acquired.

MTSL was constructed by loading the macrophage membrane onto TSLs through extrusion. Briefly, 1.0 mL of TSLs was mixed with the purified macrophage membrane from 1 × 10⁸ cells, and MTSL was acquired after the mixtures were sequentially extruded through polycarbonate membranes with pore sizes of 400 and 200 nm, respectively.
MTSLR was acquired by inserting DSPE-PEG2000-cRGD into MTSL. Namely, 1.0 mL of MTSL was mixed with 200μL of PBS containing DSPE-PEG2000-cRGD, and MTSLR was obtained after incubation at 37°C for 25 min.

**Morphology of various nanoparticles**

The nanoparticles were morphologically characterized by TEM (JEM-1010, JEOL, Tokyo, Japan) and AFM (Bruker Multimode 8, Bruker Daltonic, Billerica, MA, USA). To obtain the images from TEM, the suspensions of different nanoparticles were dropped on a copper grid and dried at room temperature to form a dry film. The samples were then negatively stained with 2% phosphotungstic acid and air-dried at room temperature again before observation under TEM. Various nanoparticles were spread onto a mica sheet and dried at room temperature for observation with AFM.

**Particle size and zeta-potential analysis**

The particle size and zeta potential of the constructed nanoparticles were determined by Anton Paar Litesizer (Litesizer 500, Austria) at 25°C. The measurements were all performed in triplicate, and the results were reported as the mean value of 50% particle distribution.

**Encapsulation efficiency**

To determine the encapsulation efficiency of different nanocarriers, siRNA loading in these formulations was first determined; that is, 200μL of 10% (W/V) Triton was added into 200μL of nanoparticle suspension to form a uniform solution containing free cy5-siRNA, and the content of cy5-siRNA was measured using a spectrophotometer. To further determine the amount of free cy5-siRNA in the nanoparticles, another 200μL of nanoparticle suspension was diluted to 2mL with distilled water, added into the ultra-filter (vivaspin2; Sartorius Biotech, USA), and then centrifuged at a speed of 7992×g for 10 min. Free cy5-siRNA in the filtrate was detected with the spectrophotometer.\(^{25,52}\) The entrapment efficiency was calculated according to Equation (1), where \(W_{\text{total drug}}\) indicates the total amount of siRNA in nanoparticles, and \(W_{\text{free drug}}\) indicates the amount of free siRNA in the nanoparticles.

\[
\text{Encapsulation efficiency} \, (\%) = \frac{W_{\text{total drug}} - W_{\text{free drug}}}{W_{\text{total drug}}} \times 100\% \quad (\text{Equation 1})
\]

**Colloidal stability**

The colloidal stability of C-siRNA/MTSLR and its dilutions with PBS (pH 7.4) or DMEM containing 10% FBS was monitored using a Turbiscan Lab expert at 37°C, with delta transmission and delta backscattering as indexes.\(^{25}\)

**DSC analysis**

To verify the thermosensitivity of the constructed system, DSC analysis was performed using a DSC Q2000 (TA, New Castle, DE, USA) for both C-siRNA/MTSLR and C-siRNA/TSL. Approximately 5mg of each sample was placed into a standard aluminum pan for the analysis. The temperature was increased from 0°C to 80°C with a speed of 10°C/min.\(^{19,33,34}\)

**In vitro release**

To further validate the temperature-triggered release in vitro, drug-release studies of C-siRNA/MTSLR and C-siRNA/TSL were conducted using a dialysis technique at 37°C and 42°C, respectively. Briefly, 0.5mL of nanoparticles was added into a dialysis bag (MWCO 50 kDa), immersed in 30mL of HEPES buffer medium and stirred at 60 RPM. Then, 0.5mL of the release sample was taken out and replaced with an equal volume of fresh release medium at predetermined intervals.\(^{19,33}\) The content of siRNA in the samples was determined by spectrophotometer, and the cumulative release of siRNA was calculated according to Equation (2):

\[
\text{Cumulative release} \, (%) = \frac{(V \times C_t + V_r \times \sum C_m)}{Dose} \times 100\%, \quad (\text{Equation 2})
\]

where \(V\) is the volume of release medium, \(C_t\) represents the determined concentration of siRNA in the collected samples at time \(t\), \(\sum C_m\) denotes the concentration sum of the collected samples, \(V_r\) corresponds to the volume of samples removed for analysis, and \(Dose\) is the amount of siRNA added into the release medium.

**CD analysis**

To investigate the changes that occurred in the secondary structure of proteins in the macrophage membrane and macrophage-membrane-disguised nanoparticles, a CD analysis was conducted on a Chirascan-Plus CD spectrometer with a 0.1 cm quartz cell. The concentrations of proteins in different formulations were all about 0.3 mg/mL. The measurements were performed in the UV region, with a wavelength range of 190–300 nm and a bandwidth of 5 nm. Each spectrum was reported as the average of three scans.\(^{34}\)

**Protein detection**

To ensure the integrity and efficacy of proteins on the nanoparticles, protein profiles and specific proteins in the purified macrophage membrane and macrophage-membrane-coated nanoparticles were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and WB analysis, respectively, as described previously.\(^{35,36}\)

For the SDS-PAGE analysis, the total membrane proteins were first extracted from the samples using the cell total protein extraction kit. Then, the extracted proteins were run on a 4%–12% Bis-Tris 10-well mini-gel in a running buffer using a Bio-Rad electrophoresis system at 80 V for 0.5 h and then at 120 V for 1 h. Finally, the resulting polyacrylamide gel was stained with SimplyBlue overnight for visualization. In contrast, nanoparticles without membrane decoration were analyzed as a negative control.

Furthermore, specific proteins including integrin α4, integrin β1, and CD47 in the purified macrophage membrane and different
nanoparticles were identified by WB analysis. In brief, the extracted proteins were collected after centrifugation at 16,100 \( \times \) g for 10 min and quantified using the Pierce BCA Protein Assay (Thermo Fisher Scientific, USA). Then, they were separated on a 10% acrylamide gel and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, USA). Thereafter, the samples were treated with a primary antibody against \( \alpha_t, \beta_1, \) and CD47, followed with HRP-labeled goat/anti-rabbit IgG. Finally, the protein signals were detected using a ChemiDoc MP imaging system (Bio-Rad, USA).

**In vitro cellular uptake**

The *in vitro* cellular uptake was first evaluated in HepG2 cancer cells using both CLSM (UltraVIEW Vox, PerkinElmer, USA) and FCM (BD FACSCalibur, Franklin Lakes, NJ, USA). For CLSM analysis, HepG2 cells were seeded at a density of \( 2 \times 10^5 \) cells/well on a Petri dish and cultured until 24 h. The media were then replaced with 2 mL of samples containing cy5-siRNA. The final concentration of cy5-siRNA for each sample was 100 nM. After incubation for 4 h, the cells treated with different samples were fixed with 4% paraformaldehyde for 20 min and stained with Hoechst 33258 for 10 min at ambient temperature. Finally, the fluorescent images were visualized under CLSM. To quantitatively analyze the cellular uptake, HepG2 cells were seeded, cultured, and treated with different samples as those in CLSM analysis. Then, the cells were detached with 0.25% trypsin solution and measured with FCM after resuspension in 0.3 mL PBS.

Similarly, the *in vitro* cellular uptake was also performed using RAW 264.7 macrophages to verify the reduction of RES phagocytosis for the nanoparticles disguised with macrophage membrane.

**Cytotoxicity**

To evaluate the cytotoxicity of different formulations, HepG2 cells were seeded in 96-well plates at a density of \( 5 \times 10^3 \) cells/well and incubated for 24 h. Then, the cells were treated with 200 \( \mu \)L of different samples including blank culture media (Control), free siRNA, C-si.NC/MTSLR, free C-siRNA, and siRNA encapsulated in different nanoparticles at a siRNA concentration of 100 nM for another 12, 24, and 48 h. Thereafter, a 20-\( \mu \)L CCK-8 agent was added into each well, and the cells were further incubated for 1 h. The absorbance of the samples at 450 nm was measured with a 96-well plate reader (Model 680, Bio-Rad, Hercules, CA, USA). The cell viability of the cell culture medium was defined as 100%.

**In vitro gene silencing**

HepG2 cells were seeded and incubated for 24 h before transfection. Then, they were incubated further with different samples including blank culture media (Control), free siRNA, free C-siRNA, siRNA encapsulated in different nanoparticles, C-si.NC/MTSLR-H, and C-siRNA/MTSLR-H at a siRNA concentration of 100 nM for 5 h. After that, the cells were further incubated for 48 h (for mRNA assay) or 72 h (for WB assay). Then, the transfected cells were collected for analysis.

The method that has been reported in the literature for performing an mRNA assay with quantitative real-time PCR was slightly modified in this study. The analysis was conducted on a real-time PCR detection system, and the relative gene expression was quantified by the \( 2^{-\Delta \Delta Ct} \) method. The primers for PCR amplification were as follows: glyceroldehyde 3-phosphate dehydrogenase (GAPDH) forward: ACATCGCTCAGACACATG; GAPDH reverse: TGTAGTGGAGTTCAATGAAGGG; Bcl-2 forward: CTAAGACACACCGCCA; Bcl-2 reverse: GAGAAATCAGACAGGC. Reaction parameters were as follows: 95°C for 15 s and then 59°C for 30 s for 40 cycles. Specificity was verified by melt curve analysis and agarose gel electrophoresis.

**Cell apoptosis assay**

To evaluate the cell apoptosis induced by different formulations, HepG2 cells were seeded in 6-well plates 24 h before the experiments. The cells were then exposed to different samples including blank culture media (Control), free siRNA, free C-siRNA, siRNA encapsulated in different nanoparticles, C-si.NC/MTSLR-H, and C-siRNA/MTSLR-H at a final siRNA concentration of 100 nM for 5 h. After 72 h, the cells were trypsinized, washed, and collected. The apoptotic cells were then treated according to the Annexin V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) and detected by FCM. The results were analyzed using FlowJo software (Treestar, Oakland, CA, USA).

**In vivo distribution**

The *in vivo* distribution was evaluated with fluorescence imaging in HepG2 tumor-bearing male nude mice. In brief, the mice bearing the HepG2 tumor with a volume of approximately 300 mm³ were first acquired after subcutaneous injection in the right axilla with \( 1 \times 10^7 \) HepG2 cells. Then, the mice were randomly divided into eight groups; the mice in each group were administered with physiological saline (control group) or different formulations containing cy5-siRNA by tail vein injections at a dose of 1.2 mg/kg conjugating to siRNA. The mice were sacrificed by cervical dislocation, and the tumors and major
organs, including the heart, liver, spleen, lung, and kidneys, were excised and imaged.

**In vivo anti-tumor efficacy**

The in vivo anti-tumor efficacy was evaluated in HepG2 tumor-bearing male nude mice. Briefly, the xenograft tumor model was established in male nude mice by a subcutaneous injection of HepG2 cells, and the tumors were allowed to grow until the volume reached approximately 100 mm³. Then, the mice were randomly divided into nine groups (n = 5 per group). The mice of eight groups were treated with physiological saline (control), free siRNA, free C-siRNA, siRNA/TSL, C-siRNA/TSL, C-siRNA/MTSL, C-siRNA/MTSLR, or C-siNC/MTSLR by intravenous injection every 2 days at a dose of 1.2 mg/kg converting to siRNA, and then the tumor regions were heated to 42°C for 30 min after injection. As controls, mice in the ninth group were treated with C-siRNA/MTSLR without heating of the tumor region.

The tumor volume was measured daily and calculated based on Equation (3), where “a” and “b” indicate the length and width of the tumor, respectively. The body weight of the animals was monitored daily during the experiments. After 14 days, the mice were sacrificed, and the tumors were excised and weighed.

\[
\text{Tumor volume} = \frac{a \times b^2}{2} \quad \text{(Equation 3)}
\]

To analyze the gene silencing in mice, the isolated tumors were homogenized in 300 μl of lysis buffer (0.05% Triton X-100 and 2 mM EDTA in 0.1 M Tris-HCl) and then centrifuged at 5,550 g for 10 min. The supernatant was used for mRNA and WB assays.

To further assess the anti-tumor efficacy, H&E staining, CD31 detection, and the TUNEL assay were performed. The collected tumor tissues were first fixed with neutral-buffered formalin (10%), embedded in paraffin, and sliced at a thickness of 0.5 μm. Then, the sections were analyzed with H&E staining, CD31 immunohistochemical detection, and a TUNEL assay, as reported in the literature.

**In vivo safety evaluation**

To investigate the safety of the constructed delivery system, healthy mice were treated with physiological saline (Control), C-siRNA, or C-siRNA/MTSLR by intravenous injection every 2 days at a dose of 1.2 mg/kg converting to siRNA. After 14 days, about 1.5 mL of a venous blood sample was collected from anesthetized animals for a hemogram assay, and the mice were then sacrificed. The main tissues, including the heart, liver, brain, lung, and kidneys, were harvested and stained by H&E for subsequent analysis.

**Statistical analysis**

All data were shown as mean ± standard deviation (SD). Student’s t test or one-way analysis of variance (ANOVA) was used for the statistical evaluation. Differences between groups were considered as statistically significant when the probability (p) was less than 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.12.016.

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**AUTHOR CONTRIBUTIONS**

C.G., Z.L., and L.L. conceived the study, supervised experiments, analyzed the data, and wrote the manuscript; J.N. and J.Z. performed the preparation, characterization, and in vitro evaluation of different formulations; J.N., J.Z., J.L., and H.L. conducted the experiments involving in vivo anti-tumor efficacy, distribution, and safety study; Y.Y., M.Y., Y.W., and W.G. revised the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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