iRGD-functionalized PEGylated nanoparticles for enhanced colon tumor accumulation and targeted drug delivery

Aim: To enhance the tumor accumulation and targeted drug delivery for colon cancer therapy, iRGD peptide was introduced to the surface of PEGylated camptothecin-loaded nanoparticles (NPs). **Methods:** Cellular uptake, targeting specificity, bio-distribution and antitumor capacity were evaluated. **Results:** The functionalization of iRGD facilitated tumor accumulation and cellular uptake of NPs by Colon-26 cells. Furthermore, the resultant iRGD-PEG-NPs remarkably improved the therapeutic efficacy of camptothecin *in vitro* and *in vivo* by inducing a higher degree of tumor cell apoptosis compared with PEG-NPs. **Conclusion:** iRGD-PEG-NP is a desired drug delivery system to facilitate the drug accumulation in orthotopic colon tumor tissues and further drug internalization by colon cancer cells.

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Ranked as the third most common malignancy worldwide, colon cancer causes more than half a million deaths each year [1]. More seriously, its prevalence rate is steadily rising in developing countries [2]. The current modalities for colon cancer therapy mainly include surgery, chemotherapy, radiotherapy and biological therapy [3,4]. Among these methods, chemotherapy aims to inhibit tumor growth and improve survival rate, which is the most common strategy in practice.

Camptothecin (CPT) is isolated from *Camptotheca acuminata* and exhibits strong anticancer effect by blocking the action of the nuclear enzyme topoisomerase I, resulting in the hindrance of tumor-related DNA replication [5]. It is one of the most commonly applied chemotherapeutic agents for clinical treatment of colon cancer [6]. Unfortunately, the therapeutic efficacy of CPT is rather limited by its hydrophobicity, lactone instability and unsatisfactory pharmacokinetic profiles (e.g., limited circulation time and nonselec-

tive biodistribution), the latter of which are usually associated with rapid blood clearance, poor accumulation in tumor tissues and undesirable adverse effects [7–9]. To address these issues, nanotechnology-based drug delivery systems (DDSs), including polymeric nanoparticles (NPs), micelles, liposomes and mesoporous silica NPs, have been widely applied in chemotherapy [10–13]. Polymeric NP-mediated DDSs have recently attracted increasing attention due to numerous advantages, including improved drug solubility and stability, controlled drug release and enhanced extravasation toward tumor tissues via the enhanced permeability and retention effect [14,15]. Although NPs are able to passively accumulate in colon tumors, their therapeutic efficacy is still far from optimum [16,17]. Therefore, researchers are seeking to develop more effective approaches to actively target to colon tumors through highly selective drug accumulation at tumor sites and efficient internalization by tumor cells.

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As previously reported, iRGD (CRGDKGPDC) is a novel tumor-homing cyclic peptide linked by a disulfide bond [18,19]. It contains an arginine-glycine-aspartic (RGD) tripeptide sequence and a C-terminal end-binding (CendR) motif that is known to increase cell internalization [20]. Upon intravenous administration of iRGD, the RGD sequence preferentially binds to $\alpha v\beta 3/\alpha v\beta 5$ integrin receptors, which are specifically and highly overexpressed on the new blood vessels in tumor angiogenesis. Subsequently, a tumor-derived protease cleaves the iRGD peptide to expose a CendR motif, which tends to bind with neuropilin-1 (NRP-1) and then induce tissue penetration/accumulation and further internalization by tumor cells [19,21–23]. As NRP-1 is highly overexpressed in colon cancer cells [24,25], it seems reasonable to hypothesize that the surface functionalization of NPs with iRGD could promote their accumulation in colon tumor tissue and colon cancer cell internalization.

Currently, the most commonly mouse model for the development of potential anticancer DDSs is the xenograft tumor model, which is simple and can be easily monitored [26]. However, the xenograft tumor model usually fails to exhibit cancer heterogeneity, neither can fully recapitulate the tumor microenvironment and also lacks clinical predictability [27,28]. It is found that carcinogen-induced mouse cancer models simulate the natural microenvironment of original tumors more closely and exhibit intact pathological characteristics [28–30]. However, such a mouse model has rarely been implemented to examine the therapeutic efficacy of DDSs.

Poly(lactic acid/glycolic acid) (PLGA) is a biodegradable and biocompatible polymer that can efficiently form NPs loaded with hydrophobic drugs [31]. It has been extensively utilized for drug delivery as reported in the literature [10,32–34]. Considering the unique advantage of iRGD as a specific peptide affiliated to colon cancer, we herein developed iRGD-functionalized PEGylated CPT-loaded PLGA NPs (iRGD-PEG-NPs). We then characterized their physicochemical properties, investigated their cellular uptake profiles and anticancer capacities, and evaluated their *in vivo* anticancer activity using an orthotopic mouse model with colon cancer.

Materials & methods

Materials

PLGA (molecular weight: 38–54 kg/mol), sodium nitrite, chitosan, poly(vinyl alcohol) (PVA, 86–89% hydrolyzed, low molecular weight), CPT, coumarin-6 (CM), azoxymethane (AOM) and Triton X-100 were supplied by Sigma-Aldrich (MO, USA). Chitosan was depolymerized using sodium nitrite as reported previ-

ously [35]. Viscosity-average molecular weight of the depolymerized chitosan was measured as 1.8×10^4 using an Ubbelohde viscometer [36]. Heterobifunctional PEG derivative (NHS-PEG-Mal, molecular weight: 2000) were purchased from Jenkem Technology (Beijing, China). Paraformaldehyde (16%) was obtained from Electron Microscopy Science (PA, USA). iRGD (sequence: CCRGDKGPDC, C2-C10 Bridge, purity > 95%) were synthesized by GL Biochem (Shanghai, China). Vybrant® MTT cell proliferation assay kit and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Vybrant Apoptosis Assay Kit were supplied by Molecular Probes (OR, USA). Hematoxylin and eosin were obtained from Richard-Allan Scientific (MI, USA). Dextran sulfate sodium (DSS, molecular weight: 36–50 kDa) was purchased from MP Biomedicals (OH, USA). A total of 10% buffered formalin was obtained from EMD Millipore (MA, USA).

Fabrication of iRGD-PEG-NPs

CPT-loaded NPs were produced by a modified oil-in-water emulsion solvent evaporation method. Briefly, PLGA (100 mg) and CPT (8 mg) were dissolved in dichloromethane-methanol co-solvent (8:2, v/v) to form oil phase, which was then added dropwise to PVA solution (5%, w/v) with depolymerized chitosan (0.5%, w/v). The mixture was immediately sonicated 60s at 40% amplitude using a Branson S-450 sonicator (CT, USA). Subsequently, this emulsion was poured into 100 ml PVA solution (0.3% PVA, w/v) with depolymerized chitosan (0.03%, w/v). Thereafter, the organic solvent in the solution was evaporated under reduced pressure. The NPs were collected by centrifugation, washed three-times and freeze-dried.

To prepare iRGD-PEG-NPs, the above CPT-loaded NPs (20 mg) were dispensed in water, and further grafted with PEG through the chemical conjugation between the amino groups of depolymerized chitosan on the surface of NPs and the NHS ester groups of NHS-PEG-Mal. The obtained Mal-PEG-NPs were washed three-times and the maleimide groups of NPs were further reacted with the sulfhydryl groups of iRGD. The molar ratio of NHS-PEG-Mal and iRGD used here was 1:3. The unreacted maleimide groups of iRGD-PEG-NPs and PEG-NPs were quenched with cysteine.

Characterization of NPs

The particle size, monodispersity and zeta potential of PEG-NPs and iRGD-PEG-NPs were determined by dynamic light scattering (DLS) using a multi-angle particle sizing method (90 Plus/BI-MAS) or DLS under an electric field (ZetaPlus analyzer, Brookhaven

Instruments). The particle size, polydispersity index (PDI) and zeta potential of PEG-NPs and iRGD-PEG-NPs were based on the average of repeated measurements.

The morphology of PEG-NPs and iRGD-PEG-NPs was characterized using transmission electron microscopy (TEM, LEO 906E, Zeiss, Germany) at an 80 kV accelerating voltage. NP suspensions were dropped onto 400-mesh carbon-coated copper grids and dried prior to analysis.

The CPT loading amount and encapsulation efficiency of NPs were measured based on the CPT fluorescence using a plate reader (Perkin Elmer, MA, USA). Briefly, PEG-NPs and iRGD-PEG-NPs (3 mg) were dissolved in acidic dimethyl sulfoxide (DMSO) solutions (0.1% HCl, v/v), which were transferred to a black 96-well plate. The fluorescence intensity was obtained at emission wavelength of 430 nm (excitation at 360 nm).

CPT release from NPs

The drug release profiles of NPs were determined using a dialysis method. In brief, PEG-NPs and iRGD-PEG-NPs were suspended in phosphate-buffered saline (PBS) and transferred into dialysis bags with molecular weight cut off of 10,000 Da. Thereafter, the closed bags was put into a centrifuge tube (50 ml) and immersed in PBS release medium containing 0.5% Tween-80. The tube was kept in a shaking water bath at 120 r.p.m. at 37°C. Fixed amount of outer release solution was collected at various time points and replaced with fresh medium. Finally, the released CPT in the outer solution was determined using the same method as presented above.

Intracellular uptake of NPs

Colon-26 cells were cultured overnight on a tissue culture plate (BD Falcon, MA, USA) before iRGD-PEG-NPs (CPT: 100 μ M) was added into the culture medium. After incubation for 3 or 5 h, Colon-26 cells were rinsed with PBS thrice to remove excess NPs, and further fixed in 4% paraformaldehyde solution for 20 min. Cell images were taken by an Olympus fluorescence microscope with a digital camera (ORCA-03G, Hamamatsu).

Quantification of cellular uptake efficiency

Colon-26 cells were cultured in 6-well plates overnight before various NPs (CPT: 25 μ M) were added into the culture medium. After incubation for 1 or 3 h, Colon-26 cells were rinsed with PBS thrice to remove excess NPs. Cells were collected using accutase before centrifugation at 2000 $\times$ g for 6 min, and transferred to polystyrene tubes (BD Falcon).

Analytical flow cytometry (FCM) was conducted through DAPI channel on the FCM Canto™ (BD Biosciences, CA, USA) based on 5000 gated cell events.

The colon cancer-targeted property of iRGD-PEG-NPs was confirmed by a competition assay. Colon-26 cells were incubated with iRGD-PEG-NPs in the presence of free iRGD (0.5 mg/ml) as a competitor, and then the cellular uptake efficiency was quantified by FCM.

In vitro anticancer test

For MTT test, Colon-26 cells were cultured in 96-well plates overnight before NPs (CPT: 0.1–32 μ M) were added in the medium. After further incubation for 24 or 48 h, cells were rinsed with PBS thrice and then incubated with MTT solution (0.5 mg/ml) for 4 h. Subsequently, the culture media were removed and DMSO (50 μ l) was added into each well before spectrophotometric analysis at 570 nm. Cells without treatment were used as negative controls, while cells treated with Triton X-100 solutions (0.5%, w/v) were positive references.

Induced cell apoptosis

Colon-26 cells were cultured in 6-well plates overnight before NPs (CPT: 8 μ M) were added in the medium. After further incubation for 2, 4 and 8 h, cells were rinsed with PBS thrice. Cells were then collected using accutase and analyzed by apoptosis assay using Annexin V-FITC/PI Vybrant Kit. The emitted green fluorescence of Annexin V and red fluorescence of PI were recorded using 525 and 575 nm band pass filters, respectively (excitation at 488 nm). The flow analysis was based on 5000 gated cell events using FCM Canto (BD Biosciences).

Bcl-2 gene expression level

Colon-26 cells were cultured in 6-well plates overnight before NPs (CPT, 8 μ M) were added to the medium. After further incubation for 2, 4 or 8 h, total RNAs were extracted using the RNeasy Plus Mini Kit (Qiagen, CA, USA). Subsequently, the complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit (Fermentas, MD, USA). Thereafter, *Bcl-2* mRNA expression levels were analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). The data were normalized relative to the internal control: 36B4. Relative *Bcl-2* mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences for qRT-PCR were listed in [Supplementary Table 1](#).

Hemolysis assay

The hemocompatibility of NPs was investigated following our previous report [10]. In brief, mice blood was collected from heart and erythrocytes were harvested by centrifugation at $2000 \times g$ for 15 min. The erythrocytes were washed thrice and re-suspended in PBS (2%, v/v). Meanwhile, NPs were dispersed in PBS and diluted according to gradient concentrations of CPT from 0.1 to 100 μM , which were divided into aliquots of 150 μl and mixed with 2% erythrocytes (150 μl) respectively. Thereafter, the obtained mixtures were incubated for 1 h at 37°C and centrifuged at $3000 \times g$ for 15 min. The released hemoglobin in the supernatant was analyzed by spectrophotometric tests at 570 nm. Untreated erythrocyte suspension was used as a negative control, whereas erythrocyte suspension treated with Triton X-100 (1%, v/v) was used as a positive control.

In vivo tissue uptake of NPs

Friend virus B-type female mice (10 weeks of age, Model Animal Research Center of Nanjing University) were used in the animal experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Southwest University. Colon cancer in the mouse model was induced following our previous report [37]. Initially, mice were intraperitoneal injected with AOM (10 mg per kg of body weight) and subjected to regular diet and water for 5 days. Subsequently, mice were treated with two cycles of 2.5% DSS (7 days for each cycle). To track the distribution of NPs in colon tumor tissues, CM was employed as a fluorescence probe. The fabrication processes of PEG-CM-NPs and iRGD-PEG-CM-NPs were exactly the same as that of PEG-NPs and iRGD-PEG-NPs, except that CPT was replaced by CM. Mice were intravenously injected with PEG-CM-NPs or iRGD-PEG-CM-NPs (3 mg CM/kg mouse) after 7 days of the second round of DSS treatment. After 12 h of injection, mice were sacrificed by CO_2 euthanasia. Colon tissues were extracted, rinsed with PBS and then embedded in Optimal Cutting Temperature compound. Eventually, tissue sections (5 μm) were stained with DAPI and Alexa Fluor 568 phalloidin. An Olympus fluorescence microscope with a Hamamatsu Digital Camera ORCA-03G was used to record the images.

In vivo antitumor activity

Orthotopic colon cancer was induced as described above. The NP-treated mice were intravenously injected with PEG-NPs or iRGD-PEG-NPs (3 mg CPT/kg mouse) every other day after 2 days of the second round of DSS treatment. After five doses of NP treatments,

mice were sacrificed, and then colonic tumors were measured and counted. The apoptosis profiles of tumor cells in colon were analyzed using a commercial apoptosis detection kit (Roche Diagnostics, IN, USA), including 4,6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). Images were taken by an Olympus fluorescence microscope with a digital camera (ORCA-03G, Hamamatsu).

Statistical analysis

Statistical analysis was conducted using ANOVA test followed by a Bonferroni post-hoc test (GraphPad Prism) or Student's *t*-test. Data were presented as mean $\pm$ standard error of mean (SEM). Statistical significance was confirmed when $*p < 0.05$ and $**p < 0.01$.

Results

Fabrication of NPs

It was reported that emulsion solvent evaporation technique was a well-established method for producing NPs [38]. Thus, it has been applied to fabricate CPT-loaded NPs here. In principle, the rapid mix of the oil phase (PLGA and CPT) and the aqueous phase (PVA and chitosan) under sonication caused immediate formation of an oil/water emulsion, which was due to the Marangoni effect and capillary break-up process [39]. After evaporation of the organic solvents (dichloromethane and methanol), CPT molecules were transferred to the hydrophobic PLGA matrix via hydrophobic interactions, and eventually solidified to form CPT-loaded NPs [40].

PVA, an amphiphilic copolymer, has been usually utilized as an emulsifier in the fabrication process of NPs [41]. It tends to present on NP surface and enables the separation of the oil phase and aqueous phase, preventing the aggregation of NPs [42]. Specifically, the hydrophobic segments of PVA insert into the hydrophobic polymeric matrix of NPs, while its hydrophilic segments surround the NPs and stabilize them through steric hindrance. On the other hand, chitosan, a natural amino group-bearing polymer, has been implemented for the surface functionalization of polymeric NPs to improve their pharmacological effects [43,44]. The action of chitosan functionalization might reflect the entanglement of its molecular chains with PVA and/or the electrostatic adsorption between positive-charged chitosan and negative-charged NP surface. This depolymerized chitosan facilitated to obtain PLGA NPs with smaller particle size in compared with original chitosan (Supplementary Figure 1), and it was thus used in the fabrication process of NPs. Further conjugation of the heterobifunctional reagent, NHS-PEG-Mal,

to chitosan on the surface of NPs provided steric stabilization, improved hemocompatibility and supplied active chemical groups for the engraftment of iRGD. Finally, the sulfhydryl group of cyclic iRGD was conjugated to the terminal maleimide group of the NHS-PEG-Mal on the NP surface, yielding iRGD-PEG-NPs. The processes of preparing CPT-loaded NPs, conjugation of NHS-PEG-Mal and grafting of iRGD were shown in **Figure 1A**.

Physicochemical properties of NPs

The diameter, PDI and zeta-potential of NPs play vital roles to determine their stability, drug release behavior, cellular uptake efficiency and *in vivo* biodistribution [39]. **Figure 1B & C** clearly showed that both PEG-NPs and iRGD-PEG-NPs typically appeared spherical, were homogeneously distributed with diameter less than 200 nm as measured by TEM. In **Table 1**, DLS analysis indicated that these NPs had hydrodynamic diameters

Figure 1. Preparation and physicochemical characterization of nanoparticles. (A) Schematic illustration of the preparation of iRGD-PEG-NPs. Representative transmission electron microscopy images of (B) PEG-NPs and (C) iRGD-PEG-NPs. Representative size distribution profiles of (D) PEG-NPs and (E) iRGD-PEG-NPs.

CPT: Camptothecin; iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; PLGA: Poly(lactic acid/glycolic acid); PVA: Poly(vinyl alcohol).

Table 1. Physicochemical characteristics of nanoparticles (mean ± SEM; n = 3).					
Nanoparticles	Particle size (nm)	Polydispersity index	Zeta-potential (mV)	Drug loading (%)	Encapsulation efficiency (%)
PEG-NPs	275.9 ± 3.0	0.06 ± 0.03	-16.2 ± 3.9	6.3 ± 0.1	91.6 ± 0.8
iRGD-PEG- NPs	279.1 ± 2.2	0.11 ± 0.01	-14.2 ± 1.2	6.1 ± 0.1	87.3 ± 1.4

NP: Nanoparticle; SEM: Standard error of mean.

ranging from 276 to 280 nm. The discrepancies in particle sizes were due to the different surface states of NPs as measured by these two methods, in which NPs were completely dehydrated (shrunk) for TEM imaging while fully hydrated (swollen) for DLS analysis [10]. The PEG-NPs and iRGD-PEG-NPs had narrow size distributions (Figure 1D & E), which were consistent with their relatively low PDI values (less than 0.11). The NPs coated with chitosan were positively charged (data not shown). In contrast, PEG-NPs and iRGD-PEG-NPs had negative zeta potentials, indicating the conjugation of the PEG derivative to the NP surface. In addition, the drug loading of both types of NPs was ranging from 6.1 to 6.3%, and their corresponding encapsulation efficiency was in the range of 87.3 to 91.6%.

In vitro drug release

CPT has proved to be an important chemotherapeutic agent for colon cancer chemotherapy. However the intravenous administration of this drug and its subsequent distribution throughout the body via blood circulation induced serious adverse effects (e.g., thrombocytopenia, leukopenia and

gastrointestinal toxicities) [45]. Controlled release and specific targeting on tumor site have been recognized as effective approaches to reduce the off-target side effects of CPT-loaded NPs [46]. It was reported that the drug release profile of polymeric NPs depends on a number of factors related to their 3D structure, surface morphology and physicochemical properties of the matrix, as well as the property of loaded drugs [47,48].

The *in vitro* CPT release behaviors of NPs were monitored in this study as a function of time. In Figure 2, both PEG-NPs and iRGD-PEG-NPs showed a slight burst of CPT release initially, and approximately 32% of the trapped CPT was released from NPs within the first 24 h. Furthermore, both types of NPs achieved more than 66% cumulative CPT release in 72 h. In a prior report, the drug release from polyester NPs was attributed to a series of events, including matrix swelling, degradation and erosion [47]. The mathematical models for *in vitro* CPT release profiles of PEG-NPs and iRGD-PEG-NPs were shown in Supplementary Tables 2 & 3, respectively. Our results implied that after a slight initial burst release, CPT migrated from the hydrophobic PLGA matrix to the surface of NPs mainly due to the matrix erosion and diffusion, and then showed a sustained release profile thereafter. Based on these observations, we may conclude that both PEG-NPs and iRGD-PEG-NPs could realize a controlled CPT release for more than 3 days. In addition, these NPs could exhibit prolonged release of CPT *in vivo* due to limited blood volume, which implied that PEG-NPs and iRGD-PEG-NPs were enabling vehicles for controlled release of chemotherapeutic drug over a long period.

Figure 2. In vitro cumulative release of camptothecin from PEG-NPs and iRGD-PEG-NPs in phosphate-buffered saline containing 0.5% Tween-80 at 37°C. Data are presented as mean ± SEM (n = 3). iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

Intracellular uptake of NPs

The intracellular uptake of anticancer drugs was a major concern for chemotherapy. Herein, we used fluorescence microscopy to characterize the internalization of CPT by Colon-26 cells at various time points (3 and 5 h). As expected, untreated Colon-26 cells exhibited no auto-fluorescence (Figure 3A), while we detected weak signals from cells treated with iRGD-PEG-NPs for 3 h and a significantly higher intracellular fluorescence intensity after 5 h of treatment. Obviously, these results confirmed that iRGD-PEG-NPs were internalized by Colon-26 cells efficiently.

Figure 3. Cellular uptake profiles of nanoparticles by Colon-26 cells. (A) Fluorescence images showing the cellular uptake of iRGD-PEG-NPs into cells after 0, 3 and 5 h of co-incubation. Scale bar = 10 μ m. Quantification of the cellular uptake efficiency of PEG-NPs and iRGD-PEG-NPs by cells. (B) Representative flow cytometry histograms of the fluorescence intensities seen in cells treated with NPs for 3 h. (C) Percentage of camptothecin-containing Colon-26 cells after treatment with PEG-NPs or iRGD-PEG-NPs (Camptothecin, 25 μ M) for 1 or 3 h. Each point represents the mean $\pm$ SEM (n = 3; *p < 0.05 and **p < 0.01, Student's t-test). CPT: Camptothecin; iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

To quantitatively and comparatively investigate the cellular uptake efficiency of PEG-NPs and iRGD-PEG-NPs, Colon-26 cells were treated with these NPs at the same CPT concentration of 25 μ M for different

time points (1 and 3 h). In Figure 3B, the average fluorescence intensity of iRGD-PEG-NP-treated Colon-26 cells was 1.9-fold higher than that of PEG-NPs-treated cells after 3 h of incubation. This indirectly indicated

Figure 4. In vitro cytotoxicity of PEG-NPs and iRGD-PEG-NPs against Colon-26 cells assessed by MTT assays.

(A) 24 h and (B) 48 h. Triton X-100 (0.5%) was used as a positive control to produce a maximum cell death rate (100%), whereas cell culture medium was used as a negative control (death rate defined as 0%). Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean $\pm$ SEM ($n = 5$; * $p < 0.05$ and ** $p < 0.01$, Student's t -test).

CPT: Camptothecin; iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

that iRGD was successfully conjugated onto the NP surface. It also suggested that the surface functionalization with iRGD enhanced the internalization efficiency of NPs by Colon-26 cells. To further verify the specific role of iRGD, we assessed the uptake efficiency of iRGD-PEG-NPs in the presence of free iRGD, which acted as a competitor of the iRGD groups in iRGD-PEG-NPs. As shown in Figure 3C, the free iRGD decreased the cellular uptake of iRGD-PEG-NPs, indicating that iRGD-PEG-NPs were internalized by Colon-26 cells via iRGD receptor-mediated endocytosis.

In vitro anticancer property

To investigate the anticancer activity of PEG-NPs and iRGD-PEG-NPs, we treated Colon-26 cells with these NPs for 24 and 48 h, respectively. Figure 4 demonstrated that both types of NPs inhibited the viability of Colon-26 cells depending on the drug concentration and time of treatment. Notably, when CPT concentration increased above 0.5 μ M, the viabilities rapidly decreased. Furthermore, the viability difference between the cells treated with iRGD-PEG-NPs and PEG-NPs appeared relatively weaker under low concentration of CPT (less than 16 μ M). However, when the CPT concentrations increased to over 16 μ M, the anticancer effect of iRGD-PEG-NPs was almost

two-times higher than that of PEG-NPs. The results indicated that iRGD-PEG-NPs demonstrated significantly stronger anticancer activities than PEG-NPs after 24 and 48 h of treatment, which might ascribed to increased cellular uptake efficiency in the presence of iRGD on the NP surface (Figure 4A & B).

To quantitatively characterize the cytotoxicity of PEG-NPs and iRGD-PEG-NPs, we measured their IC_{50} values (i.e., the drug concentrations required to induce 50% cell death within a certain period). As shown in Supplementary Table 4, the IC_{50} for iRGD-PEG-NPs was 1.67 μ M at 24 h and 0.93 μ M at 48 h, which were much lower than the corresponding values obtained for PEG-NPs. The enhanced cytotoxicity of iRGD-PEG-NPs against Colon-26 cells could be ascribed to the improved endocytosis-mediated uptake of the drug, resulting in the increase of intracellular CPT concentration and eventual cell death.

In vitro apoptosis assay

To investigate the apoptotic effects of PEG-NPs or iRGD-PEG-NPs, Colon-26 cells were treated with these NPs at the same CPT concentration of 8 μ M and then stained with Annexin V-FITC/PI Apoptosis Detection Kit. The percentages of cells corresponding to four phenotypes (viable cells, early apoptotic cells, necrotic cells and late apoptotic cells) were evaluated

Figure 5. The apoptotic profiles of Colon-26 cells treated with PEG-NPs or iRGD-PEG-NPs (Camptothecin, 8 μ M; see facing page). The percentages of the cell states are indicated in each quadrant. Lower left, viable cells; lower right, early apoptotic cells; upper left, necrotic cells; upper right, late apoptotic cells. Data are presented as means $\pm$ SEM ($n = 3$).

iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

Figure 6. Nanoparticle-induced downregulation of *Bcl-2* transcripts in Colon-26 cells *in vitro*. Cells were treated with PEG-NPs or iRGD-PEG-NPs (Camptothecin, 25 μ M) for various time periods (2, 4 or 8 h). Each point represents the mean $\pm$ SEM ($n = 3$; * $p < 0.05$ and ** $p < 0.01$, Student's *t*-test).

iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

using FCM analysis software. Figure 5 showed that the percentage of viable cells in the control group was more than 91.1%, which decreased with increasing incubation time with the NPs. It was found that similar percentages of viable cells were observed after 8 h of treatment with PEG-NPs and iRGD-PEG-NPs. However, treatment with iRGD-PEG-NPs significantly increased the percentage of apoptotic Colon-26 cells to 38.4%, whereas treatment with PEG-NPs increased this percentage to only 29.3%. Furthermore, we found that the percentage of necrotic Colon-26 cells in iRGD-PEG-NP-treated group was much lower than that of PEG-NP-treated group. As reported, necrosis would promote tumor growth [49]. These results collectively demonstrated that iRGD-PEG-NP was a more effective anticancer DDS compared with PEG-NP.

Inhibition of *Bcl-2* gene expression level

Bcl-2 regulates mitochondria-mediated apoptosis, which also plays a critical role in the cell survival in multiple malignancies [50]. Therefore, downregulating the expression level of *Bcl-2* gene was speculated to promote apoptosis and inhibit tumor growth. Figure 6 showed that Colon-26 cells treated with the different NPs exhibited varied changes in relative *Bcl-2* mRNA expression levels, in which PEG-NPs reduced *Bcl-2* expression to 34.5% after 8 h, whereas iRGD-PEG-NPs decreased *Bcl-2* expression to 16.6%. These results demonstrated that iRGD-PEG-NPs had a pronounced effect on apoptosis, which was consistent with the findings in MTT assay and apoptosis analysis.

Hemolysis assay

The hemocompatibility of NPs is an important parameter for *in vivo* application. As shown in Supplementary Figure 2A & B, there was no distinguishable evidence of hemolysis in tubes treated with PEG-NPs and iRGD-PEG-NPs under CPT concentration of 100 mM, which was much higher than the concentrations tested in the *in vitro* study. However, 1% Triton X-100 (positive control) achieved 100% hemolysis. These results indicated the excellent blood biocompatibility of both types of NPs.

Tumor tissue accumulation profiles

The accumulation of NPs in the region of a tumor is an important feature for an effective nanotherapeutic. We first obtained orthotopic colon cancer mice model with the treatment of AOM/DSS (Figure 7A). After that, mice were intravenously injected with PEG-CM-NPs or iRGD-PEG-CM-NPs at a CM concentration of 3 mg/kg. After 12 h, colon tumor tissues were excised and tissue sections were examined. As shown in Figure 7B, PEG-CM-NPs showed only minimal accumulation in colon tumor tissue. In contrast, iRGD-PEG-NPs accumulated profusely throughout the tumor tissue, indicating that iRGD-functionalized NPs had an excellent capacity for tumor accumulation.

Anticancer properties of NPs

The AOM/DSS-induced colon cancer mouse model is able to simulate the adenoma-carcinoma sequence occurred in human colon cancer [51,52]. As shown in Figure 8A, the AOM/DSS mice without NP treatment (control group) exhibited an obvious loss of body weight, while the NP-treated groups showed no significant change in body weight after two cycles of DSS treatment. Moreover, Figure 8B–D indicated that the tumor numbers per colon and tumor sizes were markedly lower in the NP-treated groups. Most importantly, the iRGD-PEG-NP-treated group had fewer tumors per mouse and smaller tumor size compared with PEG-NP-treated group. The antitumor efficacies of NPs were also determined using TUNEL assays, which can be used to detect apoptosis in tumor tissues. As shown in Figure 9, TUNEL positivity could be barely observed in the colon tumor tissues of control group, but was clearly seen in the tumors of NP-treated mice. Moreover, the tumor tissues from the iRGD-PEG-NP-treated mice exhibited more apoptotic signals than those from PEG-NP-treated mice. These results implied that iRGD-PEG-NPs represented the most efficient formulation. Finally, we found no histological evidence of damage to any major organ in the mice treated with PEG-NPs or iRGD-PEG-NPs (Supplementary Figure 3).

Discussion

To date, chemotherapy is an indispensable strategy for colon cancer therapy [53]. NP-based DDSs have been developed to enhance the therapeutic efficacy of chemotherapy [32]. Unfortunately, application of these NPs is still restrained due to some major concerns, including limited circulation time, insufficient tumor accumulation and low cellular uptake efficiency [54]. It was reported that PEG has been extensively employed as a hydrophilic corona to prolong the circulation time of NPs [55–57]. The tumor microenvironment is characterized by limited blood supply, heterogeneous vessel permeability and dense extracellular matrix, which block the accumulation and internalization of NPs in tumor tissues [58]. In recent years, various targeting ligands have been developed for colon cancer-targeted drug delivery in our group and others [33,59]. However, the accumulation of NPs remains an unresolved issue. Fortunately, iRGD has been exploited as a novel tumor-homing peptide. It could not only assist NPs to penetrate/accumulate in tumor sites, but also help them to be specific internalized by cancer cells [19]. The application of peptide as a targeting ligand also provides numerous merits, including easy manufacture, low immunogenicity and enhanced tumor penetration due to its small size. In the present study, PLGA was selected as a drug delivery matrix considering the advantages over other polymers, including excellent biocompatibility, controlled drug release and ease of

processing into NPs [32]. We previously demonstrated that CPT-loaded PLGA NPs in the absence of targeting ligand exhibited much better therapeutic efficacy against colon cancer than free CPT [10]. Thus, it was reasonable to speculate that the therapeutic outcome of these NPs might be further improved by functionalization with iRGD and PEG.

Particle size and zeta potential are crucial determinants for *in vivo* biodistribution and cellular uptake efficiency of NPs. In this study, the average diameters of PEG-NPs and iRGD-PEG-NPs were less than 280 nm. A previous study reported that NPs larger than 100 nm were able to avoid the uptake by reticuloendothelial system or rapid renal clearance, whereas NPs smaller than 400 nm were desirable for optimal enhanced permeability and retention effect [60]. Therefore, the prepared PEG-NPs and iRGD-PEG-NPs had a favorable size range for tumor penetration and cellular internalization. In addition, we found that the zeta potentials of both types of NPs were around -15 mV, showing the desirable slightly negative surface charge that could facilitate NP accumulation in tumor tissue [61]. Moreover, no significant variation on the particle size and zeta potential was observed following the surface functionalization with iRGD, which was in good agreement with the prior report [62].

Enhanced cellular internalization by targeted cells is necessary for increased therapeutic index. The surface

Figure 7. Colon tumor accumulation of PEG-CM-NPs and iRGD-PEG-CM-NPs *in vivo*. (A) Protocol for colon cancer induction. Mice were administered NPs (3 mg CPT/kg mouse) every 2 days in the treatment groups. (B) Colon tumor tissue uptake profiles of PEG-CM-NPs and iRGD-PEG-CM-NPs. NPs (3 mg CM/kg per mouse) were intravenously injected, and colon tumor tissues were collected at 12 h postinjection. Fixed tissues were stained with Alexa Fluor 568 phalloidin and DAPI for visualization of actin (red) and nuclei (purple), respectively. Scale bar = 20 μ m.

AOM: Azoxymethane; CM: Coumarin-6; DSS: Dextran sulfate sodium; FITC: Fluorescein isothiocyanate; iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle.

functionalization with iRGD remarkably increased the cellular uptake efficiency of NPs. In the presence of free iRGD, a dramatic drop in cellular uptake of iRGD-PEG-NPs was observed, indicating that iRGD blocked the interaction between iRGD receptors and the iRGD groups in iRGD-PEG-NPs. These results also implied that iRGD-PEG-NPs entered cells via receptor-mediated endocytosis. The *in vitro* MTT assay suggested that iRGD enhanced the cytotoxicity of CPT-loaded NPs evidently with decreased IC_{50} value. The cell apoptosis and RT-PCR tests further demonstrated that the functionalization with iRGD induced higher apoptosis in Colon-26 cells, which was ascribed to the enhanced cellular uptake capacity of iRGD. Hemocompatibility evaluation is usually a prerequisite before intravenous injection [63]. Hemolysis assay showed that both types of NPs exhibited excellent hemocompatibility. Previous reports dem-

onstrated that co-delivery of free iRGD and NPs/free anticancer drug via intravenous injection improved chemotherapeutic efficacy of colon cancer [29,64]. To the best of our knowledge, no attempt has been conducted to examine the therapeutic outcomes of intravenous administered iRGD-functionalized NPs for colon cancer treatment. In the present study, the anticancer effect of iRGD-PEG-NPs has been further investigated based on an orthotopic colon cancer mice model. *In vivo* biodistribution study showed improved tumor accumulation of iRGD-PEG-NPs compared with PEG-NPs, as indicated by the increased green fluorescence (NPs) in the colon tumor tissue. The superior performance of iRGD-PEG-NPs was verified using orthotopic mice model with colon cancer. As expected, iRGD-PEG-NPs exhibited much better growth inhibition efficiency and tissue apoptotic profiles of colon cancer in comparison to PEG-NPs,

Figure 8. Anticancer effects of PEG-NPs and iRGD-PEG-NPs *in vivo*. (A) Mouse body weight over time, normalized as a percentage of the day-zero body weight and given as the mean of each treatment group. (B) Representative colon samples were obtained from each experimental group at Day 48. (C) Number of tumors per mouse. (D) Tumor size was determined using a dissecting microscope fitted with an ocular micrometer. The tumor size distribution was graphed. Each point represents the mean $\pm$ SEM ($n = 6$ or 7). Statistical significance was assessed using ANOVA followed by a Bonferroni post-hoc test (* $p < 0.05$ and ** $p < 0.01$). iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle; SEM: Standard error of mean.

Figure 9. Representative images of double-fluorescence labeling with DAPI (nuclei, blue) and TUNEL (green) in colon tumors from the various mouse groups. Scale bar = 20 μ m.

iRGD-PEG-NP: iRGD-functionalized PEGylated CPT-loaded PLGA NP; NP: Nanoparticle.

which was also consistent with the *in vitro* results. It was worth noting that the resultant NPs have been washed for three-times after their fabrication process, which ensured that there was no free iRGD in the drug formulation. Therefore, we can reasonably conclude that the improved tumor accumulation and enhanced anticancer efficiency were attributed to the successful modification of PEG and iRGD. Additionally, histological analysis on the major organs of the experimental mice after treatment showed no obvious organ damage, indicating that both types of NPs have good biocompatibility.

Conclusion

Here, we employed iRGD-functionalized PEGylated polymeric NP as a drug carrier for colon cancer chemotherapy. The prepared iRGD-PEG-NPs exhibited a desirable particle size and negative surface charge that effectively facilitated cellular uptake. We further found that iRGD-PEG-NPs could enhance the apoptosis of colon cancer cells and downregulate the *Bcl-2* mRNA expression level more effectively than their nonfunctionalized counterparts (PEG-NPs). *In vivo* experiments demonstrated that iRGD-PEG-NPs had a stronger capacity to accumulate in tumor tissues and a much better therapeutic efficacy against orthotopic colon tumors in mice as compared with PEG-NPs. These results unambiguously demonstrated the promising potential of iRGD-

PEG-NPs as a nanotherapeutic for colon cancer-targeted chemotherapy.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2017-0107

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Ethical conduct of research

The authors state that all animal experiments were conducted according to the Regulation on Experimental Animals of Southwest University. The authors also state that

they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Summary points

- To improve the tumor penetration and cell-specific drug delivery of nanoparticles (NPs), iRGD-functionalized PEGylated camptothecin-loaded NPs (iRGD-PEG-NPs) were designed and fabricated using a well-established approach.
- iRGD-PEG-NPs were less than 280 nm with spherical morphology, and they had slightly negative zeta-potential and controlled drug release profile.
- iRGD-PEG-NPs were internalized by Colon-26 cells via an iRGD receptor-mediated endocytosis, which was much faster than nonspecific endocytosis of PEG-NPs.
- iRGD-PEG-NPs showed much higher antitumor efficiency and proapoptotic capacity than PEG-NPs *in vitro*.
- iRGD-PEG-NPs induced effective tumor accumulation and significantly enhanced chemotherapeutic efficiency with no obvious organ damage *in vivo*.
- This study has shown that iRGD-PEG-NP is a suitable formulation for colon cancer therapy.

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•• of considerable interest

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